Author:
Rees, Philip

Title:
The role of notch signalling in colorectal cancer

THE ROLE OF NOTCH SIGNALLING IN COLORECTAL CANCER

A thesis submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the Faculty of Medical & Veterinary Sciences, Department of Cellular & Molecular Medicine.

PHIL REES

February 2010

DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original, except where indicated by special reference in the text, and no part of the dissertation has been submitted for any other academic award. Any views expressed in the dissertation are those of the author:

SIGNED: 

DATED: 1st March 2010

ABSTRACT

Colorectal cancer (CRC) continues to be one of the leading causes of death in the western world and the molecular basis of this disease remains the focus of intensive research. Notch is a highly conserved embryogenic pathway, which is also essential for homeostasis in adult tissues and is increasingly implicated in many human malignancies, including CRC. Evidence for the expression and function of Notch in the intestinal epithelium has largely been derived from analysis in animal models and highlights both Notch1 (N1) and Hes1 respectively as the principal receptor and effector in this tissue. Analysis in the murine epithelium also strongly suggests a central role for these factors in intestinal tumorigenesis; however, the expression and/or function of N1 in the human colorectal epithelium and CRC remains poorly defined. Therefore, the aim of the present study was to define the expression of N1 in these tissues and to describe possible changes in expression with the malignant progression of disease. Furthermore, this study also aimed to model these changes *in vitro* and to evaluate a functional role for active Notch signalling in the human CRC phenotype.

From *in vivo* analysis, it was found that N1 is expressed in the normal colorectal epithelium and that expression is frequently altered in human colorectal tumours. Crucially, N1 expression was comparatively low and intermittent in adenomas but was significantly increased in adenocarcinoma tissues. No significant difference was observed between the different adenoma or adenocarcinoma subtypes but N1 protein expression was found to correlate strongly with the malignant progression of this disease. Furthermore, these findings were closely reflected using colorectal tumour cell lines *in vitro* and it was found that N1 expression increases with both cell cycle progression and tumour de-differentiation. Although a causal role for N1 in these tumour features was not established, these findings allude to a complex and context-dependent basis for the Notch pathway in these tissues. Moreover, while these findings are important in furthering the potential for Notch signalling as a therapeutic target in CRC, this study goes on to highlight several important but unappreciated roles for N1 in the non-neoplastic colorectal epithelium. Namely, N1 was found to be expressed in both differentiating cells of the normal colonic crypt and in regions of active tissue repair in inflammatory bowel disease. In summary, the results of this study confirm that altered N1 expression represents important changes in the human colorectal epithelium, including colorectal carcinogenesis; however, findings presented here also suggest that Notch signalling may be responsible for more functions in these tissues than currently described.

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my heartfelt gratitude to my supervisors Prof. Massimo Pignatelli and Dr David Qualtrough for making this possible, as well as their insightful guidance and continued support throughout. To David (*aka Chief*) in particular, I am extremely indebted. I would like to express appreciation to him for many things, but primarily for being an excellent mentor for many years now, for his unfailing patience and for often supporting me above and beyond the call of duty. Many thanks also for his excellent taste in music, for climbing and for fixing the clutch on my car.

Appreciation must go to Prof. Chris Paraskeva, Dr Ann Williams and all members of the CP group who have known me (past and present). In particular I would like express my appreciation to: Chris for his valuable advice, caring support and weekend chats; Ann for picking me up, helping me find the way and for all much appreciated help in guiding this thesis; Tray and Dianne for putting up with me, for everything lab related and for all the caring things they have done; Amy for going through this with me and for being a good friend in general; Abdul for early insight and food; Katy P for her excellent role as guardian angel and proof-reader extraordinaire (...what?).

I would like to take this opportunity to thank Jenny Baker for all her training and guidance with the immunohistochemistry, especially for help with various staining events and tissue sectioning. Thank you also for being a lovely person and for your good company. A huge 'thank you' must go to Dr Nahida Banu for invaluable help in the scoring of these tissues. I also thank Ginny, Kieran and Wingman for their varying involvement and support. Particular appreciation must go to Dr Helena Smartt for her excellent assistance and training in all things 'Weiser'. I was also grateful for help given by Dr Karim Malik and Dr Anthony Dallosso in the art of PCR, DNA sub-cloning and gene promoter analysis. Furthermore, I would also like to thank Dr Andy Herman and Tom 'Lumberjack' Curry for their endless help with the flow cytometry and cell cycle analysis. Special thanks to Tom for staying past his bed time on many occasions and for all mountain biking fun (including the hospital). I am also grateful to Dr Angela Hague and Dr Keith Brown for their involvement as my internal assessors and for all useful guidance and discussions.

Separately, many thanks go to my friends who have tolerated me through this venture of mine. Kuci and Vicky especially, 'hey you guys'. Much appreciation also goes to my family for their continued understanding and patience throughout. In particular I must express a very special 'thank you' to my mum for her unending support, interest and encouragement from start to finish, without which this would not have been possible.

DEDICATION

To Joy, for her continual support and belief.

For all things lost and gained along the way.

ABBREVIATIONS

α-SMA - α-smooth muscle actin
ACF - Aberrant crypt foci
ADAM - A Disintegrin and Metalloprotease
AGS - Alagille syndrome
ANK - Ankyrin
APC - Adenomatous Polyposis Coli
APH - Anterior pharynx defective
APP - Amyloid precursor protein
APS - Ammonium persulfate
Atoh1 - Atonal homologue 1
β-TrCP - β-transducin repeat containing protein
BCC - Basal cell carcinoma
Bcl-Xl - B-cell lymphoma-extra large
bHLH - basic helix loop helix
Bmi1 - B lymphoma Mo-MLV insertion region 1
BMP - Bone morphogenic protein
BSA - Bovine serum albumin
CAF - Cancer associated fibroblasts
CADASIL - Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
Ca-R - Calcium receptor
CBC - Crypt base columnar cell
CCK - Cholecystokinin
CD - Crohn's disease
CDK - Cyclin dependent kinase
CDKI - Cyclin-dependent kinase inhibitor
CK1 - Casein-kinase-1
CR - cystein-rich
CRC - Colorectal cancer
CRT - Catenin related transcription
CSC - Cancer stem cell
CSL - CBF1/RBP-J in mammals, Su(H) in *Drosophila*, Lag-1 in *C. Elegans*
CTR - Control
DAB - 3-3'-Diaminobenzidine
DABCO - 1,4-Diazabicyclo(2.2.2)octane
DAPI - 4',6-diamidino-2-phenylindole
DCC - Deleted in colon cancer
Dhh - Desert hedgehog
Dkk1 - Dickkopf homologue 1
dKO - Double Knock-out
Dll1/3/4 - Delta-like ligand 1/3/4
DMEM - Dulbecco modified eagles medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DNase - Deoxyribonuclease
dnTCF4 - dominant negative TCF-4
DRM - Diverticulitis resection margin
Dsh - Dishevelled
DSL – Delta/Serrate/LAG-2
E-cadherin - Epithelial cadherin
ECL - Enhanced chemiluminesence
EDTA - Ethylenediaminetetraacetic acid
EGTA - Ethylene glycol tetraacetic acid
EGF - Epidermal growth factor
EMT - Epithelial-mesenchymal transition
Eph - Ephrin receptor
ER - Endoplasmic reticulum
FACS - Fluorescence-activated cell sorting
FAP - Familial adenomatous polyposis
FBS - Foetal bovine serum
FGF - Fibroblast growth factor
FLV - Feline leukaemia virus
Fng - Fringe
FSC-A - Forward scatter
Gfi - Growth factor independent transcription repressor
GI - Gastrointestinal
Gli - Glioma-associated oncogene
GSI - γ-secretase inhibitor
GSK-3α/β - Glycogen synthase kinase 3α/β
H_2O_2 - Hydrogen peroxide
H&E - Haematoxylin and Eosin
Hath1 - Human atonal homologue 1
HDAC - Histone deacetylase
Herp - Hes related protein
Hes - Hairy/Enhancer of split
Hh - Hedgehog
HNPCC - Hereditary non-polyposis colorectal cancer
HRP - Horseradish peroxidase
IBD - Inflammatory Bowel Disease
ICC - Immunocytochemistry
Id - Inhibitor of DNA binding
IEC - Intestinal epithelial cell
IEG - Immediate early gene
IHC - Immunohistochemistry
Ihh - Indian hedgehog
JAK - Janus kinases
Jag1/2 - Jagged1/2
JPS - Juvenile polyposis syndrome

kDa - kilodalton
Klf4 - Kruppel-like factor 4
KO – Knock-out
Lag1/2 - Larval arrest gene 1/2
LEF - Lymphoid enhancer factor
Lfng - Lunatic fringe
Lgr5 - Leucine-rich-repeat containing G protein coupled receptor 5
LI - Large intestine
LOH - Loss of heterozygosity
LRC - Label retaining cell
LRP5/6 - Low density lipoprotein receptor-related protein 5/6
Luc - Luciferase
Maml1 - Mammalian Mastermind-like
Mash - Mammalian achaete-scute homolog
Math1 - Mouse atonal homologue 1
MD - Moderately differentiated adenocarcinoma
MET - Mesenchymal-epithelial transition
Mfng - Manic fringe
Mib - Mind bomb
Min - Multiple intestinal neoplasia
MINT - Msx2-interacting nuclear target protein
miRNA - microRNA
MMP - matrix metalloproteinases
MMR - Mismatch repair
MSI - Microsattelite instability
Msi-1 - Musashi-1
MMTV - Mouse mammary tumour virus
M-MuLV - Moloney murine leukaemia virus
Muc2 - Mucin 2
N1/2/3/4 - Notch1/2/3/4
$N^{\Delta E}$ - membrane-tethered Notch
N^{EC} - Extracellular Notch
N^{FULL} - Full length Notch
N^{IC}/NIC/ICD - Intracellular Notch
N^{TM} - Transmembrane Notch
ncRNA - Non-coding RNA
NCT - Nicastrin
NSC - Neural stem cell
NeuroD - Neurogenic differentiation
Ngn3 **-** Neurogenin3
NLS - Nuclear localisation sequence
NRARP - Notch regulated ankyrin repeat protein
***O*-fut1 -** *O*-fucosyltransferase 1
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
PD - Poorly differentiated adenocarcinoma
PDGF - Platelet-derived growth factor
PEN - Presenilin enhancer
PEST - proline, glutamate, serine, threonine rich
PFA - Paraformaldehyde
PI - Propidium Iodide
PI3K - Phosphoinositide 3-kinase
PLB - Passive lysis buffer
PS1 - Presinilin1
Ptch - Patched
PTEN - Phosphatase and tensin homolog
PVDF - Polyvinylidine difluoride
RAM - RBP-Jκ/CBF1-associated module
RAS-MAPK - RAS-mitogen-activated kinase
Rfng - Radical fringe
RIP - Regulated intra-membrane proteolysis
RNA - Ribonucleic acid
RNAi - RNA interference
RNase - Ribonuclease
RPB-Jκ - Recombinding protein of suppressor of Hairless
RT-PCR - Reverse transcriptase PCR
S1 - Notch protein cleavage site 1 (furin-like convertase mediated)
S2 - Notch protein cleavage site 2 (ADAM/TACE mediated)
S3 - Notch protein cleavage site 3 (γ-secretase mediated)
SCC - Squamous cell carcinoma
SCFA - Short-chain fatty acids
SD - Sondylocostal dysostosis
SDS-PAGE - Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SEM - Standard error of the mean
SEMF - Sub-epithelial myofibroblast
SFM - Serum free medium
Shh - Sonic hedgehog
SI - Small intestine
siRNA - Small interfering ribonucleic acid
Smo - Smoothened
SSC-A - Side scatter
STAT - Signal Transducers and Activators of Transcription
TA – Tubular adenoma
TACE - Tumour Necrosis Factor Alpha Converting Enzyme
TAD - Transcription activation domain

T-ALL - T-cell acute lymphoblastic leukaemia
TACE - Tumour Necrosis Factor Alpha Converting Enzyme
TAN1 - Translocation-associated Notch homologue
TCF - T-cell factor
TCRβ - T-cell-receptor-β
TEMED - N,N,N',N'-Tetramethylethylenediamine
TFBS - Transcription factor binding site
TGF-β - Transforming growth factor β
TLE - Transducin-like enhancer-of-split
TNM - Tumour, Node, Metastasis
TRM - Tumour resection margin
Tris - 2-amino-2hydroxymethyl-1,3-propanediol
TVA – Tubulovillous adenoma
Tween - Polyoxyethylene sorbitan monolaurate
UC - Ulcerative colitis
UTR - Untranslated region
UV - Ultraviolet
VA – Villous adenoma
VE-cadherin - vascular-endothelial cadherin
WD - Well differentiated adenocarcinoma
Wg – Wingless
Wnt - Wingless-type MMTV integration site family

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

1.1 THE ORIGINS OF COLORECTAL CANCER

1.1.1 THE EPIDEMIOLOGY OF COLORECTAL CANCER

Colorectal cancer (CRC) is one of the leading causes of morbidity and mortality in the western world and is the third most frequently diagnosed form of cancer in the UK, after that of the lung and breast (Cancer Research UK, Statistics 2008). CRC accounts for around 13-15% of all cancer cases and equates to more than 37,500 new UK diagnoses annually, or over 100 people every day. Although surgical intervention can normally remove all macroscopic traces of this disease, approximately half of patients will demonstrate some form of recurrence due to micro-metastases in the surrounding tissue. As such, CRC is associated with only about a 50% five year survival rate for men and women, and approximately 16,000 people die of this disease each year in the UK alone (Midgley and Kerr, 1999). Despite the unquestionable significance of this disease, adjuvant therapies are still hugely limited because of the complex molecular and genetic alterations involved. Therefore, a greater understanding of the cellular-molecular factors involved in both the initiation and progression of colorectal tumours is now essential to help improve survival in these patients.

1.1.2 THE AETIOLOGY OF COLORECTAL CARCINOGENESIS

The vast majority (~80%) of CRC cases arise in an age-related, sporadic manner from a step-wise accumulation of genetic mutations (reviewed in Fearnhead *et al.*, 2002). These enable colorectal epithelial cells to progressively escape normal homeostatic control and progress through well defined stages of colorectal carcinogenesis. Due to the time taken for this, CRC usually arises in patients aged sixty onwards and is uncommon under the age of fifty. By the age of 70, however, at least 50% of the western population will have developed a benign colorectal tumour and about 1 in 10 of these will progress to malignant disease (Cancer Research UK, Statistics 2008; Kinzler and Vogelstein, 1996).

The aetiology of colorectal tumorigenesis is complex but it is now well established that CRC incidence correlates strongly with geographical location and is heavily impacted by associated environmental and cultural factors (reviewed in Boyle and Langman, 2000). It has been long been known that incidence is highest in developed, industrialised nations and associates strongly with the 'western' lifestyle, particularly dietary practices (Armstrong and Doll, 1975). It is now widely

accepted that a diet containing excessive fat and red meat, in combination with physical inactivity, imparts the greatest risk for colorectal tumorigenesis (Sandhu *et al.*, 2001). Conversely, a diet rich in fruit, vegetables and dietary fibre offers considerable protection from all cancers, but particularly to epithelial cells of the alimentary tract (Steinmetz and Potter, 1991). In addition to increasing faecal bulk and reducing transit time, some dietary fibre may also be degraded into physiologically important constituents that directly regulate epithelial homeostasis (Wong *et al.*, 2006). The occurrence of CRC in countries with low incidence increases with greater affluence, industrialisation and adoption of western habits, while people migrating from low- to high-incidence countries are seen to approximate to the rates in the new region within one to three generations (Boyle and Langman, 2000). It might therefore be suggested that a large proportion of CRC incidence is theoretically avoidable but, as western habits spread across globe, incidence levels continue to increase steadily and CRC looks set to remain one of the most frequently occurring cancers worldwide.

1.1.3 STRUCTURE AND FUNCTION OF THE SMALL AND LARGE INTESTINE

The regions of the intestinal tract (gut) can be broadly divided into the proximal small intestine (SI) and distal large intestine (LI). Although much of the intestine shows similarities in structure and function, regional variation exists due to differences in required function (reviewed in Ponz de Leon and Di Gregorio, 2001). The LI is composed of the colon and rectum (colorectum) and can be anatomically subdivided, starting at the caecum (junction with the SI), into the right (ascending), middle (transverse), left (descending) and sigmoid colon, with the rectum and anal canal positioned most distally. Similarly, the SI can be anatomically subdivided into the duodenum, jejunum and ileum.

The primary function of the intestinal tract, aside from continued digestion, is the absorption of nutrients, salts and water. The small intestine contains numerous finger-like projections into the lumen, termed 'villi', which dramatically increase the absorptive surface area associated with nutrient uptake. In contrast, the large intestine is flat and devoid of villi, being mostly concerned with water and salt absorption. The wall of both the small and large intestine, termed the 'mucosa', is composed of three distinct layers surrounding the lumen; the outer smooth muscle (muscularis externae), the middle stroma/mesenchyme (lamina propria and submucosa) and the inner epithelium (*FIGURE 1-1*). This single cell thick columnar epithelium is separated from the underlying lamina propria by a basement membrane and provides a protective and functional interface with the lumenal environment.

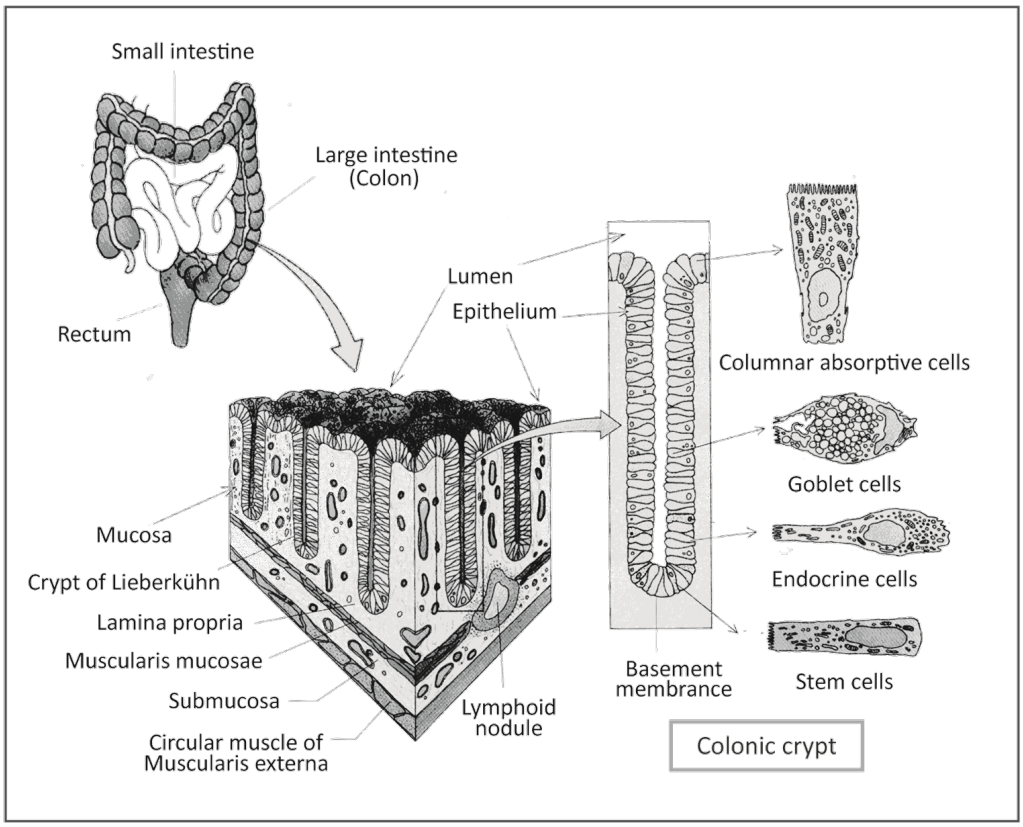


Figure 1-1| ***Schematic representation of the colonic mucosa.*** The large intestine is a long hollow organ lined with mucous membrane (mucosa) wrapped in multiple layers of connective and muscularis tissues. The outermost layer of the colonic mucosa is a single cell thick, rapidly dividing epithelium which is characterised by multiple invaginations into the mucosal surface, termed 'crypts'. These crypts provide the germinal centre of the epithelium and a number of intestinal stem cells are found located at their base. These pluripotent stem cells give rise to the three distinct functional cell lineages found in the epithelium; the absorptive columnar cells, the mucin secreting goblet cells and the hormone secreting enteroendocrine cells (Gartner and Hiatt, 1997).

1.1.4 ABSORPTIVE AND SECRETORY CELLS CONSTITUTE THE INTESTINAL EPITHELIUM

Due to the extreme functional and protective requirements of the intestinal epithelium, it is widely considered to be one of the most dynamic and rapidly self-renewing of all adult mammalian tissues, demonstrating an exceptionally high cell turnover rate (Heath, 1996). The surface of both the SI and LI also comprises of numerous tubular invaginations of the epithelium into the underlying submucosa, termed the 'crypts of Lieberkühn'. Millions of these crypt structures are found throughout the entire length of the human colon, each being approximately 80 cells deep and containing about 2000 cells in total (*FIGURE 1-1*) (Potten *et al.*, 1992).

It has long been known that the epithelium is composed of three to four functionally distinct intestinal epithelial cell (IEC) types which can be broadly classified as either absorptive or secretory (Cheng and Leblond, 1974). The most abundant are the absorptive columnar cells

(Enterocytes in the SI/Colonocytes in the LI), which constitute approximately 80% of the entire intestinal epithelial population (Karam, 1999). The secretory cells can be further subcategorised as goblet cells, enteroendocrine cells or Paneth cells, depending on specific function and cell morphology. Goblet cells are responsible for the secretion of protective mucins and trefoil proteins that facilitate movement of the increasingly compacted lumenal contents and protect the underlying mucosa against shear stress and toxin-mediated damage. The proportion of goblet cells in the intestine increases distally from the duodenum (~4%) to the descending colon (~16%) due to increasing protective requirement (Karam, 1999). Enteroendocrine cells constitute only 1% of the total intestinal epithelium but comprise of approximately 15 different functional subtypes (Schonhoff *et al.*, 2004). Enteroendocrine cells are responsible for the secretion of specific intestinal peptide hormones and play an essential role in coordinating gut function. Paneth cells are found almost exclusively in the SI, but do occur in the proximal colon, and are localised at the very base of the crypt (Porter *et al.*, 2002). Paneth cells function in innate immunity through the secretion of antimicrobial peptides (cryptidins and defensins), which govern the gastrointestinal flora.

Analysis in animal models (Bjerknes and Cheng, 1999; Ponder et al., 1985) and the human colon (Novelli *et al.*, 1996) has shown that intestinal crypts are clonal structures, suggesting each is maintained by a small number of its own pluripotent stem cells. These stem cells divide asymmetrically to give rise to progenitors of all cell lineages found in the intestinal epithelium (Marshman *et al.*, 2002) and the base of each crypt forms the 'stem cell niche', which is maintained by a complex interplay between the stem cells, their immediate progeny and the underlying pericryptal myofibroblasts (Yen and Wright, 2006). The epithelial sheet is therefore in a state of continual cellular motion, where new cells derived from stem cells at the crypt base constantly move upwards while rapidly proliferating and undergoing cell fate determination, before functionally differentiating and senescing towards the lumenal surface.

There is a gradual loss of stemness and proliferation along the crypt axis, with the peak location for proliferation being positions 15-17 and few cell divisions occurring after position 35 (Potten *et al.*, 1992). Following several rounds of division, progenitor cells undergo a process of commitment to one of the functional cell lineages, before exhausted cells undergo apoptosis and are lost into the gut lumen (Gavrieli et al., 1992; Hall et al., 1994). Genes associated with proliferation are therefore located basally along the crypt axis, while those associated with terminal differentiation and apoptosis are located apically (Kosinski *et al.*, 2007). This process

takes only 4 days from genesis to attrition in the human colon (Potten *et al.*, 1992), although it appears that cellular fate in the crypt is not entirely dependent on autonomous mechanisms but is instead heavily regulated by cell position and environmental context (Hermiston *et al.*, 1996). Crucially, this tightly controlled balance is now known to be governed by a complex network of signals exchanged within the epithelium and with the underlying myofibroblasts, the exposure to which changes extensively along the crypt axis (Crosnier *et al.*, 2006).

1.1.5 LOCATION AND MAINTENANCE OF THE INTESTINAL CRYPT STEM CELLS

Although all evidence points to the existence of a stem cell niche at the base of the crypt, the precise location of these cells has been hotly debated over the past two decades and remains highly controversial (Potten *et al.*, 2009). The transcriptional signature of these stem cells has been partially characterised (Stappenbeck *et al.*, 2003) but, so far, only a few candidate molecular markers have been proposed (Barker and Clevers, 2007). Based on the proposal that adult ISCs are generally quiescent but give rise to rapidly cycling progenitor cells, original identification was based on long term label retention techniques. By this method, an annulus of label retaining cells (LRC) undergoing minimal rounds of divisions were identified four cells up from the crypt base in the small intestine (+4 LRC), just above the Paneth cell zone (Potten *et al.*, 2003; Potten *et al.*, 2002). These have long been widely regarded as the ISCs.

Alternatively, it has been proposed that a fifth epithelial cell type, the crypt base columnar cell (CBC), might constitute the intestinal stem cell population (Chwalinski and Potten, 1989). CBCs are located at the base of the crypt (position 1-4 in SI) and an ISC role for these cells has received recent support from work by Clevers and colleagues (Barker *et al.*, 2007) using the novel stem cell marker *Lgr5* (*Leucine-rich-repeat containing G protein coupled receptor 5*). They demonstrate the presence of about six long lived, but actively cycling, *Lgr5*$^{+}$ cells at the bottom of the murine small and large intestinal crypts, which fulfil all the criteria required of an intestinal stem cell. The presence of these cells has also been confirmed in the human SI and LI through analysis of Lgr5 protein expression (Becker *et al.*, 2008). However, in contrast to popular belief, these putative ISCs are not quiescent but are instead highly proliferative (Barker *et al.*, 2007). It has recently been shown that single *Lgr5*$^{+}$ cells are capable of rebuilding the entire intestinal crypt (Sato *et al.*, 2009) and it now appears that *Lgr5* is a highly valid stem cell marker in a number of other tissues (Barker *et al.*, 2008; Jaks *et al.*, 2008).

Interestingly, both theories find support from analysis using previously proposed stem cell markers, such as *Musashi-1* (*Msi-1*), a neural stem cell marker that localises to both the CBCs and

the +4 LRC (Kayahara *et al.*, 2003), and *B lymphoma Mo-MLV insertion region 1 (Bmi1*), a neural and haematopoietic stem cell marker that is found exclusively in the +4 LRC crypt position (Sangiorgi and Capecchi, 2008). In possible explanation, it has been proposed that the CBCs and the +4 LRCs may represent two distinct but highly interactive ISC sub-populations, whereby +4 LRCs act as a quiescent reserve to replenish the actively cycling CBCs (Scoville *et al.*, 2008). Thereby, +4 LRCs and CBCs are both ISC populations but in different stem cell niches, with cells migrating from one to the other to maintain the fidelity of the crypt.

1.1.6 A STEM CELL ORIGIN FOR COLORECTAL CARCINOGENESIS

Analysis of intestinal stem cells also highlights one area of controversy in the field of CRC research; the true cellular origin of intestinal tumours. At present, it continues to be hotly debated whether tumours derive from stem cells that retain their self-renewing capabilities, but acquire further tumorigenic properties, or whether tumour stem cells are proliferative progenitors that have acquired the capacity of longevity and self-renewal. It has been proposed that the increased environmental exposure and rapidly cycling nature of proliferative progenitor cells renders them vulnerable to carcinogenic mutation, leading to clonal expansion back down the crypt in a 'Top-Down' model of carcinogenesis (Shih *et al.*, 2001). Alternatively, the crypt stem cells divide much more slowly but already have the propensity to self-renew and are thought to be the only cells that persist long enough for successive mutation to occur. Therefore, it has also been proposed that the tumours constitute mutant clones arising in the base of the crypt, which spread upwards in a 'Bottom-Up' fashion (Preston *et al.*, 2003).

More definitive evidence for a stem cell origin to colorectal tumours has recently been provided by a mouse model of intestinal tumorigenesis driven by *Lgr5* (Barker *et al.*, 2009). It was eloquently shown that the transformation of proliferative progenitor cells may produce tumour-like lesions, but that these are not sustained and rarely result in the production of full-blown intestinal tumours. In contrast, transformed *Lgr5*$^{+}$ stem cells accumulate in the base of the crypt and result in the formation of many large intestinal tumours. This suggests that intestinal tumorigenesis relies on genetic mutation in crypt stem cells, not progenitors, and lends further weight to a 'Bottom-Up' theory of tumour origin. Moreover, *Lgr5*$^{+}$ cells remain distributed throughout the resulting adenomas, suggesting that a stem and progenitor cell hierarchy is retained in early neoplastic lesions (Barker *et al.*, 2009).

It is increasingly well accepted that tumour growth and progression is maintained by a distinctive sub-population of cancer stem cells (CSCs) that aberrantly recapitulate the homeostatic

mechanisms of the parent tissue (reviewed in Visvader and Lindeman, 2008). The first evidence for the existence of these CSCs came from analyses in acute myeloid leukaemia, in which only a small subset of cells (0.1-1%) are capable of inducing leukaemia when transplanted into immunodeficient mice (Bonnet and Dick, 1997; Lapidot *et al.*, 1994). This distinct sub-population was isolated by their high expression of a particular cell surface glycoprotein, CD34. Similar markers, such as CD44 and CD133, have now been used to isolate tumour initiating CSC populations in solid tumours, including breast (Al-Hajj *et al.*, 2003), brain (Bao *et al.*, 2006; Beier *et al.*, 2007; Singh *et al.*, 2004), pancreatic (Hermann *et al.*, 2007) and colorectal cancer (O'Brien *et al.*, 2007; Ricci-Vitiani *et al.*, 2007). Mathematical analysis indicates that colorectal tumour formation must arise as a consequence of increased stem cell number (Boman *et al.*, 2001). Therefore CSCs in advanced tumours may not occur as a small fraction (Kern and Shibata, 2007) and, in agreement, it has been shown that the $CD133^+$ CSC population in colorectal tumours may constitute up to 25% of the total tumour population (O'Brien *et al.*, 2007). These CSCs have received further interest because, in addition to maintaining the tumour population, they are also now believed to be the key mediators of both CRC invasion (Brabletz *et al.*, 2005b) and chemoresistance (Dylla *et al.*, 2008).

1.1.7 THE HALLMARKS OF CANCER

Accordingly, the origins and mechanisms of cancer formation have been hotly debated and have remained the focus of extensive research over previous years (reviewed in Hahn and Weinberg, 2002). Importantly, virtually all mammalian cells are governed by similar molecular mechanisms controlling their survival, proliferation, differentiation and apoptosis. Therefore, it should not be surprising that, in addition to the CSC theory, many defining features are common to nearly all cancer types. These 'hallmarks of cancer' have been eloquently summarised in an infamous paper by Hanahan and Weinberg (2000) where it was proposed that cancer can be defined by just six distinctive properties (*FIGURE 1-2*). Primarily, cancer cells must be self sufficient in growth promoting signals (usually limiting in controlled growth of normal cells) and insensitive to the growth inhibitory and pro-apoptotic mechanisms, which maintain the equilibrium between cellular genesis and attrition in the normal tissue. Like stem cells, cancer cells must possess the ability to limitlessly replicate and evade the exhaustive limitations imposed on normal progenitors. Together these characteristics bias the homeostatic dynamics present in the normal tissue towards a phenotype that promotes significant cellular proliferation and tumour expansion.

However, increasing tumour size is not without its own limitations and the subsequent increased requirement for both nutrients and oxygen must be overcome through new blood vessel growth and increasing angiogenic capabilities. Tumour cells might escape these emerging tumour constraints by detaching from the primary tumour site and invading into the surrounding tissue, the defining difference between the benign and malignant phenotype. The generation of CRC in humans therefore involves a complex succession of pre-malignant and malignant events through the acquisition various hallmark cancer features.

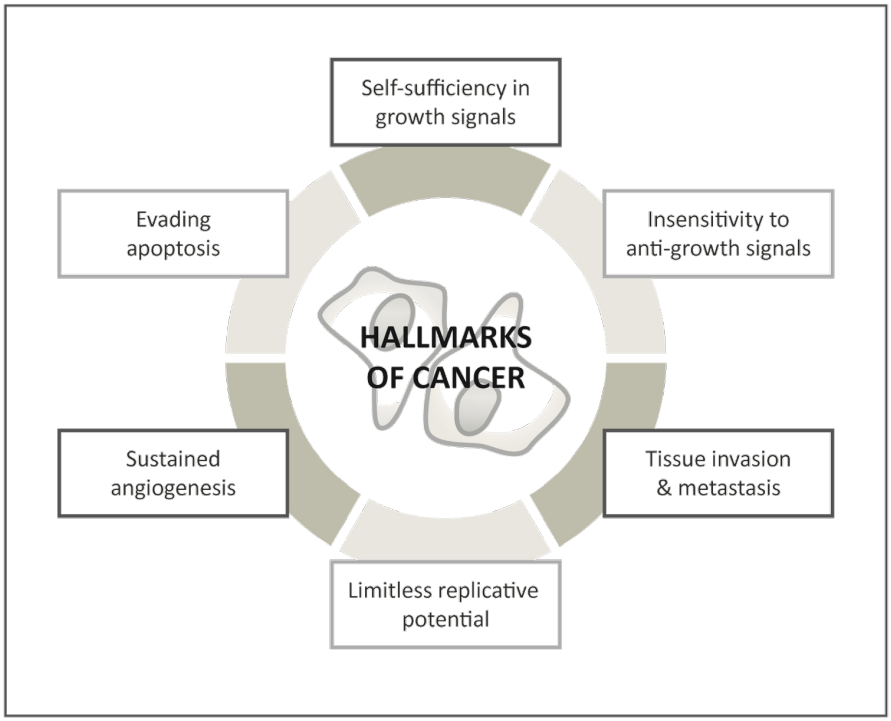


Figure 1-2| *The hallmarks of cancer*. A similar cohort of six acquired functional capabilities is observed in most if not all cancers, albeit through various mechanisms (Hanahan and Weinberg, 2000). These enable altered cellular clones to evade the homeostatic constraints of the normal tissue and permit the cells to adapt to the changing environment of the growing tumour tissue, leading to further tumour expansion and malignant dissemination.

1.1.8 THE ADENOMA-ADENOCARCINOMA ROUTE OF TUMOUR PROGRESSION

It has long been known that the transformation of normal cells into malignant cancer cells proceeds along a defined route of tumour progression in many tissues, often referred to as the multi-stage theory of carcinogenesis (Armitage and Doll, 1954). This process is particularly evident and well characterised during colorectal carcinogenesis and CRC continues to be a paradigm for tumour formation and malignant progression in general (Luebeck and Moolgavkar, 2002). Due to the glandular and secretory nature of the intestinal epithelium, neoplasia arising within this tissue is denoted by the prefix *Adeno-* (meaning glandular) and can be broadly

categorised as either adenoma (benign) or adenocarcinoma (malignant). Most evidence supports the existence of a step-wise adenoma-adenocarcinoma sequence of tumour progression, for which the histological (Ponz de Leon and Di Gregorio, 2001) and many molecular-genetic (Fearon and Vogelstein, 1990) features have already been deciphered in detail.

The earliest stages of colorectal carcinogenesis occur in the normal mucosa as a result of aberrant cellular replication and hyper-proliferation (reviewed in Renehan *et al.*, 2002). As described above (*SECTION 1.1.6, p7*), increasing evidence suggests that the initiating event for intestinal tumorigenesis occurs in the crypt stem cell population, producing transformed cells with an enhanced capacity to proliferate and survive (Barker *et al.*, 2009). Expansion of the crypt base population leads to an upward shift in the proliferative compartment, which eventually occupies the entire crypt axis (Boman *et al.*, 2004). These aberrant crypts (monocryptal adenomas) therefore represent the earliest state of neoplasia in the colon (Woda *et al.*, 1977) and increasing stem cell number initiates a process of crypt duplication (fission) resulting in clusters of enlarged abnormal crypts (Greaves *et al.*, 2006; Preston *et al.*, 2003).

These aberrant crypt foci (ACF) are frequently observed in individuals predisposed to CRC and in rodents exposed to colon-specific carcinogens (Whiteley *et al.*, 1996). They are characterised by an increase in crypt size, but also a thickened epithelium and an altered lumenal opening. Given that the abnormal cells occupying ACF represent increased proliferative potential, these lesions also demonstrate a notable decrease in cellular differentiation, most evident by reduced goblet cell number (Pretlow *et al.*, 2003). Therefore, a large proportion of ACF compose of cells with an abnormally differentiated phenotype, termed ‘dysplasia’ (Siu *et al.*, 1997), while the increasing outgrowth of these abnormal cells leads to the formation of ‘neoplasia’. The dysplastic crypts found in ACF thus represent the precursors of intestinal adenomas (polyps) and may increase in size to form micro- then macro-adenomas (Takayama *et al.*, 1998).

Intestinal adenomas can be defined as masses of dysplastic epithelial cells with uncontrolled cell division. These often protrude above the normal mucosa and can be classified on the basis of size, histological presentation and degree of dysplasia. The vast majority of adenomas are less than 1cm in diameter but intermediate and large adenomas may exceed 2-3cm (Houlston, 2001). Varying degrees of distinctive crypt-like or villi-like histological architecture are usually present and enable categorisation as either tubular, tubulovillous (intermediate) or villous adenomas. Adenomas often present with heterogenous grades of dysplasia, with areas of severe dysplasia being considered a marker for the increased risk of malignant progression. In the past, adenomas

composed entirely of highly dysplastic cells without evidence of invasion have been referred to as 'carcinoma in situ' due to the propensity of these tissues to become malignant. However, an adenoma is only truly considered malignant (an adenocarcinoma) once cells pass through the muscularis mucosae and infiltrate into the submucosa (*FIGURE 1-3*). Although nearly all adenocarcinomas are derived from benign adenomas this does not mean that all adenomas will progress to malignancy. In fact, severe dysplasia is found in 5-10% of intestinal adenomas and only about 5% of all adenomas are believed to progress to malignancy (Midgley and Kerr, 1999).

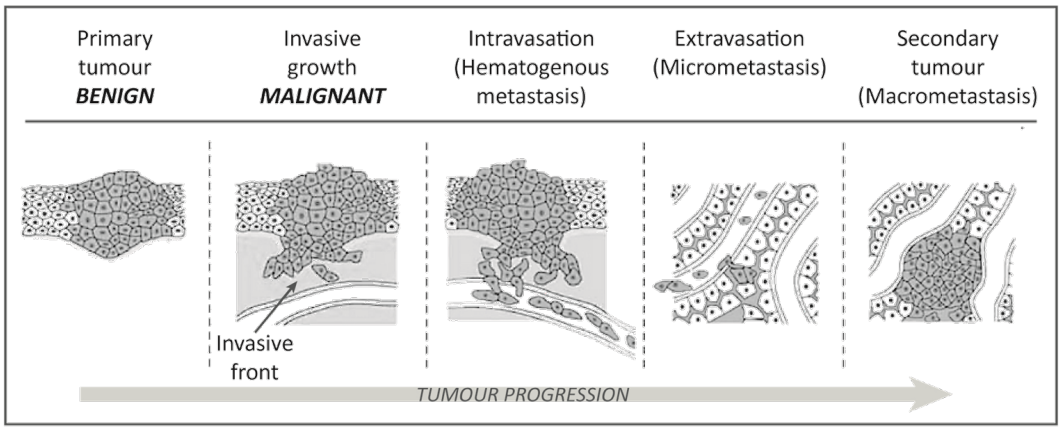


Figure 1-3| *The stepwise process of colorectal tumour progression.* Colorectal tumours frequently progress along a well defined route of histological alteration from aberrant crypt foci to benign adenoma. With increasing intrinsic and extrinsic alterations these benign tumours progress to form malignant colorectal adenocarcinomas. Invasive tumour cells detach from the 1° tumour and spread into the surrounding tissue before entering the associated blood (and lymph) supply. Following intravasation into the blood supply, colorectal cancer cells then disseminate around the body before adhering and undergoing extravasation to form a metastases (2° tumour) at distant sites around the body.

Adenocarcinomas often resemble the normal epithelium less closely than their adenoma counterparts owing to increased molecular alterations and can be roughly categorised as well, moderately or poorly differentiated (tumour grade). Well differentiated malignancies retain recognisable features of crypt formation, but this is progressively lost in moderately and poorly differentiated tissues. Poorly differentiated adenocarcinomas are commonly associated with a more aggressive phenotype and poorer patient prognosis (Purdie and Piris, 2000) and tissue de-differentiation has been shown to be a major feature of tumour cells at the invasive front (Brabletz *et al.*, 2001). Following invasion, tumour progression continues in a stepwise manner, leading to entry into the lymph or blood supply and dissemination to form distant metastases (reviewed in Woodhouse *et al.*, 1997). Not every cell in an adenocarcinoma has the ability to invade or metastasise, which instead arises from a significant change in cell phenotype and gradual clonal selection (reviewed in Grunert *et al.*, 2003). Colorectal adenocarcinomas therefore

present with differing degrees of invasion and metastatic potential, depending on the duration of malignancy and aggressiveness of the cancer phenotype (Fidler, 1991).

1.2 A MOLECULAR BASIS FOR COLORECTAL CARCINOGENESIS

1.2.1 THE GENETIC BASIS OF COLORECTAL CANCER

CRC is often referred to as an 'environmental disease' owing to its sporadic origin in the vast majority of patients (Boyle and Langman, 2000). It has long been understood, however, that a genetic (Vogelstein and Kinzler, 2004) and epigenetic (Feinberg *et al.*, 2006) basis underlie the progressive transition of a normal epithelial cell to invasive and metastatic CRC. Cells may acquire successive mutations in either oncogenes, with dominant gain of function, or tumour suppressor genes, with recessive loss of function (reviewed in Weinberg, 1995); both affording the cell a growth and survival advantage over surrounding normal tissue. It can be rationalised that loss of tumour suppressor function is equally as important as oncogenic gain of function, since both may have the same functional outcome. Moreover, a single genetic defect is not normally sufficient for tumorigenesis and it has been shown that at least two cooperating genetic events are often required for aberrant growth of truly normal cells (Land *et al.*, 1983). Subsequent conversion into malignant cancer cells requires alteration of multiple genes and cell processes in order to evade the homeostatic mechanisms constraining normal growth, morphology and localisation (reviewed in Hahn and Weinberg, 2002). In fact, kinetic analyses have shown that between four and seven rate-limiting events must occur during the carcinogenesis of most tissues (Renan, 1993) and a plethora of data exists to suggest that human cancer is a multi-stage genetic disease (Armitage and Doll, 1954). Tumour initiation is thought to require deregulation of a key 'gate-keeping' pathway with additional genetic hits conferring a growth advantage to tumour cells, leading to successive rounds of clonal outgrowth. The sequence of histological changes during colorectal carcinogenesis is accompanied by an increasingly well defined sequence of genetic alterations where the same changes are repeatedly observed in colorectal tumours from different patients (Fearon and Vogelstein, 1990).

1.2.2 LESSONS FROM HEREDITARY COLORECTAL CANCER

Although the vast majority of colorectal tumours arise in a sporadic manner, presentation in younger individuals might be attributed to one of a number of dominantly inherited genetic conditions, (reviewed in Kinzler and Vogelstein, 1996). Patients with a familial risk account for approximately 20% of all CRC cases and about 5-10% are inherited in an autosomal dominant

fashion. The two major forms of hereditary CRC are Familial Adenomatous Polyposis (FAP; *OMIM #175100*) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC; *OMIM #120435*), which together provide important clues towards the molecular basis governing both colorectal tumour initiation and progression.

FAP is an autosomal dominant disorder affecting about 1 in 7000 people and results in the dramatic development of multiple (hundreds to thousands) adenomatous colorectal polyps during the second and third decades of life. When left untreated, full CRC development is almost inevitable in the third to the fifth decade of life (Haggitt and Reid, 1986). A notable feature in the colonic epithelium of FAP patients is an abundance of irregular levels of crypt fission and ACF formation (Bjerknes *et al.*, 1997; Wasan *et al.*, 1998). This is suggestive of an aberrant increase in ISC number and a shift from asymmetrical to symmetrical ISC division (Boman *et al.*, 2004). FAP has long been known to occur via the inheritance of one defective allele of the *adenomatous polyposis coli (APC)* tumour suppressor gene (Groden *et al.*, 1991; Joslyn *et al.*, 1991; Kinzler *et al.*, 1991), where mutation results in the loss or truncation of the 300kDa APC protein product (Fodde, 2002). Somatic inactivation of the second allele leads to loss of heterozygosity (LOH) (Ichii *et al.*, 1992) in a manner consistent with Knudson's classical two hit hypothesis (Knudson, 1971). It is therefore believed that the *APC* gene provides an essential 'gatekeeper' function in the colonic epithelium and its loss significantly increases the degree of tumour initiation within FAP patients. Interestingly, FAP patients do not develop uniform clinical features and significant variation even exists between patients with identical *APC* aberrations. This can be explained in part since the severity of disease is highly dependent on the site of mutation, leading to varying degrees of truncation and functional inactivation (Nagase *et al.*, 1992; Scott *et al.*, 1995). In line with the multi-stage theory of tumour progression, additional genetic alterations collaborate with *APC* mutations and are necessary for the continued progression of these tumours (Fearon and Vogelstein, 1990). These further genetic alterations are therefore a key determinant in the heterogenous extent of colorectal tumour progression in these patients, highlighting that continued genetic instability is a major factor in the progression of colorectal carcinogenesis.

In contrast to FAP, HNPCC is an autosomal dominant predisposition syndrome that does not result in excessive polyposis of the colonic epithelium but instead demonstrates normal levels of tumour formation followed by greatly accelerated tumour progression to malignancy (reviewed in Lynch and de la Chapelle, 1999). In addition to a clinical association with CRC, HNPCC patients also demonstrate increased development of other tumours, including those of the endometrium,

ovary, hepatobiliary tract and skin, suggesting a more widespread mutational profile. HNPCC is now known to occur as a result of mutation in any number of DNA mismatch repair (MMR) genes (reviewed in Boland, 2000), the protein products of which are essential for the surveillance and elimination of replicative errors that occur during DNA strand duplication (Kobayashi *et al.*, 2005). The vast majority of HNPCCs can be attributed to mutation in the MMR genes *hMSH2* (60% of cases) and *hHLH1* (Bronner *et al.*, 1994; Leach *et al.*, 1993; Papadopoulos *et al.*, 1994). The frame-reading mistakes that arise contribute greatly to an elevated gene mutation rate and micro-satellite instability (MSI), an indicator of genetic instability as a whole. HNPCC therefore results in cells with a 'mutator phenotype' and the mutation rates in patients with a MMR deficiency are two to three times higher than in normal cells (Eshleman *et al.*, 1995). Therefore, the increased rate of malignant progression observed in HNPCC patients arises through the greater propensity for epithelial cells in these patients to acquire the additional genetic aberrations required for the successive stages of colorectal tumour progression.

Although both FAP and HNPCC predispose patients to the development of CRC, they present very different stage-specific clinical outcomes. FAP can be defined by greatly increased tumour initiation but normal progression (tumour initiation disorder), while HNPCC is classically associated with normal initiation but greatly accelerated tumour progression (tumour progression disorder) (Kinzler and Vogelstein, 1996). They also add further weight to a multi-step genetic basis for CRC, implicating *APC* mutation as a major tumour initiating feature alongside other genetic changes necessary for tumour progression.

1.2.3 APC MUTATION RESULTS IN ABERRANT WNT PATHWAY ACTIVATION

The tumour suppressor role of the APC protein is mediated largely through its action as an essential regulatory component of the Wnt (Wingless in *Drosophila*) developmental signalling pathway (reviewed in Hlsken and Behrens, 2000). APC acts to negatively regulate the Wnt pathway by associating with and promoting degradation of the key effector, β-catenin (Munemitsu *et al.*, 1995; Rubinfeld *et al.*, 1993). APC forms part of a multi-protein complex termed the β-catenin 'destruction complex' in combination with two serine/threonine kinases Glycogen synthase kinase-3β (GSK-3β) and casein-kinase-1 (CK1), plus the scaffold proteins Axin (Axin1) and Conductin (Axin2). Wild-type APC possesses several β-catenin binding domains and recruits free cytosolic β-catenin to the destruction complex where it is sequentially phosphorylated by both CK1 and GSK-3β (Liu *et al.*, 2002; van Noort *et al.*, 2002). Phosphorylated β-catenin is then targeted for proteasomal degradation via the action of the E3 ubiquitin ligase β-

TrCP (β-transducin repeat containing protein) (Winston *et al.*, 1999), ensuring that basal β-catenin levels are kept low in the cytoplasm (Hart *et al.*, 1998) (*FIGURE 1-4A*).

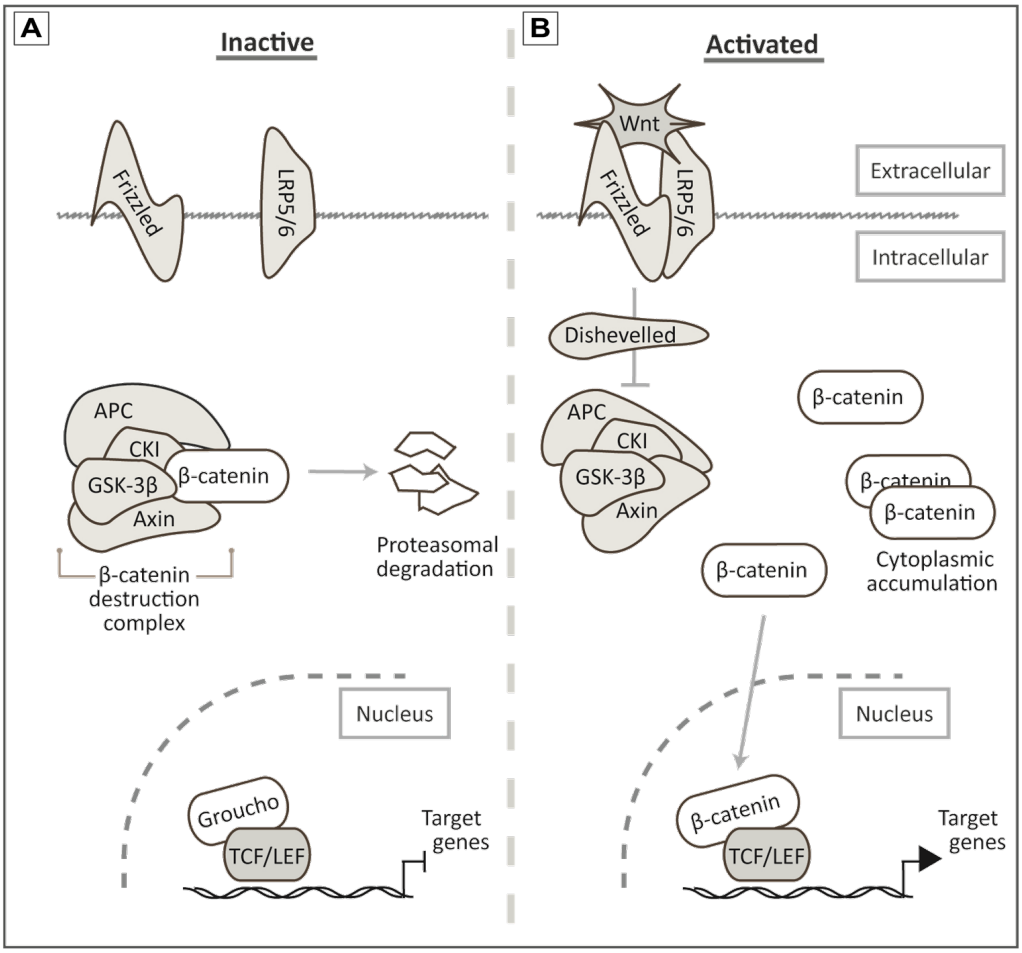


Figure 1-4| *The Wnt signalling pathway*. A schematic representation of the Wnt pathway. **[A]** In the absence of mitogenic Wnt stimulation, cytoplasmic levels of β-catenin are kept at a minimum by the action of the β-catenin 'destruction complex'. This multi-protein functional complex is composed of APC, the two serine/threonine kinases Glycogen synthase kinase-3β (GSK-3β) and casein-kinase-1 (CK1), plus the scaffold proteins Axin (Axin1) and Conductin (Axin2). Wild type APC recruits free cytoplasmic β-catenin to the complex where it is sequentially phosphorylated by CK1 and GSK-3β, which targets it for ubiquitin-mediated destruction in the proteasome. **[B]** Stimulation of the transmembrane Frizzled/LRP receptor complex by the diffusible Wnt ligand results in inactivation of the 'destruction complex' via the protein Dishevelled, leading to enhanced stabilisation of cytoplasmic β-catenin. Accumulating β-catenin translocates to the nucleus where it forms a transcriptional activation complex with the LEF/TCF family of transcription factors to promote downstream Wnt pathway activation.

The Wnt family of secreted glycoproteins (about 20 in mammals) act as diffusible ligands that activate signalling via engagement with the 7-span transmembrane Frizzled receptor (FZD1-8) in conjunction with LRP5/6 (low density lipoprotein receptor-related protein 5/6), leading to inactivation of the destruction complex through the downstream action of the intracellular

Dishevelled (Dsh) protein (*FIGURE 1-4B*) (reviewed in Hlsken and Behrens, 2000). Stabilised β-catenin accumulates in the cytoplasm before rapidly translocating to the nucleus, where it forms a transcriptional activation complex with the LEF/TCF (Lymphoid enhancer factor/T-cell factor) family of transcription factors (Behrens *et al.*, 1996; Huber *et al.*, 1996; Korinek *et al.*, 1998b). LEF/TCF remains associated with specific Wnt target genes through the presence of highly conserved optimal binding motifs located in the promoter or enhancer regions (Hallikas *et al.*, 2006; van de Wetering *et al.*, 1997). In the absence of nuclear β-catenin, these LEF/TCF factors recruit transcriptional co-repressors, such as Groucho (Cavallo *et al.*, 1998; Roose *et al.*, 1998). β-catenin acts as a transcriptional co-activator, displacing co-repressor proteins from the LEF/TCF complex and mediating a tightly controlled process of activation vs. a default of active repression. The β-catenin-LEF/TCF complex is now known to target a wide spectrum of genes, although *Cyclin D1* (Tetsu and McCormick, 1999) and *c-Myc* (He *et al.*, 1998) remain the best characterised. DNA array technology has made it possible to identify Wnt target genes on a genome-wide scale, usually following pathway manipulation (Gaspar *et al.*, 2008; Hallikas *et al.*, 2006; Sansom *et al.*, 2006; van de Wetering *et al.*, 2002; Van der Flier *et al.*, 2007) and a comprehensive list can now be found at the Wnt homepage (http://www.stanford.edu/%7ernusse/wntwindow.html) maintained by Roeland Nusse.

Active Wnt signalling is crucial for the promotion of growth and tumorigenesis in many tissues, in part through its potent downstream effects on the cell cycle (reviewed in Ilyas, 2005). Cyclin D1 is a key cell cycle regulatory protein that is directly involved in initiating new rounds of cellular division (G1-S transition) (Ewen *et al.*, 1993). c-Myc is also known to promote the G1-S transition, but indirectly through the transcriptional repression of an important cell cycle inhibitory protein p21 (encoded by *CDKN1A*) (van de Wetering *et al.*, 2002). One of the main tumour suppressing abilities of APC therefore resides in its capacity to down-regulate β-catenin levels and suppress the expression of these Wnt target genes. APC truncation is known to result in impaired action through the loss of the axin/conductin binding motifs alongside some or all of its β-catenin binding domains, depending on the degree of truncation (Morin *et al.*, 1997). APC also possesses further tumour suppressor functions through its ability to shuttle β-catenin out of the nucleus, which may also be lost in APC mutant cells (Rosin-Arbesfeld *et al.*, 2003). Different *APC* mutations therefore result in varying degrees of β-catenin stabilisation and nuclear localisation, but in general cause cells to become ligand-independent and constitutively activated (Korinek *et al.*, 1997).

1.2.4 ANIMAL MODELS OF INTESTINAL TUMORIGENESIS

In order to unravel the functional significance of *APC* mutation in human CRC and to generate a system to functionally study intestinal neoplasia, a number of mouse models have been created over the past two decades (reviewed in: Clarke, 2006; Kucherlapati *et al.*, 2001). Primarily, a mouse strain demonstrating a hereditary form of multiple intestinal neoplasia (Min) was generated by mutagenesis studies (Moser *et al.*, 1990). It was subsequently shown that these mice harbour a heterozygous mutation in the mouse *Apc* gene ($Apc^{+/Min}$) that results in a chain termination mutation at codon 850 and production of a truncated form of the protein (Su *et al.*, 1992). These mice therefore represent the murine counterpart of hereditary FAP in humans and spontaneously develop multiple intestinal tumours at around 6 months of age. Analogous to FAP patients, the crypts in these $Apc^{+/Min}$ mice undergo fission at a much greater rate than wild-type mice (Wasan *et al.*, 1998). Several different *Apc* knock-out mouse models have since been generated that harbour distinct *Apc* mutations and are representational of the different intestinal tumour formation patterns and disease severity found in the human condition (Fodde *et al.*, 1994; Oshima *et al.*, 1997; Smits *et al.*, 1999). Using these mouse models it was further shown that, irrespective of mutation site, the mutant Apc protein does not act in a dominant negative fashion (Oshima *et al.*, 1997) but instead loss of tumour suppressor function results from a second somatic mutation within the second allele (Luongo *et al.*, 1994).

At the molecular level, the various murine systems are good models for human adenoma formation and have provided invaluable information for the genetic basis of human CRC. However, it should be noted that murine studies are not completely analogous to the human and one important distinction remains: adenoma formation in mice preferentially localises in the SI (Boivin *et al.*, 2003) whereas in humans they are found almost exclusively in the colon (LI) in both sporadic and familial cases. Many studies of intestinal neoplasia have therefore been performed in the SI of animal models, although the exact reason for this disparity between the human and murine scenario remains unknown and may be attributable to differences in environmental factors and the preferential site for LOH (Tanaka, 2009). Interestingly, introduction of an inducible homozygous mutation into adult mice results in rapid adenoma development in both the small and large intestine, providing functional proof that *Apc* mutation is a key tumour initiating event in the colorectal epithelium as well as the SI (Shibata *et al.*, 1997). Similarly, inducible systems targeting Apc knock-out in the LI have specifically demonstrated that neoplasia occurs readily in the colonic epithelium under these conditions (Hinoi *et al.*, 2007). Most importantly, analysis using the various animal models outlined above has provided invaluable

functional evidence that Apc has an essential 'gatekeeper' function and its mutation is a critical early event sufficient to initiate tumorigenesis in the epithelium of the intestine.

1.2.5 WNT ACTIVATION IS A CAUSATIVE FACTOR IN SPORADIC COLORECTAL TUMOURS

Although familial conditions such as FAP and HNPCC constitute only a small number of CRC cases, the key molecular changes identified in these are known to be essential features for sporadic (non-familial) CRC. Apart from the number and age of onset, colorectal adenomas in FAP patients do not show any differences when compared to common sporadic adenomas and following the identification of germline *APC* mutations in FAP patients, it was found that similar somatically acquired mutations are also highly prevalent in sporadic colorectal tumours. It was shown that approximately 85% of sporadic CRC cases show inactivation of both copies of the *APC* gene and its inactivation is observed in ACF, the earliest dysplastic lesions that can be examined in humans, suggesting an APC mutation is one of the earliest steps in colorectal tumorigenesis (Jen *et al.*, 1994; Miyoshi *et al.*, 1992; Powell *et al.*, 1992). Despite this, alterations to *APC* are not an absolute requirement of colorectal tumorigenesis and tumours possessing intact *APC* are often found to carry activating mutations in the β-catenin encoding gene *CTNNB1* (Ilyas *et al.*, 1997; Morin *et al.*, 1997), or in *AXIN2* (Lammi *et al.*, 2004; Liu *et al.*, 2000) instead. Stabilising *CTNNB1* mutations are found in about 7% of sporadic human colon adenocarcinomas and commonly delete residues critical for CK1 and GSK-3β-mediated phosphorylation (Iwao *et al.*, 1998; Morin *et al.*, 1997; Sparks *et al.*, 1998).

The functional outcome of these different genetic lesions is the same, resulting in increased stabilisation and nuclear accumulation of the β-catenin protein. It can therefore be assumed that it is the consequent aberrant activation of the Wnt target gene program and not the specific site of mutation that is important for driving the formation of benign intestinal adenomas (Korinek *et al.*, 1997; Morin *et al.*, 1997; van de Wetering *et al.*, 2002). In recent years it has been shown, through sequencing analysis as part of the 'cancer genome project', that despite similarities in histogenesis, very little overlap in candidate genetic mutations is observed between different colorectal tumour tissues (Sjoblom *et al.*, 2006), suggesting that a complex mutational landscape is instead associated with tumour progression to malignancy. Although the cohort of mutations may differ extensively between individual tumours, it is clear that alterations to seemingly disparate genes may in fact yield functionally equivalent outcomes by deregulating the same molecular pathway. It is therefore believed that all cancers actually arise and progress by the deregulation of a relatively small number of common pathways and/or processes.

1.2.6 A MOLECULAR BASIS FOR COLORECTAL TUMOUR PROGRESSION

In colorectal cancer particularly, a number of important cellular signalling features are found to be frequently deregulated in tumours of both sporadic and familial origin and, in addition to *APC* (residing on chromosome 5q), deregulation of genes located at 17p and 18q are frequently altered during colorectal tumour development and progression to malignancy (Vogelstein *et al.*, 1988). Furthermore, these alterations are also generally seen to arise in a highly stage-specific and preferential manner, consistent with a multi-hit hypothesis of colorectal carcinogenesis (Fearon and Vogelstein, 1990) (*FIGURE 1-5*).

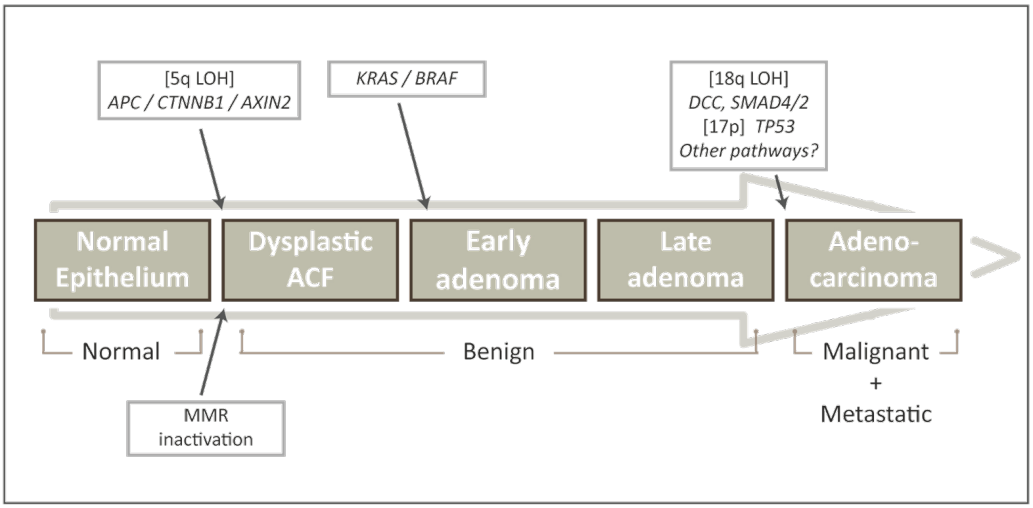


Figure 1-5| *A genetic basis for colorectal carcinogenesis.* A schematic representation of the common genetic alterations observed during the formation of colorectal cancers. Following inactivation of the *APC* tumour suppressor gene on chromosome 5q, colorectal tumours frequently proceed through a series of progressive genetic alterations in additional oncogenes and/or tumour suppressors (found on chromosomes 17p and 18q). While *RAS* mutations often occur at an early stage in colorectal carcinogenesis, deletions at 17p and 18q (or functionally equivalent mutations) usually take place at a later stage and may be associated with adoption of the malignant phenotype.

The RAS proteins of the RAS-MAPK (RAS-mitogen-activated kinase) pathway are a large superfamily of GTPases and the genes encoding *HRAS*, *KRAS* and *NRAS* frequently acquire activating mutations in a large proportion of cancers (Bos, 1989). Of these, *KRAS* is most frequently mutated and it has been shown that approximately 50% of colorectal tumours harbour mutations in *KRAS* (Bos *et al.*, 1987; Forrester *et al.*, 1987), while another 10-20% harbour mutations in the downstream kinase, *BRAF* (Davies *et al.*, 2002). Mutations may also occur in a number of other downstream pathway components and, like *KRAS* and *BRAF*, the net result of these seemingly distinct mutations is equivalent, resulting in constitutive pathway activation (reviewed in Sebolt-Leopold and Herrera, 2004). RAS-MAPK deregulation appears to

occur at a later stage than *APC,* since RAS-MAPK associated mutations occur in 58% of human adenomas larger than 1cm but are found in only 9% of smaller adenomas (Vogelstein *et al.*, 1988) and are not observed in dysplatic neoplasia without the presence of *APC* mutations (Jen *et al.*, 1994). Similarly, compound *Apc* and *Kras* mutations in mice increases the size, number and invasiveness of tumours arising in the intestinal epithelium but *Kras* mutations alone do not alter normal intestinal homeostasis (Sansom *et al.*, 2006).

The p53 protein (encoded by the *TP53* tumour suppressor gene, chromosome 17p) is an essential cell sensor of DNA damage that can potently mediate cell cycle arrest and facilitate DNA repair during replication (reviewed in Vousden and Lane, 2007). p53 has been dubbed as the 'guardian of the genome' and hence its role has pivotal consequences for both the accumulation of mutations and tumour progression (Lane, 1992). The p53 protein is frequently lost in many human cancers and may occur by mutations in *TP53* itself (60% of cases) or by loss of cell signalling mechanisms upstream and downstream of wild-type p53 expression (Bourdon, 2007). Alterations to p53 are considered to be a major feature of colorectal carcinogenesis and are observed in approximately 75% of CRC; however, these alterations are rarely detectable in colorectal adenomas and instead occur during the later stages of colorectal carcinogenesis (Baker *et al.*, 1990). Loss of p53 function is therefore considered to be a major factor in the malignant progression of colorectal tumours but, in support of a stage-specific role, patients with germline *TP53* mutations are not predisposed to developing intestinal tumours (Garber *et al.*, 1991).

Other late-stage alterations are frequently observed at the chromosome 18q locus (Vogelstein *et al.*, 1988). A candidate tumour suppressor gene, termed '*deleted in colon cancer*' (*DCC*), was identified at 18q21 and several point mutations in *DCC* have now been identified in CRC (Chan *et al.*, 1996; Keino-Masu *et al.*, 1996). Interestingly, loss of a second tumour suppressor gene was also identified at chromosome 18q and was subsequently identified as *SMAD4*, an essential co-factor of transforming growth factor-β (TGF-β) superfamily signalling, which includes TGF-β, bone morphogenic protein (BMP) and activin pathways (reviewed in Shi and Massague, 2003). These factors have been shown to be essential mediators of differentiation in the intestinal epithelium (Avery *et al.*, 1993; Barnard *et al.*, 1989; Hardwick *et al.*, 2004; He *et al.*, 2004) and most colorectal cancers harbour mutations in at least one component of the TGF-β pathway such as the receptor *TGF-βR2* (Markowitz *et al.*, 1995), *SMAD2* and *SMAD4* (Eppert *et al.*, 1996; Thiagalingam *et al.*, 1996). In agreement with the promotion of late stage carcinogenesis, $Apc^{+/-}$

/*Smad4*$^{+/-}$ compound heterozygous mice have been shown to demonstrate intestinal tumours with increased malignant potential, compared to *Apc*$^{+/-}$ mutation alone (Takaku *et al.*, 1998).

From these studies it is clear that despite a diverse array of genetic mutations and a complex genetic landscape, the functionally deregulated features of CRC may be limited to only a handful of common cellular processes, which arise in a stage- and context-dependent fashion. It is increasingly clear that normal post-natal tissues rely on mechanisms common to their development in the embryo and therefore embryogenic (developmental) signalling pathways feature heavily in both homeostasis and neoplasia (Kelleher *et al.*, 2006). In-depth analysis of intestinal homeostasis in particular has revealed an extensive requirement for developmental signalling and has been the subject of several recent reviews (Crosnier *et al.*, 2006; Sancho *et al.*, 2004; Scoville *et al.*, 2008; van den Brink and Offerhaus, 2007). Although some of these pathways, including Wnt and TGF-β, are known to be commonly mutated in colorectal carcinogenesis, predicted roles for others are only just beginning to be described.

1.3 THE MORPHOGENETIC CODE IN HOMEOSTASIS AND CANCER

1.3.1 EMBRYOGENIC PATHWAYS PERSIST IN THE ADULT INTESTINAL EPITHELIUM

During embryonic morphogenesis, cellular phenotype is most heavily governed by the relative location of a cell within a developing tissue or embryo (Lewis, 2008). The mechanisms underlying this 'positional context' are indispensible for correct functional morphogenesis and, as such, have remained the focus of extensive research for well over a century (Wolpert, 1996). From these studies it became apparent that embryogenesis is orchestrated by only a handful of cellular pathways that are able to mediate a hugely complex and diverse array of events through combinatorial control and reiterative, context-dependent modes of action. In addition to Wnt, the other major developmental signalling pathways in mammals are Hedgehog, TGFβ, BMP, Phosphoinositide 3-kinase (PI3K), Ephrins and Notch, all of which have emerged as master regulators of position-dependent cell fate in the embryo and are highly conserved from flies through to man. In most cases, these pathways largely dictate cell position through the 'morphogenic' action of their ligands, which readily diffuse to form tightly controlled and localised gradients within a tissue (Cinquin, 2006; Lawrence and Struhl, 1996). Cell fate is thereby determined in a concentration-dependent manner, where the synergistic and antagonistic action of the different morphogens culminates in a 'morphogenetic code', dictating both a positional and a phenotypic context to a cell within an embryo (Hogan, 1999).

It is widely accepted that these same developmental features persist throughout life to maintain correct histological and functional homeostasis in mature post-natal tissues. This tightly controlled process is most evident in the highly ordered, rapidly renewing tissues of the body, as exemplified by the epithelium of the intestinal tract (van den Brink and Offerhaus, 2007). As with embryonic morphogenesis, it has been shown that proliferation and differentiation in the adult intestine is not cell-autonomous but is heavily dependent on the position of a cell, from the base of the crypt to the lumenal surface (Hermiston *et al.*, 1996). This is in part regulated by autocrine signalling between cells of the epithelium but it is also heavily governed by reciprocal paracrine signalling between the epithelium and underlying stromal fibroblasts of the lamina propria (mesenchyme) (Kedinger *et al.*, 1986). It is now known that this interaction is inherently mediated by developmental signalling pathways and that a concerted morphogenetic code underlies normal intestinal homeostasis (Crosnier *et al.*, 2006) and intestinal tumorigenesis (van den Brink and Offerhaus, 2007) (*FIGURE 1-6*).

1.3.2 WNT SIGNALLING MEDIATES THE CRYPT PROGENITOR PHENOTYPE

Of these various pathways, it is clear that deregulated Wnt signalling constitutes one of the most significant features of intestinal tumorigenesis. As described previously, aberrant pathway activation by *APC* mutation has been shown to be the key initiating factor in the human colon and is observed in the vast majority of CRC cases (*SECTION 1.2.3, p14*). Various studies have shown that the prominence of the Wnt signalling pathway in intestinal tumorigenesis arises directly from its essential role in normal intestinal homeostasis, where it is a dominant force behind the proliferative crypt-progenitor phenotype (van de Wetering *et al.*, 2002).

Although Wnt expression patterns change during development of the intestinal epithelium (Kim *et al.*, 2007; Lickert *et al.*, 2001), expression in the mature intestinal epithelium remains largely restricted to the proliferative and stem cell region at the base of the crypt (Gregorieff *et al.*, 2005). Nuclear β-catenin, indicative of pathway activation, is also found predominantly at the base of the crypt and becomes increasingly membranous towards the lumenal surface (van de Wetering *et al.*, 2002). This is supported by gene expression profiles consistent with Wnt activity towards the base of the crypt in both the murine small intestine (Mariadason *et al.*, 2005; Stappenbeck *et al.*, 2003) and human colon (Kosinski *et al.*, 2007).

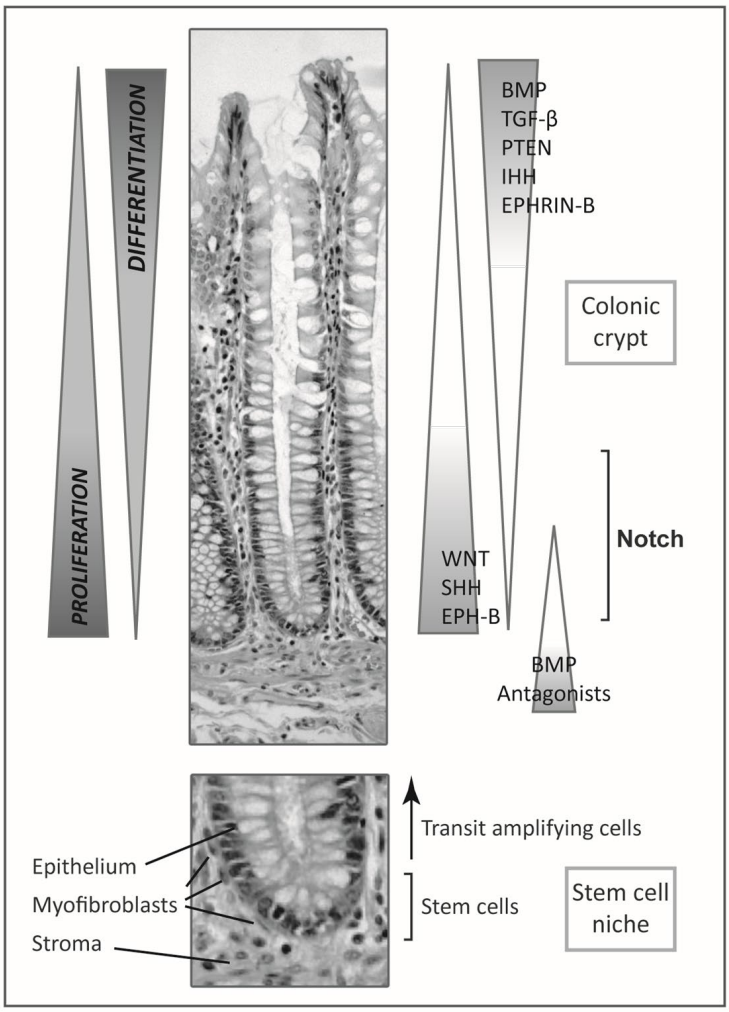


Figure 1-6| *Normal intestinal homeostasis is maintained by developmental signalling pathways*. The complex processes behind embryonic morphogenesis are coordinated by a handful of highly conserved developmental signalling pathways. These pathways are also known to persist in the mature post-natal tissues to maintain correct tissue structure and function (homeostasis). As with many tissues, development and homeostasis in the intestinal epithelium is coordinated by the Hedgehog, TGFβ, BMP, PI3K, Ephrins and Notch pathways. Many of these factors act as diffusible morphogenic gradients, mediating autocrine signalling within the epithelium and paracrine signalling with the underlying myofibroblasts. Disruption of these pathways therefore deregulates normal crypt physiology and homeostasis, contributing significantly to intestinal homeostasis.

The development of animal models that harbour various germline *Apc* mutations have proved invaluable in determining a fundamental role for aberrant Wnt signalling in intestinal over-growth and tumour formation (*SECTION 1.2.4, p17*). The use of conditional genetic models has enabled analysis of both the immediate and extended effects of biallelic *Apc* deletion in the adult murine intestine (Andreu *et al.*, 2005; Sansom *et al.*, 2004) and has therefore begun to rationalise our understanding of a physiological role for Wnt signalling in normal intestinal homeostasis. From these studies it can be seen that loss of *Apc* in the naïve adult epithelium results in rapid

accumulation of β-catenin in the nucleus and many cells enter S-phase, leading to expansion of the proliferative progenitor compartment along the entire length of the crypt (Andreu *et al.*, 2005; Sansom *et al.*, 2004). Coincidentally, cell movement within the crypt is also hindered and there is a general failure of cellular differentiation throughout, suggesting an increased affinity for the stem cell niche and expansion of the progenitor cells. Therefore, *Apc* mutation alone is sufficient to dramatically alter normal intestinal homeostasis and impose a pro-tumorigenic phenotype; however, similar results are also achieved using stabilised β-catenin (Harada *et al.*, 1999) and pathway agonists (Kim *et al.*, 2005), further underscoring the importance of pathway deregulation rather than specific genetic alterations during intestinal tumorigenesis.

Conversely, genetic manipulation in mouse models has also enabled similar studies following Wnt inhibition. In a seminal paper by Korinek *et al.* (1998a) it was shown that Wnt inactivation by knock-out of Tcf4 (*Tcf7/2*$^{-/-}$), the predominant intestinal LEF/TCF family member, results in mortality shortly after birth and a significant loss of all proliferative epithelial cells (Korinek *et al.*, 1997). Similar results have also been demonstrated following intestine-specific ablation of β-catenin (Fevr *et al.*, 2007; Ireland *et al.*, 2004), over-expression of the diffusible Wnt inhibitor protein Dkk1 (Dickkopf homologue 1) (Hoffman *et al.*, 2004; Kuhnert *et al.*, 2004; Pinto *et al.*, 2003) or knock-out of *c-Myc* (Muncan *et al.*, 2006). Moreover, it has since been shown that active Wnt signalling through β-catenin/Tcf4 inhibits intestinal differentiation and maintains the proliferative 'crypt progenitor' phenotype largely through its canonical effects on c-Myc and p21 expression (van de Wetering *et al.*, 2002). Wnt signalling therefore constitutes a master switch between proliferation and differentiation in the intestinal epithelium and appears to be a very highly conserved feature of this pathway (Lin *et al.*, 2008).

1.3.3 PI3K-AKT SIGNALLING ENHANCES STEM CELL SELF-RENEWAL

PI3K is an important component in the signal transduction cascade initiated by various receptor tyrosine kinase-specific growth factors (reviewed in Kapeller and Cantley, 1994). These include the fibroblast growth factor (FGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), which are all essential features of embryonic development and adult tissue maintenance. Stimulation of the PI3K pathway leads to activation of the AKT family of serine/threonine kinases and is negatively regulated by PTEN (phosphatase and tensin homolog). Constitutive PI3K pathway activation occurs in a large range of sporadic tumour types (reviewed in Cully *et al.*, 2006) and approximately 40% of all human CRCs show alterations to the PI3K/AKT pathway, mainly due to reduced or absent function of PTEN (Nassif *et al.*, 2004; Parsons *et al.*,

2005). Inherited mutations in *PTEN* have been linked to a CRC predisposing condition known as Cowden syndrome (Liaw *et al.*, 1997) (CS; *OMIM #158350*) and *PTEN* deletion leads to intestinal polyposis in mice (He *et al.*, 2007).

Predictably, several strong links exist between PI3K-AKT and Wnt signalling. Primarily, active AKT phosphorylates β-catenin and promotes its nuclear accumulation and transcriptional activity (He *et al.*, 2007; He *et al.*, 2004; Persad *et al.*, 2001), suggesting a significant role in maintenance of the crypt progenitor phenotype (He *et al.*, 2004). Conversely, PTEN hinders the nuclear localisation of β-catenin (Persad *et al.*, 2001) and is most strongly expressed towards the lumenal surface away from the progenitor pool (Kim *et al.*, 2002b). In addition to Wnt, the PI3K-AKT pathway also interacts with another key developmental pathway in the intestinal epithelium, the bone morphogenic protein (BMP) pathway, and it is now believed that integration of these three pathways together is essential for intestinal homeostasis (He *et al.*, 2004).

1.3.4 BMP SIGNALLING INHIBITS DE NOVO CRYPT FORMATION

Like other TGF-β superfamily members, activation of the BMP receptors leads to a subsequent phosphorylation and activation of SMAD effector proteins (reviewed in Shi and Massague, 2003). An essential role for BMP in intestinal homeostasis is highlighted by the identification of deregulatory *BMPR-1A* (Howe *et al.*, 2001) and *SMAD4* (Houlston *et al.*, 1998; Howe *et al.*, 1998) mutations in patients with the CRC predisposing condition, juvenile polyposis syndrome (JPS; *OMIM #174900*).

BMP signalling in the normal intestine is complex, since BMPs and their receptors are expressed in both the epithelium and stroma, suggesting that BMP signalling is a central mediator of bi-directional paracrine signalling between the two mucosal compartments (Auclair *et al.*, 2007; Haramis *et al.*, 2004; Hardwick *et al.*, 2004; He *et al.*, 2004; Kosinski *et al.*, 2007). The principal intestinal BMP ligands, BMP-2 and BMP-4, are predominantly expressed in the interconnecting stroma, including the sub-epithelial myofibroblasts adjacent to the epithelial crypt. In contrast to Wnt, BMP signals appear to be excluded from the proliferative compartment of the intestinal crypt since the main receptor, Bmpr1a, and actively phosphorylated SMADs are located predominantly in the upper crypt region of the murine intestine and human colon. Moreover, BMP antagonists such as Noggin and Gremlin are expressed in the sub-epithelial myofibroblasts surrounding the stem cell compartment and are believed to provide protection from the physiologically effects of any active signalling (He *et al.*, 2004; Kosinski *et al.*, 2007).

Importantly, inhibition of endogenous BMP signalling by *Bmpr1a* knock-out or forced expression of Noggin in the murine intestinal epithelium results in expansion of the progenitor population and ectopic crypt formation, phenocopying human patients with JPS (Haramis *et al.*, 2004; Hardwick *et al.*, 2004; He *et al.*, 2004). BMP is also known to cross-talk extensively with other pathways in a range of contexts (reviewed in Herpin and Cunningham, 2007) and it is believed that BMP signalling restricts stem cell number and prevents polyposis in the intestinal epithelium by restraining Wnt signalling to the base of the crypt (Haramis *et al.*, 2004; He *et al.*, 2004). This has been shown to occur, at least in part, through modulation of PI3K/AKT and altered stability of PTEN (He *et al.*, 2004; Waite and Eng, 2003). Nuclear β-catenin localisation is significantly increased following BMP inactivation and active Wnt signalling is a prominent feature of the resulting tumours (Haramis *et al.*, 2004; He *et al.*, 2004). Surprisingly, it has been proposed that active BMP signalling is an essential feature of stem cell self-renewal in the crypt base (He *et al.*, 2004). This reflects a possible context-dependent role for BMP signalling in the intestinal epithelium and from these studies it has been proposed that active BMP signalling in the mesenchyme maintains crypt morphogenesis, while signalling in the epithelium may mediate both stem cell self-renewal and differentiation (Auclair *et al.*, 2007).

1.3.5 THE TGF-β PATHWAY MEDIATES CELLULAR DIFFERENTIATION

In a similar fashion to BMP, the expression of TGF-β pathway components is observed in the epithelium and stroma, where it potentially mediates paracrine and autocrine signalling and is associated with the differentiated upper portion of the intestinal crypt epithelium, suggesting an important role in the control of growth and differentiation in the intestinal epithelium (Avery *et al.*, 1993; Barnard *et al.*, 1989; Winesett *et al.*, 1996). In addition to *SMAD2* and *SMAD4*, other TGF-β specific mutations, such as in *TGF-βR2*, are heavily implicated in colorectal carcinogenesis (*SECTION 1.6.2, p47*) (Grady *et al.*, 1999; Markowitz *et al.*, 1995). Interestingly, acquisition of these mutations and the loss of responsiveness to TGF-β-mediated growth inhibition during intestinal tumorigenesis appear to be highly stage-specific. In addition to the normal epithelium, it has been shown that TGF-β treatment also inhibits the growth of early colorectal adenoma cells but that adenocarcinomas have become refractory to its effect (Engle *et al.*, 1999; Manning *et al.*, 1991). In contrast to its tumour suppressor role, it is believed that TGF-β demonstrates a switch in functional outcome and aberrant signalling in late stage tumours that may actually promote their proliferation and invasion (Roman *et al.*, 2001), leading to a poorer prognosis in those patients with increased expression of TGF-β pathway components (Friedman *et al.*, 1995; Robson *et al.*, 1996).

1.3.6 CORRECT CRYPT ARCHITECTURE IS MAINTAINED BY HEDGEHOG SIGNALLING

The growth inhibitory effects of BMP/TGF-β in the intestinal epithelium are believed to be further complemented by the action of another key developmental morphogenic factor, the Hedgehog signalling pathway (Hh). Hh signalling comprises of the action of three different diffusible ligands, Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh), of which Shh and Ihh are known to play a fundamental role in intestinal homeostasis (reviewed in van den Brink, 2007). Contrary to many other interactions between the epithelium and mesenchyme, the direction of Hh signalling is relatively reversed. In animal models, the ligands Shh and Ihh are predominantly secreted by cells in the epithelium but their receptors and effectors are located in the sub-epithelial myofibroblasts (Bitgood and McMahon, 1995; Madison *et al.*, 2005; Nielsen *et al.*, 2004; Oniscu *et al.*, 2004; Ramalho-Santos *et al.*, 2000; van den Brink *et al.*, 2004).

Mutations in *Shh* and *Ihh* lead to a variety of gross developmental gut malformations (Madison *et al.*, 2005; Ramalho-Santos *et al.*, 2000; van den Brink *et al.*, 2004) and appear to be essential for correct mesoderm formation (Apelqvist *et al.*, 1997; Sukegawa *et al.*, 2000). In addition, Hh signalling is believed to limit precursor cell expansion, since Ihh is highly expressed in differentiated cells at the lumenal interface in both human and animal models, and is believed to restrict Wnt signalling to the crypt base (Jones *et al.*, 2006; Nielsen *et al.*, 2004; Oniscu *et al.*, 2004; van den Brink *et al.*, 2004). In contrast, Shh appears to localise to the crypt base and is believed to function in ISC maintenance (Nielsen *et al.*, 2004; Oniscu *et al.*, 2004). Accordingly, human gastrointestinal tumours are associated with increased Shh (Berman *et al.*, 2003; Oniscu *et al.*, 2004; Thayer *et al.*, 2003) and Ihh expression has been shown to be lost early in colorectal tumorigenesis (van den Brink *et al.*, 2004). Moreover, treatment of CRC cell lines with Hh inhibitors has been shown to result in increased differentiation and apoptosis (Qualtrough *et al.*, 2004), highlighting the Hh pathway as a potential therapeutic target in CRC, but also suggesting this pathway to be essential for growth of these tumour tissues.

1.3.7 AN ESSENTIAL ROLE FOR EPHRIN SIGNALLING IN COMPARTMENTALISATION

It is clear that the ordered transition of cell fate along the intestinal crypt axis is heavily dependent on positional cell context, as dictated by various epithelial-mesenchymal interactions and morphogenic gradients. As a key mediator of epithelial migration and crypt compartmentalisation, the Ephrin signalling pathway has also been shown to heavily impact on normal intestinal homeostasis (Holmberg *et al.*, 2006). Both Ephrin-B ligands and EphB receptors are membrane-bound proteins and require direct cell-cell contact for their interaction, leading to

mechanisms of cellular repulsion (Xu *et al.*, 1999). Interestingly, EphB3 expression is restricted to the CBCs and Paneth cells located at the crypt base with its expression decreasing in a gradient towards the lumenal surface (Batlle *et al.*, 2002; Holmberg *et al.*, 2006). In contrast, both the Ephrin-B1 and Ephrin-B2 ligands are expressed in an opposing gradient. Cells in the crypt therefore migrate upwards due to progressive changes in Ephrin-B/EphB expression along the crypt axis, while continued EphB3 expression in the CBC and Paneth cell compartment prevent these cells from migrating, maintaining the crypt base compartment (Batlle *et al.*, 2002). As a result, deletion of *EphB2* or *EphB3* in the murine intestine results in loss of normal epithelial compartmentalisation and both proliferative progenitors and Paneth cells become abnormally distributed along the length of the crypt (Batlle *et al.*, 2002).

It is known that the opposing gradients of EphB and Ephrin-B arise as a consequence of their respective positive and negative regulation by β-catenin/TCF, providing a further mechanism by which active Wnt signalling modulates intestinal proliferation and differentiation in the intestinal crypt (Batlle *et al.*, 2002). This explains the general loss of cell migration observed in the crypt following loss of *Apc* and is believed to extend the time that mutated cells are exposed to the stem cell/progenitor niche, promoting tumour initiation (Andreu *et al.*, 2005; Sansom *et al.*, 2006). However, continued Ephrin signalling restricts the spread of colorectal tumour cells and acts to suppress tumour progression (Cortina *et al.*, 2007). Therefore, loss of EphB2 receptor is a common feature of intestinal malignancies (Batlle *et al.*, 2005) and is known to be an indicator of poor prognosis in CRC patients (Jubb *et al.*, 2005; Lugli *et al.*, 2007).

1.3.8 AN INCREASING APPRECIATION FOR NOTCH SIGNALLING IN THE INTESTINE

The Notch signalling pathway also relies on transmembrane ligands and receptors, requiring direct cell-cell contact, and Notch pathway components are known to be expressed throughout the developing and adult intestine (Sander and Powell, 2004; Schroder and Gossler, 2002). Functional analysis has shown that active Notch signalling is essential for normal intestinal homeostasis (Fre *et al.*, 2005; Jensen *et al.*, 2000; van Es *et al.*, 2005). Furthermore, Notch has been strongly implicated in other human malignancies (reviewed in Koch and Radtke, 2007; & Leong and Karsan, 2006) and analysis in animal models suggests a role for the Notch pathway activation in intestinal tumorigenesis (van Es *et al.*, 2005). However, unlike many of the pathways outlined above, at present the expression and/or functional contribution of Notch signalling in CRC remains largely unknown and has become the focus of intensive research in this field.

1.4 THE NOTCH SIGNALLING PATHWAY

1.4.1 NOTCH PATHWAY - DISCOVERY AND COMPONENTS

Notch is a highly conserved developmental signalling pathway, which was originally discovered through *Drosophila* mutational studies and derives its name from the resultant 'notched' wing phenotype (Morgan, 1917). Subsequently, inactivation of this pathway in the *Drosophila* eye was shown to give rise to a 'neurogenic' phenotype, in which cells normally destined to become epidermal switch fate and undergo neural differentiation (Poulson, 1937). This was the first of many studies demonstrating an essential role for Notch in cell fate determination and it is now known that this is a highly conserved feature of the Notch pathway in many tissues, from flies through to man (Artavanis-Tsakonas *et al.*, 1999).

The *Notch* gene was cloned in the mid 1980s and found to encode a ~2500 amino acid (300kDa) single-pass transmembrane protein (Wharton *et al.*, 1985), the receptor of the Notch signalling pathway (reviewed in Baron, 2003). Following translation and entry into the trans-Golgi apparatus, the full length Notch protein is enzymatically cleaved by a Furin-like convertase at the first cleavage site, S1 (Logeat *et al.*, 1998). This produces the Notch transmembrane (N^{TM}) and extracellular (N^{EC}) subunits, which constitute the heterodimeric transmembrane receptor at the cell surface (Sanchez-Irizarry *et al.*, 2004). The N^{EC} portion contains 36 EGF-like repeats, which participate in ligand binding (Rebay *et al.*, 1991), followed by three cysteine rich Notch/Lin12 repeats (LN), which prevent signalling in the absence of ligand. The N^{TM} domain contains an RBP-Jκ/CBF1-associated module (RAM) domain, six ankyrin (ANK) repeats, two nuclear localisation signals (NLS), a transcription activation domain (TAD) and a PEST (proline-, glutamate-, serine-, threonine-rich) sequence (Wharton *et al.*, 1985). These distinct domains provide a platform for the modifications and specific regulatory events that coordinate Notch signalling. In *Drosophila*, the Notch receptor is activated by its interaction with either Serrate or Delta transmembrane ligands presented on adjacent cells (Rebay *et al.*, 1991), leading to downstream pathway activation. With the discovery of homologues in other animals, such as Lag2 in *C. elegans*, these ligands became collectively known as DSL (Delta/Serrate/LAG-2) (Lissemore and Starmer, 1999).

It is now known that the *Drosophila* Notch pathway is highly conserved and component homologues have since been shown to be essential in nearly all metazoans investigated (Artavanis-Tsakonas *et al.*, 1999; Greenwald, 1998). Mammals possess four Notch receptor homologues, Notch1-4 (N1-4) (del Amo *et al.*, 1993; Lardelli *et al.*, 1994; Uyttendaele *et al.*, 1996; Weinmaster *et al.*, 1992) and five ligands; three Delta-like ligands (Dll1, -3 and -4) (Bettenhausen

et al., 1995; Dunwoodie *et al.*, 1997; Shutter *et al.*, 2000) and two Serrate-like Jagged ligands (Jag1 and -2) (Lindsell *et al.*, 1995; Shawber *et al.*, 1996a) (*FIGURE 1-7*). Since both Notch ligands and receptors are transmembrane anchored proteins, cell-cell contact is an important pre-requisite to ligand interaction and pathway activation.

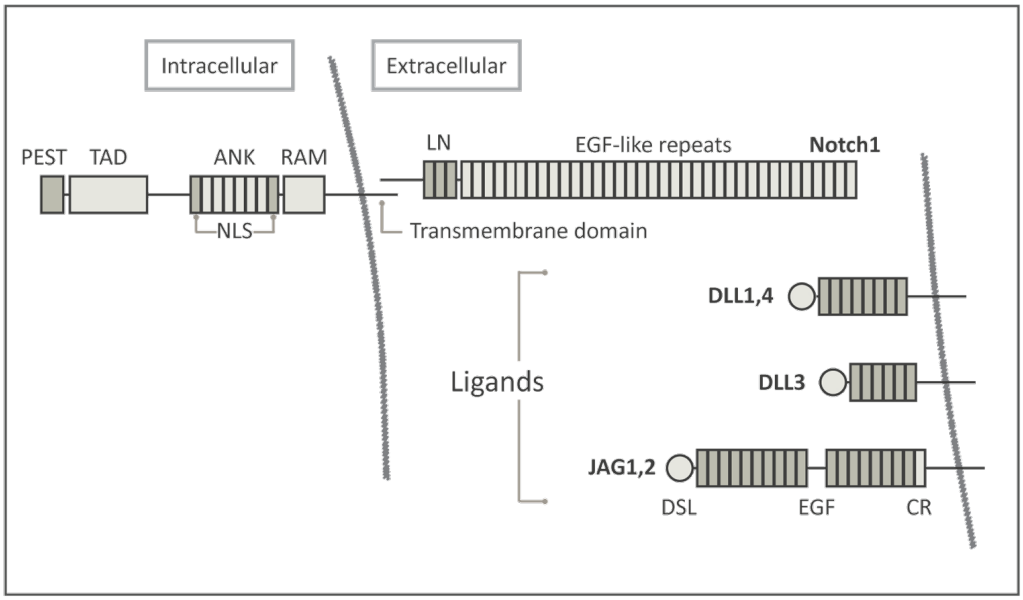


Figure 1-7| *Schematic representation of the Notch1 protein and associated ligands.* The four mammalian Notch receptors are similar in structure to Notch1, differing only in the number of EGF repeats (N1/N2 = 36, N3=34, N4=29) and TAD affinity (N1=strong, N2=weak, N3/N4=absent). The Delta-like (Dll1,3,4) and Jagged (Jag1/2) ligands each contain a Delta/Serrate/LAG-2 (DSL) domain, required for Notch transactivation or cis-inhibition, but differ in their number of EGF repeats and by the presence of a cysteine rich (CR) region close to the membrane in both Jag1 and Jag2 ligands (Lissemore and Starmer, 1999).

1.4.2 NOTCH PATHWAY ACTIVATION

The complex processes behind Notch pathway activation have been described extensively in several recent reviews (Bray, 2006; Fiuza and Arias, 2007; Tien *et al.*, 2009) but shall be summarised here. Primarily, receptor-ligand interaction results in a proteolytic cascade to produce a soluble intracellular form of N^{IC} (also known as NICD, ICD or NIC) from N^{TM}, which translocates to the nucleus and mediates transcriptional activation (*FIGURE 1-8*). This process is known as 'regulated intra-membrane proteolysis' (RIP), whereby the Notch receptor functions as a dual-address protein, acting firstly as a receptor and then as a nuclear transcription factor (Brown *et al.*, 2000). Notch ligand to receptor binding is extremely strong (Ahimou *et al.*, 2004) and the ligand plus the N^{EC} portion are known to be subsequently endocytosed by the ligand-expressing cell (Nichols *et al.*, 2007; Parks *et al.*, 2000). The second Notch cleavage site (S2) remains deeply embedded and protected prior to ligand stimulation and current theory suggests

that the force generated by N^{EC} endocytosis leads to a conformational change in the receptor (Gordon *et al.*, 2007; Nichols *et al.*, 2007), leading to S2 exposure (*FIGURE 1-8*). The resulting cleavage is mediated by ADAM/TACE enzymes (a disintegrin and metalloprotease/tumour necrosis factor-alpha converting enzyme) and releases a membrane-tethered form of the intracellular portion ($N^{\Delta E}$) (Brou *et al.*, 2000; Lieber *et al.*, 2002), prior to a final activating cleavage at S3. This S3 cleavage is mediated by the γ-secretase complex (De Strooper *et al.*, 1999; Song *et al.*, 1999; Struhl and Greenwald, 1999), which comprises of four subunits, presenilin (PS1), nicastrin (NCT), anterior pharynx defective (APH-1) and presenilin enhancer-2 (PEN-2). Cleavage at S3 thereby releases a soluble form of the intracellular portion, N^{IC} in *Drosophila* and $N1^{IC}$-$N4^{IC}$ in mammals.

Although N^{IC} contains a transcriptional activation domain (TAD), it has no DNA binding domain and must instead bind to a sequence-specific transcription factor, CSL (CBF1/RBP-Jκ in mammals, Su(H) in *Drosophila*, Lag-1 in *C. elegans*) (Henkel *et al.*, 1994). In the absence of Notch signalling, CSL acts to repress the transcription of immediate Notch target genes by binding DNA and recruiting co-repressors, such as histone deacetylase (HDAC) (Dou *et al.*, 1994; Kao *et al.*, 1998). N^{IC} displaces these co-repressors and converts CSL to a transcriptional activator, while simultaneously recruiting co-activators such as the nuclear protein Maml-1 (homologue of *Drosophila* Mastermind) (Fryer *et al.*, 2002; Kurooka and Honjo, 2000). Importantly, endogenous N^{IC} proteins are known to exert their transcription factor effects at very low nuclear concentrations (Conboy *et al.*, 2005; Schroeter *et al.*, 1998) and the mechanism of activation vs. a default of active repression enables extremely tight control of the resultant Notch target genes (Barolo and Posakony, 2002; Barolo *et al.*, 2002). Furthermore, transcriptional activity is also regulated by mechanisms of rapid N^{IC} degradation and, therefore, sustained Notch activation is essential for continued signalling (Fryer *et al.*, 2004).

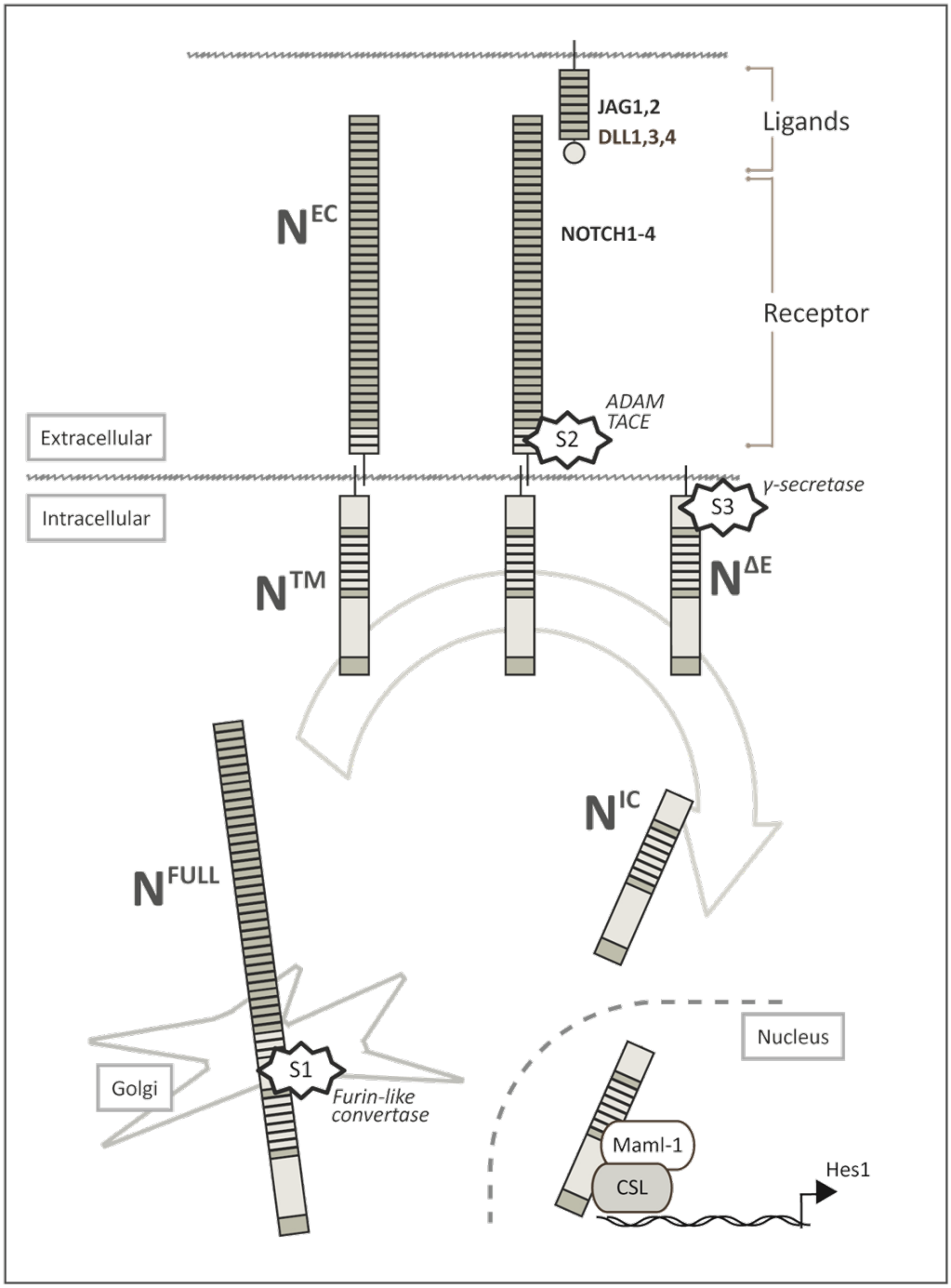


Figure 1-8| ***Processing and activation of the Notch signalling pathway***. As the full length Notch protein passes through the cell excretory system, it undergoes a proteolytic cleavage by a Furin convertase, at site S1, yielding the 180kDa extracellular and 120kDa transmembrane portion, which are calcium bonded to form the heterodimer receptor (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998). Receptor activation causes S2 cleavage to occur approximately 12 amino acids outside the transmembrane domain, which is strictly dependent on both ligand binding and the ADAM family of metalloproteases. S2 cleavage results in a membrane tethered form of Notch, $N^{\Delta E}$, which is then the substrate for the final cleavage at S3 by the γ-secretase complex, releasing the soluble intracellular portion (N^{IC} in *Drosophila*, $N1^{IC}$-$N4^{IC}$ in mammals) for translocation to the nucleus.

1.4.3 HES AND HERP GENES ARE IMMEDIATE TARGETS OF ACTIVE NOTCH SIGNALLING

The downstream effects of N^{IC} and CSL are not yet fully defined, but in mammals the genes for two distinct families of basic helix-loop-helix (bHLH) transcription factors, *Hes* (Homologues of *Hairy/Enhancer of Split* in *Drosophila*) (Sasai *et al.*, 1992) and the more recently discovered *Herp* (*Hes-related proteins*) (Iso *et al.*, 2001a) are identifiable as immediate transcriptional targets of active Notch signalling (reviewed in Iso *et al.*, 2003). To date, seven mammalian *Hes* members and three mammalian *Herp* members have been identified, of which the genes *Hes1, -5, -6 and -7*, plus all three *Herp* members appear to be targets of active Notch signalling (Iso *et al.*, 2003).

The bHLH family of proteins are important transcription factors and bind DNA as homodimers or heterodimers (Iso *et al.*, 2001b) to positively or negatively regulate the transcription of downstream target genes (Dawson *et al.*, 1995), including other bHLH transcription factors (Massari and Murre, 2000). In *Drosophila*, Hairy recruits the co-repressor Groucho and inhibits the transcription of genes involved in neuronal differentiation (Fisher and Caudy, 1998a; Fisher and Caudy, 1998b). The mammalian homologues of Groucho are the transducin-like Enhancer of split (TLE) proteins 1-4 (Stifani *et al.*, 1992), which are also known to be required for many mammalian developmental processes mediated by Notch signalling (reviewed in Chen and Courey, 2000), suggesting a similar mode of action. Although the canonical Notch pathway relies on a direct mechanism of target gene transactivation, it should be noted that increasing evidence exists for various 'non-canonical' Notch signalling mechanisms, including *Hes*/*Herp* and CSL-independent events (Martinez Arias *et al.*, 2002). Furthermore, it is becoming increasingly evident that the Notch receptors and downstream signalling events are heavily modulated, adding further levels of complexity to this once seemingly simple pathway.

1.4.4 SPECIFIC MODULATION OF THE NOTCH PATHWAY

In addition to the core pathway components, a number of modulatory factors have been identified that can positively or negatively influence Notch activation and operate primarily at the level of ligand-receptor interaction (Bray, 2006; Kadesch, 2004; Panin and Irvine, 1998). For example, Notch receptors are heavily modified by post-translational glycosylation, and elongation of *O*-linked fucose residues is mediated by *O*-fucosyltransferase 1 (*O*-fut1), as well as the Fringe family of proteins (reviewed in Haines and Irvine, 2003). Addition of the principal fucose residues by *O*-fut1 is essential for the generation of a functional receptor and its loss in both flies and mice results in severe Notch-related defects (Okajima *et al.*, 2003; Shi and Stanley, 2003). Following addition of this first residue, the carbohydrate chains are then extended by

other glycosyl-transferases, namely members of the Fringe family. The *Fringe* (*Fng*) gene was first identified in *Drosophila* through its profound effect on wing development (Irvine and Wieschaus, 1994) and the Fringe protein cell-autonomously modulates Notch receptor-ligand interactions by inhibiting Notch receptor activation by Serrate but potentiating its activation by Delta (Fleming *et al.*, 1997; Panin *et al.*, 1997). To date three mammalian fringe genes have been identified, *Lunatic fringe* (*Lfng*), *Manic fringe* (*Mfng*) and *Radical Fringe* (*Rfng*) (Cohen *et al.*, 1997; Johnston *et al.*, 1997; Moran *et al.*, 1999), of which the essential functions of *MFng* and *RFng* remain unknown. *LFng* function is known to be essential for proper somite segmentation during vertebrate development (Evrard *et al.*, 1998). Although the role of O-fucosylation and Fringe proteins in mammals is less well defined, it appears to be highly reminiscent of function in *Drosophila* and is believed to differentially bias activation of the Notch receptor by Delta-like ligands (Hicks *et al.*, 2000).

A number of post-activation modulators have also been discovered, of which the function of the Numb protein has been most well defined. Numb associates with N^{IC} and antagonises Notch signalling in a cell-autonomous fashion (Guo *et al.*, 1996; Spana and Doe, 1996; Zhong *et al.*, 1996) by interfering with the nuclear translocation of N^{IC} (Wakamatsu *et al.*, 1999) and promoting its degradation (McGill and McGlade, 2003). Importantly, asymmetric segregation of the Numb protein during cell division functions to inhibit Notch signalling in the receiving cell and biases the cell fate between seemingly identical daughter cells (McGill and McGlade, 2003). In contrast, Musashi-1 (Msi-1), an RNA binding protein, associates with the 3'-untranslated region of *Numb* mRNA and blocks its translation (Imai *et al.*, 2001). Msi-1 has been shown to be required for the asymmetric division of sensory neural precursor cells (Nakamura *et al.*, 1994; Okabe *et al.*, 2001) and is suggested to be a marker of the mammalian neural stem cell population (Kaneko *et al.*, 2000; Sakakibara *et al.*, 2002; Sakakibara and Okano, 1997).

1.4.5 NOTCH SIGNALLING IS A GENERAL ARBITER OF CELL FATE DETERMINATION

Notch signalling was originally identified through its role as a key developmental signalling pathway in *Drosophila* and it has been shown that mutation of the Notch receptors, ligands and downstream effectors results in widespread developmental abnormalities in both invertebrate and vertebrate models (Artavanis-Tsakonas *et al.*, 1999; Lai, 2004). From these studies it is known that Notch signalling plays a crucial role at some level in the development of almost all organs and tissues, with at least one orthologue of each component being found to be widely expressed in all metazoan species studied to date.

In embryogenesis, Notch signalling is classically known for its ability to direct equivalent cells (equivalence groups) to acquire different cell fates, ensuring the correct number and distribution of specialised cells in a tissue (Artavanis-Tsakonas *et al.*, 1999; Lai, 2004). This is most clearly described during *Drosophila* neurogenesis, through the paradigm of 'lateral inhibition' (*FIGURE 1-9A*). By this mechanism, isolated nerve cells in the eye discs derive from a sheet of identical and equipotent epithelial cells and neighbouring cells are inhibited from following a neural fate by Notch (Heitzler *et al.*, 1996; Oellers *et al.*, 1994). Therefore, in Notch mutants all cells are able to follow the default neural lineage, resulting in generation of the 'neurogenic' phenotype (Poulson, 1937). This occurs because the pro-neural genes *Achaete-scute* and *Atonal* are downstream targets of the Enhancer of split complex and their repression is dependent on active Notch signalling (Heitzler *et al.*, 1996).

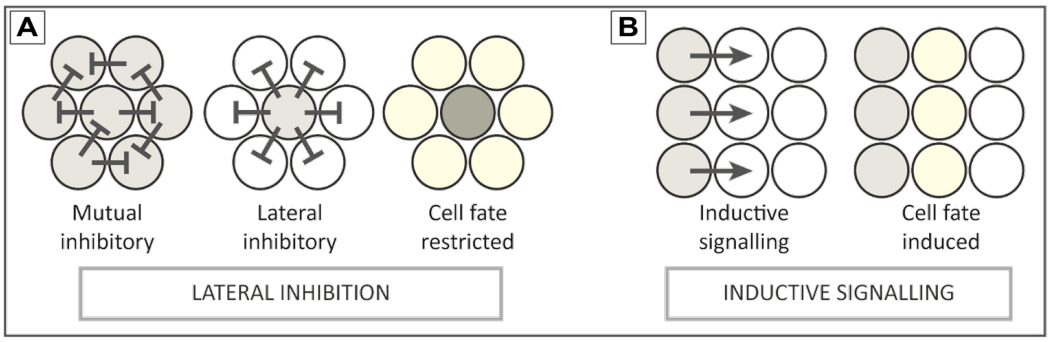


Figure 1-9| *Schematic representation of 'inductive signalling' and 'lateral inhibition'.* [A] Inductive signalling enables the formation of boundaries between developmentally distinct cells, where one group predominantly express the Notch receptor while others predominantly express the ligand. These boundaries are maintained by negative feedback mechanisms operating in these cells, where Notch activation leads to a subsequent repression of ligand expression. **[B]** Lateral inhibition is most classically defined by the neurogenic phenotype of the *Drosophila* eye. In the developing eye discs, isolated nerve cells are derived from a sheet of identical and equipotent epithelial cells. Notch is activated in the surrounding cells, preventing them from following the same cell fate and ensuring a correct number and distribution of neurons throughout the tissue.

Alternatively, the mechanism of 'inductive signalling' also mediates cell fate determination by Notch in *Drosophila*, but occurs between developmentally distinct cell types (*FIGURE 1-9B*). Inductive signalling is therefore frequently observed in the formation of cellular boundaries and is used to promote the development of specific body regions. This is most clearly illustrated during *Drosophila* wing development, in which signalling between the dorsal and ventral parts of the future wing are essential for proper development of the wing margin (Couso *et al.*, 1995; Kim *et al.*, 1996). Disrupted signalling therefore results in the 'notched' wing phenotype observed in the early mutagenic studies, from which the Notch name was originally derived (Morgan, 1917).

1.4.6 NOTCH SIGNALLING IS ESSENTIAL FOR NORMAL VERTEBRATE DEVELOPMENT

Notch receptors, ligands and effectors are also widely expressed during mammalian organogenesis (Artavanis-Tsakonas *et al.*, 1999; Lai, 2004) and are essential in the development of tissues derived from all three primary germ layers (Vooijs *et al.*, 2007). Insights into the *in vivo* role of vertebrate Notch have come primarily from experimental manipulation in mice. Targeting of *N1* (Conlon *et al.*, 1995; Swiatek *et al.*, 1994) or *N2* (Hamada *et al.*, 1999) leads to embryonic lethality, while *N3* (Krebs *et al.*, 2003) and *N4* (Krebs *et al.*, 2000) null mice show no apparent embryonic phenotype. Interestingly, mice null for both *N1* and *N4* show a more extreme phenotype than *N1* alone, suggestive of possible functional redundancy between these different receptors (Krebs *et al.*, 2003). Furthermore, mice lacking the common co-factor *CSL* are embryonic lethal and closely resemble that of *N1* nulls, strongly suggesting that N1 is the principal Notch ligand in mammals (de la Pompa *et al.*, 1997; Oka *et al.*, 1995). Mice lacking *Dll1* or *Jag1* are also embryonically lethal (Hrabe de Angelis *et al.*, 1997; Xue *et al.*, 1999), while mice lacking *Dll3* remain viable but exhibit severe skeletal defects (Dunwoodie *et al.*, 2002; Kusumi *et al.*, 1998).

Notch also mediates mechanisms analogous to both lateral inhibition and inductive signalling during vertebrate development. In a similar manner to lateral inhibition in *Drosophila*, mammalian Notch homologues feature heavily in binary cell fate determination, including mammalian neurogenesis (reviewed in Lewis, 1996). Mammalian neurogenesis also relies heavily on pro-neural factors homologous to *Achaete-scute* and *Atonal* in *Drosophila,* which are known as Mash1 (Mammalian achaete-scute homolog 1, Hash1 in humans; expressed by *Ascl1*) (Johnson *et al.*, 1990) and Math1 (Mammalian atonal homolog-1, Hath1 in humans; expressed by *Atoh1*) (Akazawa *et al.*, 1995) respectively (these proteins are referred to as Mash1 and Math1 only here forth for simplicity). Several other Atonal homologues also exist in mammals and include both *NeuroD* (*Beta2*) and the *Neurogenins* (*Ngn1-3*) (Lee, 1997). All of these are transcriptionally repressed by active Notch signalling as targets of Hes1 and Hes5 (Ohtsuka *et al.*, 1999) and ectopic *Hes1* expression represses neuronal differentiation (Ishibashi *et al.*, 1994; Tomita *et al.*, 1996) while *Hes1* knock-out causes premature neurogenesis (Ishibashi *et al.*, 1995). The function of *Hes5* is less well defined but *Hes1*/*Hes5* double knock-out mice are associated with a more severe phenotype, suggestive of possible redundancy (Ohtsuka *et al.*, 1999). Consistent with the reiterative use of Notch signalling in development, similar mechanisms also mediate binary cell fate decisions in other mammalian tissues, such as T- *vs.* B-lymphoid cells in lymphogenesis (Pui

et al., 1999), exocrine *vs.* endocrine cells in pancreagenesis (Apelqvist *et al.*, 1999) and arteries *vs.* veins during vasculogenesis (Lawson *et al.*, 2001).

Notch is also known to mediate processes in mammalian development that are reminiscent of inductive signalling, as most clearly illustrated by somite boundary formation during vertebrate somitogenesis (Bessho and Kageyama, 2003). As with most organisms, the segmented body plan in mammals is formed in early embryogenesis, from periodic oscillations of developmental signalling cascades and Notch signalling appears to be central to this segmentation 'clock' through Hes-mediated target genes oscillations (Conlon *et al.*, 1995; Palmeirim *et al.*, 1997). It is clear therefore that Notch signalling is highly conserved as a result of its integral requirement during the development of nearly all organisms but it is also evident that Notch mediates multiple different functions within each organism and suggests a highly reiterative and context-dependent basis to this pathway in general.

1.5 NOTCH GOVERNS NORMAL INTESTINAL HOMEOSTASIS

1.5.1 NOTCH COMPONENT EXPRESSION IN THE DEVELOPING AND ADULT INTESTINE

Information on the expression of selective Notch receptors, ligands and target genes in the intestinal epithelium has largely been derived from analysis of mRNA expression in the small intestine of animal models (*TABLE 1-1*). From these experiments, it has been shown that N1 is the principal Notch receptor expressed in both the developing and adult intestine, where signalling appears to be mediated by Dll1, Jag1, and Hes1 (Crosnier *et al.*, 2005; Jensen *et al.*, 2000; Sander and Powell, 2004; Schroder and Gossler, 2002). Consistent with a potential role in stem or progenitor cells, the expression of these components in adult tissues is largely, but not exclusively, restricted to the proliferative crypt compartment. More recently, several groups have also begun to characterise expression in the normal human intestine and also reveal a gene profile consistent with Notch activation towards the base of the crypt (Kosinski *et al.*, 2007; Reedijk *et al.*, 2008), however this continues to remain poorly defined.

Component	Origin / Stage	Detail / Localisation	Technique	Reference
N1	Mouse SI - P25	Epithelial cells of the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
	Mouse SI - Adult	Epithelial cells of the crypt	*In situ* hybridisation	Riccio et al., 2008
	Mouse SI/LI - Adult	Crypt and villi - inc. mature goblet	Cre/β-gal	Vooijs et al., 2007
	Rat SI/LI - Adult	Increased in LI compared to SI	Northern blotting	Sander and Powell, 2004
	Rat SI - Adult	Lower third of the crypt	*In situ* hybridisation	Sander and Powell, 2004
	Rat LI - Adult	Proliferative region (crypt mid 3rd)	*In situ* hybridisation	Sander and Powell, 2004
	Human SI/LI - Adult	Expressed - Tissue array	Immunohistochemistry	Baldi et al., 2004
	Human SI/LI - Adult	Base of the crypt	Microarray analysis	Kosinski et al., 2007
	Human LI - Adult	Base of the crypt	*In situ* hybridisation	Reedijk et al., 2008
N2	Mouse SI - P25	Weak & diffuse in the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
	Mouse SI - Adult	Epithelial cells of the crypt	*In situ* hybridisation	Riccio et al., 2008
	Rat SI - Adult	Few crypt cells	*In situ* hybridisation	Sander and Powell, 2004
	Rat LI - Adult	Absent	*In situ* hybridisation	Sander and Powell, 2004
	Human LI - Adult	Base of the crypt	Microarray analysis	Kosinski et al., 2007
N3	Mouse SI - P25	Scattered cells of villi	*In situ* hybridisation	Schroder and Gossler, 2002
	Rat SI/LI - Adult	Increased in LI compared to S	Northern blotting	Sander and Powell, 2004
	Human LI - Adult	Base of the crypt	Microarray analysis	Kosinski et al., 2007
CSL	Human LI - Adult	Base of the crypt	Microarray analysis	Kosinski et al., 2007
Dll1	Mouse SI - P25	Individual cells in the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
	Mouse SI - Adult	Secretory cells of the crypt & villi	β-gal	Crosnier et al., 2005
Dll4	Mouse SI - P25	Individual cells in the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
Jag1	Mouse SI - P25	Individual cells in the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
	Rat SI - Adult	Lower third of the crypt	*In situ* hybridisation	Sander and Powell, 2004
	Rat LI - Adult	Proliferative region (crypt mid 3rd)	*In situ* hybridisation	Sander and Powell, 2004
	Human LI - Adult	Top of the crypt	Microarray analysis	Kosinski et al., 2007
Jag2	Rat SI - Adult	Lower third of the crypt	*In situ* hybridisation	Sander and Powell, 2004
	Rat LI - Adult	Uniformly in the crypt	*In situ* hybridisation	Sander and Powell, 2004
Lfng	Mouse SI - P25	Crypt and villous epithelium	*In situ* hybridisation	Schroder and Gossler, 2002
Mfng	Mouse SI - P25	Scattered cells	*In situ* hybridisation	Schroder and Gossler, 2002
Rfng	Mouse SI - P25	Crypt and villous epithelium	*In situ* hybridisation	Schroder and Gossler, 2002
Hes1	Mouse SI - E17	Villous epithelium	Immunohistochemistry	Jensen et al., 2000
	Mouse SI - Adult	Base of crypt - proliferating cells	Immunohistochemistry	Jensen et al., 2000
	Mouse SI - P25	Epithelial cells of the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
	Mouse SI - pre P14	Epithelial cells of the crypt	Immunohistochemistry	Kayahara et al., 2003
	Mouse SI - P21	Mid to lower crypt cell nuclei (Not secretory cells)	Immunohistochemistry	Kayahara et al., 2003
	Mouse SI - Adult	Epithelial cells of the crypt	*In situ* hybridisation	van Es et al., 2005
	Mouse SI – Adult	Epithelial cells of the crypt	Immunohistochemistry	Riccio et al., 2008
	Human LI - Adult	Base of the crypt	*In situ* hybridisation	Reedijk et al., 2008
Hes5	Mouse SI - P25	Crypt and villous epithelium	*In situ* hybridisation	Schroder and Gossler, 2002
	Mouse SI - Adult	Paneth cell compartment	*In situ* hybridisation	van Es et al., 2005
Hes6	Mouse SI - P25	Epithelial cells of the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
	Mouse SI - Adult	Cells directly above Paneth cells	*In situ* hybridisation	van Es et al., 2005
Hes7	Mouse SI - P25	Epithelial cells of the crypt	*In situ* hybridisation	Schroder and Gossler, 2002
Math1	Mouse SI - Adult	Epithelial cells of the crypt	*In situ* hybridisation	Akazawa et al., 1995
	Mouse SI/LI - Embryo	Epithelial cells of the crypt	β-gal	Yang et al., 2001
	Mouse SI/LI - Adult	Crypt region & scattered cells in villi	β-gal	Yang et al., 2001
	Human SI/LI – Adult	Goblet cells of the crypt	Immunohistochemistry	Leow et al., 2004
	Human SI/LI - Adult	Secretory cells of the crypt	Immunohistochemistry	Park et al., 2006
	Human SI/LI - Adult	Increased in LI compared to SI	Gene Logic Database	Leow et al., 2004
	Human SI/LI - Adult	Increased in LI compared to SI	Northern blotting	Tsuchiya et al., 2007

Table 1-1| ***Notch signalling components are expressed in the developing and adult intestine.***

Pharmacological and genetic manipulation of Notch signalling in animal models has begun to unravel a fundamental functional role for Notch signalling during intestinal development and homeostasis (Fre *et al.*, 2005; Jensen *et al.*, 2000; Milano *et al.*, 2004; van Es *et al.*, 2005; Yang *et al.*, 2001a). From these studies it appears that, consistent with component expression towards the base of the crypt, Notch signalling plays an essential role in both the proliferation and cell fate determination of early intestinal progenitors. Interestingly, it appears that cell fate in the intestine is governed by lateral inhibition mechanisms and, in further analogy to neurogenesis, this appears to result from Notch-mediated repression of bHLH factors related to *Atonal* in *Drosophila,* namely *Math1* (*Atoh1*) (Schonhoff *et al.*, 2004).

1.5.2 PHARMACOLOGICAL INHIBITION PROMOTES SECRETORY DIFFERENTIATION

Initial evidence for Notch-mediated cell fate specification in the mammalian GI tract has been derived from toxicological studies of γ-secretase inhibitors (GSIs) in animal models. GSIs were originally developed to inhibit cleavage of the amyloid precursor protein (APP), a major causative factor in Alzheimer's disease (reviewed in Citron, 2004). As described previously, the final stage in Notch receptor activation is also dependent on cleavage by the γ-secretase complex (*SECTION 1.4.2*, *p30*). Therefore, Notch activity is also potently inhibited by these agents (Citron, 2004; De Strooper *et al.*, 1999; Kopan and Goate, 2000) and treatment in rodents was shown to halt intestinal proliferation while increasing the size and number of mucous secreting goblet cells, an undesirable side effect of these drugs (Milano *et al.*, 2004; Searfoss *et al.*, 2003; van Es *et al.*, 2005; Wong *et al.*, 2004). These results therefore begin to allude to crucial roles for Notch in both the proliferation and cell fate determination of this tissue.

1.5.3 NOTCH MAINTAINS INTESTINAL PROLIFERATION AND DETERMINES CELL FATE

In addition to generalised Notch pathway inactivation by GSI therapy, a number of approaches using genetic manipulation have also been employed and have highlighted essential functions for specific Notch pathway components within the developing and adult intestine (*TABLE 1-2*). Analogous to GSI treatment, Notch inactivation by conditional knock-out of the common co-factor *CSL* (*RBP-J*), or *N1* and *N2,* (Riccio *et al.*, 2008; van Es *et al.*, 2005) results in the rapid and complete conversion of the proliferative crypt compartment into post-mitotic goblet cells at the expense of absorptive cells. Similar results have also been achieved by knock-out of Delta1 (*DeltaD*$^{-/-}$) or mind bomb (*Mib*$^{-/-}$; required for all Delta signalling) in zebrafish larvae, which have been shown to faithfully model the mammalian intestine, suggesting Delta signalling to be an essential mediator of cell fate determination in the intestinal epithelium (Crosnier *et al.*, 2005).

Furthermore, these studies gain additional support from analysis of the upper GI tract, since mice lacking either *Dll1* or *CSL* exhibit accelerated endocrine differentiation in the developing pancreas (Apelqvist *et al.*, 1999). These findings are therefore reminiscent of excessive neural differentiation following Notch inhibition during neurogenesis and suggest that lateral inhibitory mechanisms are an essential feature of cell fate determination in the intestinal epithelium.

Component	Origin / Stage	Method	Reference	General phenotype
Notch signalling	Mouse & Rat SI/LI - Adult	GSI Treatment	Searfoss et al., 2003 Milano et al., 2004 Wong et al., 2004 van Es et al., 2005 Riccio et al., 2008	• *Excess of goblet cells* • *Halted cell proliferation* • *Decreased Hes1* • *Increased Math1* • *Increased p27 & p57*
N1/N2	Mouse SI - Adult	Knock-out (Ind-Cond; *Villin-Cre*)	Riccio et al., 2008	• *Excessive secretory differentiation* • *Loss of proliferative compartment* • *Decreased Hes1* • *Increased Atoh1* • *Increased p27/p57*
CSL	Mouse SI - Adult	Knock-out (Ind-Cond; *Cyp1A-Cre*) Knock-out (Ind-Cond; *Villin-Cre*)	van Es et al., 2005 Riccio et al., 2008	
DeltaD/Mib1	Zebrafish - Larvae	Knock-out (Constitutive)	Crosnier et al., 2005	
Hes1	Mouse SI - Embryo	Knock-out (Constitutive)	Jensen et al., 2000 Suzuki et al., 2005	• *Excessive secretory differentiation* • *Loss of proliferative compartment* • *Increased Atoh1, NeuroD & Ngn3*
Math1	Mouse SI - Embryo	Knock-out (Constitutive) Knock-out (Constitutive) Knock-out (Conditional; *Fabpl-Cre*)	Yang et al., 2001 Shroyer et al., 2005 Shroyer et al., 2007	• *Ablation of all secretory differentiation* • *Increased absorptive cells* • *Loss of NeuroD expression*
Gfi1	Mouse SI - Embryo	Knock-out (Constitutive)	Shroyer et al., 2005	• *Reduced Paneth & goblet cells* • *Increased enteroendocrine cells*
Rac1	Mouse SI - Embryo	Knock-out (Constitutive)	Stappenbeck et al., 2000	• *Reduced Paneth & goblet cells*
Klf4	Mouse SI - Embryo	Knock-out (Constitutive)	Katz et al., 2002	• *Reduced goblet cells*
Ngn3	Mouse SI - Embryo	Knock-out (Constitutive)	Jenny et al., 2002	• *Loss of all enteroendocrine cells* • *Loss of NeuroD expression*
NeuroD	Mouse SI - Embryo	Knock-out (Constitutive)	Naya et al., 1997	• *Loss of Secretin & Cholecystokinin (CCK) enteroendocrine cells specifically*
Activated N1	Mouse SI - Embryo	Knock-in (Conditional; *Villin-Cre*)	Fre et al., 2005	• *Expansion of the proliferative crypt compartment* • *Reduced secretory cells* • *Increased Hes1 expression* • *Decreased Atoh1 expression*
	Mouse SI - Adult	Knock-in (Conditional; *Fabpl-Cre*)	Stanger et al., 2005	
	Mouse SI - Adult	Knock-in (Ind-Cond; *Cyp1A-Cre*)	Zecchini et al., 2005	• *Increased post-mitotic goblet cell number* • *Increased Hes5 expression*

Table 1-2| *Functional modulation of Notch signalling in the intestinal epithelium.*

1.5.4 HES1 REPRESSES THE SECRETORY CELL LINEAGE

Although several Notch target genes have now been identified, it remains widely accepted that *Hes1* is the principal Notch target in many tissues, including the intestine (Iso *et al.*, 2003). *Hes1*-deficient mice (*Hes1*$^{-/-}$) die perinatally due to aberrant expression of pro-neural factors and severe neuronal hypoplasia (Ishibashi *et al.*, 1995); however, these mice are also associated with a similar hypoplasia phenotype in the GI tract due to precocious secretory cell differentiation (Jensen *et al.*, 2000; Suzuki *et al.*, 2005). It was shown that *Hes1*$^{-/-}$ deletion in the intestinal epithelium results in increased expression of *Dll1, Dll3* and *Math1,* as well as other pro-neural factors, including *Mash1*, *Ngn3* (*Atoh5*) and *NeuroD1* (*Beta2*) (Jensen *et al.*, 2000). This is consistent with the negative regulation of these factors by Hes1 during mammalian neurogenesis (Akazawa *et al.*, 1995; Kageyama and Nakanishi, 1997) and further highlights extensive similarities between the cell fate determination of these two highly distinct tissues. Moreover, decreased *Hes1* and increased *Atoh1* expression is observed following Notch inhibition by either *CSL* or *N1/N2* knock-out in the intestinal epithelium (Riccio *et al.*, 2008; van Es *et al.*, 2005), confirming that these features are tightly controlled by canonical Notch signalling in this tissue.

Taken together, these results suggest that *Hes1* operates as a central regulator of the binary-fate decision between the absorptive and secretory phenotype in the intestinal epithelium, acting to suppress both pro-neural gene expression and the secretory cell fate. The observed increase in *Dll* expression is reminiscent of negative-feedback mechanisms underlying *Drosophila* neurogenesis (Heitzler *et al.*, 1996) and further suggests a central role for lateral inhibition type mechanisms in the intestine. Furthermore, *Hes5* is also a direct target of active Notch signalling (Nishimura *et al.*, 1998; Ohtsuka *et al.*, 1999) and is known to be expressed in the intestinal epithelium (Schroder and Gossler, 2002; van Es *et al.*, 2005; Zecchini *et al.*, 2005). Hes1 and Hes5 are known to carry out overlapping functions in the developing neuro-epithelium (Ohtsuka *et al.*, 1999), however the intestinal phenotype of *Hes5* null mice remains to be determined.

1.5.5 MATH1 IS REQUIRED FOR SECRETORY CYTODIFFERENTIATION

As described above, Math1 is a neurogenic bHLH transcription factor that is strongly expressed in the developing central nervous system (Akazawa *et al.*, 1995). Math1 is known to play a critical role in terminal differentiation of various tissues, including neurons (Gowan *et al.*, 2001), brain (Ben-Arie *et al.*, 1997), skin (Leonard *et al.*, 2002) and the auditory system (Bermingham *et al.*, 1999). It is also strongly expressed in the normal intestine, where positive cells are scattered throughout the colonic crypts and are identifiable as secretory cells (Akazawa *et al.*, 1995; Leow

et al., 2004; Tsuchiya *et al.*, 2007; Yang *et al.*, 2001a). Expression of Atoh1 in the proliferative mid crypt region is strongly suggestive of a function in early cell fate specification and *Atoh1* knock-out mice die neonatally, with loss of all three secretory lineages in the intestine (Yang *et al.*, 2001a). These mice also demonstrate decreased *Dll* expression, consistent with lateral inhibition (Yang *et al.*, 2001a), plus a complete loss of the alternative *Atonal* homologue, *NeuroD*, suggesting that *Atoh1* acts upstream of *NeuroD* in the intestine and other tissues (Miyata *et al.*, 1999; Yang *et al.*, 2001a). Atoh1 is therefore believed to act as a general arbiter of secretory cell fate in the intestine but these results also suggest that subsequent secretory specialisation occurs through the expression of other pro-secretory factors.

1.5.6 NGN3 AND NEUROD ACT DOWNSTREAM OF MATH1

A number of additional factors are also known to act downstream of *Hes1* and *Math1* to mediate differentiation of the distinct cellular subtypes present within the mature epithelium. For example, it has been shown that all enteroendocrine cells are derived from *Ngn3*-expressing progenitors in the developing intestine and in the adult crypts (Jenny *et al.*, 2002). *Ngn3* (*also Atoh5*) is another *Atonal* homologue (Sommer *et al.*, 1996) and *Ngn3*$^{-/-}$ mice die postnatally from diabetes due to an absence of endocrine cells in the pancreas (Gradwohl *et al.*, 2000; Jensen *et al.*, 2000). *Ngn3* expression is also detectable in the embryonic and adult mouse intestine, where it is restricted to immature proliferating cells of the adult crypt (Jenny *et al.*, 2002). As in the pancreas, *Ngn3* knock-out mice demonstrate complete ablation of enteroendocrine cells, while all other cell lineages persist. In fact, goblet cell number increases, suggesting that both cell types may be derived from a common bi-genic precursor cell (Jenny *et al.*, 2002). Furthermore, *NeuroD* expression is lost in *Ngn3*$^{-/-}$ intestine, while *Atoh1* expression is readily detectable (Jenny *et al.*, 2002) and *NeuroD* is known to be a transcriptional target of Ngn3 in other contexts (Huang *et al.*, 2000), suggesting that NeuroD may act downstream of both *Atoh1* and *Ngn3* in the intestine.

NeuroD (*Beta2*) was originally discovered as an important factor in the activation of insulin gene transcription and neuronal differentiation (Lee *et al.*, 1995; Naya *et al.*, 1995) but is also expressed by differentiated endocrine cells in the intestine (Rindi *et al.*, 1999). Accordingly, *NeuroD* deficient mice exhibit pancreatic endocrine defects but, in contrast to *Ngn3*$^{-/-}$, lack only secretin and cholecystokinin (CCK) producing enteroendocrine sub-populations in the intestine (Naya *et al.*, 1997; Rindi *et al.*, 1999). Enteroendocrine cells consist of at least 15 different cell types classified on the basis of hormonal content and geographical distribution (Hocker and

Wiedenmann, 1998) and it was shown that all other enteroendocrine cell types continue to develop in the absence of *NeuroD* (Naya *et al.*, 1997; Rindi *et al.*, 1999).

Alternatively, *Gfi1* (*Growth factor independent repressor-1*) is a zinc-finger transcriptional repressor that functions in the differentiation of immune cells (Karsunky *et al.*, 2002), lung neuroendocrine cells (Kazanjian *et al.*, 2004) and sensory hair cells (Wallis *et al.*, 2003). *Gfi1*$^{-/-}$ mice demonstrate reduced numbers of Paneth cells and goblet cells but increased enteroendocrine cells (Shroyer *et al.*, 2005). Similarly, loss of *Rac1* (*ras-related C3 botulinum toxin substrate 1*) also affects the differentiation of both Paneth and goblet cell but has no effect on enteroendocrine cells (Stappenbeck and Gordon, 2000), suggesting that both *Gfi1* and *Rac1* are essential for the divergence of immature goblet and Paneth cells from a common intestinal secretory progenitor. In addition, *Klf4* (*Kruppel-like factor 4*) encodes a zinc-finger transcription factor expressed in the differentiated epithelial cells of several organs, including the intestine (Garrett-Sinha *et al.*, 1996; Shields *et al.*, 1996; Ton-That *et al.*, 1997). *Klf4*$^{-/-}$ mice die perinatally due to epidermal defects (Segre *et al.*, 1999) but also demonstrate significant reduction in goblet cell number while all other cell lineages differentiate normally (Katz *et al.*, 2002). This strongly suggests that *Klf4* acts downstream of *Math1*, *Gfi1 and Rac1* to specifically promote goblet cell differentiation in the intestinal epithelium.

1.5.7 A PUTATIVE MODEL FOR NOTCH-MEDIATED CELL FATE SPECIFICATION

It is clear that Notch acts in a highly conserved fashion to govern binary cell fate decisions in a vast range of organisms and tissues, where pathway inhibition frequently leads to an excess of one class of cells at the expense of others, for example neurons in the CNS (Haddon *et al.*, 1998; Jiang *et al.*, 1996; Schier *et al.*, 1996), hair cells in the ear (Haddon *et al.*, 1999; Haddon *et al.*, 1998) and, more recently, secretory cells in the intestine (*SECTIONS* 0-*1.5.4, p39-41*). Active Notch signalling therefore influences cell fate decisions through the reiterative use of bHLH transcription factor cascades that follow a tightly controlled hierarchy of activation (Kageyama and Nakanishi, 1997) *(FIGURE 1-10).* As described above, the factors implicated in intestinal cell fate decisions are mostly related to the *Drosophila Atonal* gene and show strong mechanistic correlation to that observed in neurogenesis (reviewed in Schonhoff *et al.*, 2004).

Analogous to *Drosophila* and mammalian neurogenesis, Notch activation in the early intestinal progenitor cells suppresses the default secretory fate and drives cells along the absorptive lineage. Immature secretory cells arising within this group of equivalent progenitors express Dlls and induce Notch signalling in neighbouring cells, thus preventing them from following the same

secretory cell fate (Crosnier *et al.*, 2005; van Es *et al.*, 2005). Notch activation suppresses the secretory lineage through the immediate transcriptional target *Hes1* (Fre *et al.*, 2005; van Es *et al.*, 2005). Crucially, expression of the pro-neural (pro-secretory) factors is suppressed by Hes1, but so is expression of the Dlls, resulting in positive feedback between these cells and their restriction to either ligand or receptor expressing phenotypes. Cells expressing *Hes1* repress *Atoh1* and all downstream secretory effectors, becoming enterocytic (Jensen *et al.*, 2000) and those expressing *Math1* instead become secretory (Yang *et al.*, 2001a). Following this pre-patterning, enteroendocrine cells then derive from *Ngn3 and NeuroD* expressing cells (Jenny *et al.*, 2002; Naya *et al.*, 1997), while *Gfi1, Rac1* and *Klf4* promote differentiation along the goblet and Paneth cell lineages (Katz *et al.*, 2002; Shroyer *et al.*, 2005; Stappenbeck and Gordon, 2000).

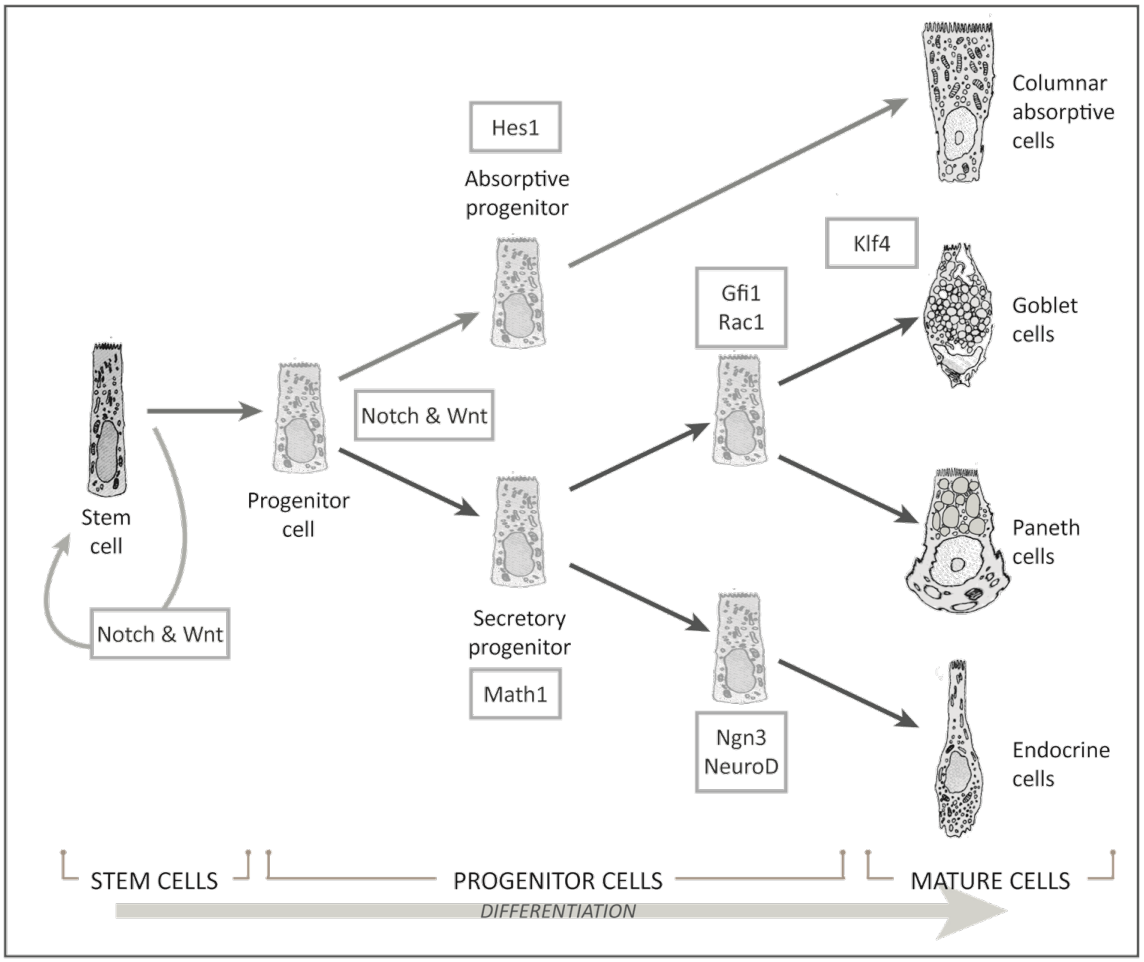


Figure 1-10| ***Notch governs correct cell fate specification in the intestine*****.** Notch positive 'receptor' cells exhibit *Hes* following receptor activation, leading to subsequent Hes1-mediated repression of *Math1* (Jensen et al., 2000; van Es et al., 2005b; Yang et al., 2001a). Cells expressing ligand do not exhibit Notch activation or *Hes1* expression, releasing *Math1* from repression. *Math1* positive cells then adopt a secretory fate (Yang et al., 2001a), which is further specified by downstream expression of transcription factors such as *Ngn3* or *NeuroD* (Jenny et al., 2002).

1.5.8 NOTCH EXPRESSION IN INTESTINAL HOMEOSTASIS IS HIGHLY CONSERVED

As with all developmental signalling pathways, Notch is known to be highly conserved from flies to man (del Amo *et al.*, 1993). It now appears that development and homeostasis in the *Drosophila* gut is coordinated in a highly analogous fashion to the mammalian tissue (reviewed in Wilson and Kotton, 2008). Notch is essential for normal *Drosophila* gut morphogenesis (Rasmussen *et al.*, 2008) and is known to play an essential role in cell fate determination during homeostasis of the developing and adult epithelium (Fusse and Hoch, 2002; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Tanaka *et al.*, 2007). As in the mammalian intestine, the *Drosophila* midgut is maintained by a sub-population of pluripotent stem cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) whose self-renewal is also strongly promoted by Wingless (Wg), the *Drosophila* homologue of mammalian Wnt (Lin *et al.*, 2008). These stem cells divide asymmetrically, giving rise to both absorptive enterocyte and secretory enteroendocrine progeny, and it has been shown that this binary cell fate decision is governed by Notch signalling. In analogy to the mammalian intestine, over-activation of Delta-mediated Notch signalling in these tissues leads to altered proliferation and bias against the secretory fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), confirming this function to be a highly conserved feature of the Notch pathway.

1.5.9 NOTCH PLAYS A PIVOTAL ROLE IN INTESTINAL PROLIFERATION

Although the size and location of the intestinal stem cell population in mammals is known to be controlled by Wnt and BMP4 signalling (Haramis *et al.*, 2004; He *et al.*, 2004; Sancho *et al.*, 2004), loss of the proliferative compartment in the Notch pathway mutants described above (Riccio *et al.*, 2008; van Es *et al.*, 2005) also highlights a fundamental role for Notch in the intestinal stem/progenitor phenotype. In support of this, functional studies have shown that forced expression of $N1^{IC}$ in foetal mouse intestine to inhibits differentiation and results in expansion of the proliferative progenitor pool (Fre *et al.*, 2005; Stanger *et al.*, 2005). This is associated with high levels of nuclear Hes1, significant repression of *Math1* and compromised secretory cell number. Therefore, these findings confirm that activation of N1 alone is sufficient to induce aberrant proliferative expansion in these tissues. Furthermore, it has also shown that Hes1 co-localises with the Notch-associated stem cell marker Msi-1 in the CBC population at the base of the crypt (Kayahara *et al.*, 2003), alluding to further roles for active Notch signalling in stem cell maintenance. Therefore, in addition to cell lineage determination, active Notch signalling may also be an essential feature of both progenitor proliferation and/or stem cell maintenance in the

intestine, although how Notch might mediate these disparate outcomes in such close proximity remains to be determined.

1.5.10 NOTCH AND WNT ARE EQUALLY REQUIRED FOR INTESTINAL PROLIFERATION

Analysis in the normal murine intestine has, therefore, shown that the proliferative progenitor phenotype in this tissue is highly dependent on the actions of both Notch and Wnt (reviewed in Nakamura *et al.*, 2007). These studies have shown that exogenous Wnt activation in the intestinal epithelium induces proliferative expansion (Andreu *et al.*, 2005; Kim *et al.*, 2005; Sansom *et al.*, 2004) and pathway inhibition leads to premature differentiation along the absorptive lineage (Ireland *et al.*, 2004; Korinek *et al.*, 1998a; Pinto *et al.*, 2003). In a highly similar fashion, it is clear that over-activation of N1 also leads to expansion of these proliferative progenitors (Fre *et al.*, 2005; Stanger *et al.*, 2005), although pathway inhibition instead induces differentiation along the secretory lineage (van Es *et al.*, 2005). This implies that, while both pathways have an equally central role in promoting intestinal proliferation, each may have opposing functions in determining the subsequent cell fate of these progenitors. Most importantly, these findings also suggest that the concerted action of both Notch and Wnt may also be essential for proliferative expansion during intestinal tumorigenesis and over-activation of either pathway may be sufficient to initiate the formation of these tumours. While aberrant changes in the Wnt signalling pathway have become one of the most well described molecular features of CRC (*SECTION 1.3.2, p22*), however, a putative role of Notch has barely been addressed.

1.6 A ROLE FOR NOTCH SIGNALLING IN INTESTINAL TUMORIGENESIS

1.6.1 NOTCH SIGNALLING IN CONGENITAL DISEASE

During development the outcome of active Notch signalling is known to be highly sensitive to small changes in Notch component expression and/or pathway activation (Harper *et al.*, 2003). Although mutants for Notch components are largely non-viable, several human diseases have now been linked to genes involved in this pathway (reviewed in Gridley, 2003) and include the following; CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; *OMIM #125310*) is an autosomal-dominant vascular disorder and has been linked to various mutations in *N3*, mostly affecting the extracellular EGF repeats (Joutel *et al.*, 2000). Alagille syndrome (AGS; *OMIM #118450*) is an autosomal dominant disease associated with *Jag1* mutations and is characterised by developmental defects in a range of organs including the liver, heart, eye and skeleton (Li *et al.*, 1997; Oda *et al.*, 1997). Sondylocostal dysostosis (SD;

OMIM #122600) is a disease of defective vertebral segmentation and frequently presents as rib abnormalities and short trunk dwarfism, one form of which is caused by a mutation in *Dll3* (Bulman *et al.*, 2000; Kusumi *et al.*, 1998). Therefore, it is clear that subtle changes in normal Notch signalling may have profound effects on a diverse array of tissues and suggests that altered Notch signalling may also have important consequences in a non-congenital setting.

1.6.2 CONSTITUTIVE NOTCH ACTIVATION IS ONCOGENIC

Given the importance of the Notch signalling pathway in maintenance of normal tissue development and homeostasis it should not be surprising to find that this pathway also has a prominent role in many human cancers (reviewed in: Koch and Radtke, 2007; Leong and Karsan, 2006). In fact, the *N1* gene was originally identified as a promoter of human malignancy through its role in a subset of human T-cell acute lymphoblastic leukaemia's (T-ALL) bearing chromosome 7 to chromosome 9 translocations, t(7;9)(q34;q34.3) (Reynolds *et al.*, 1987). Chromosome 9 contains the *T-cell-receptor-β* (*TCRβ*) gene, which is strongly active in T-cells, while chromosome 7 contains a gene which was found to be very similar to *Drosophila Notch* and was named *Translocation-associated Notch homologue (TAN1*) (Ellisen *et al.*, 1991). *TAN1* subsequently became known as *Notch1* and it was shown that t(7;9) results in expression of a truncated and constitutively active protein form, equivalent to $N1^{IC}$ (Ellisen *et al.*, 1991).

Since all T-ALL with t(7;9) exhibit this feature, $N1^{IC}$ was proposed to be the causative factor and its expression was subsequently shown to result in the development of T-cell leukaemia in mice (Aster *et al.*, 2000; Pear *et al.*, 1996). Despite the potent T-ALL initiating effects of $N1^{IC}$, less than 1% of human T-ALLs actually exhibit a t(7;9) translocation (Ma *et al.*, 1999); however, other somatic mutations in N1 are frequently observed in both childhood and adult patients with this disease (Lee *et al.*, 2005b) and account for more than 50% of all human T-ALL cases (Weng *et al.*, 2004). Furthermore, these sporadic mutations are also a common feature in murine models of induced T-ALL (Palomero *et al.*, 2006) and deregulated N1 signalling is now widely accepted to be an integral feature of leukemic initiation (Armstrong *et al.*, 2009). While this is consistent with a role in promoting the proliferation of these cells, it should be noted that the expansive T-cell growth induced by constitutive N1 is also accompanied by a decrease in B-cell number (Radtke *et al.*, 1999). Since T-cells and B-cells derive from the same hematopoietic stem cell, this suggests a 'dual-role' for N1 in these tissues as both a promoter of proliferation and a controller of differentiation (reviewed in Radtke *et al.*, 2004).

1.6.3 NOTCH PROMOTES TUMORIGENESIS IN SOLID TISSUES

Further evidence for Notch involvement in leukaemogenesis is also provided by the frequent insertion of proviral promoters into the *N1* or *N2* genes by viruses such as Moloney murine leukaemia virus (M-MuLV) or Feline leukaemia virus (FLV), leading to pathway over-activation and T-cell leukaemia (reviewed in Allenspach *et al.*, 2002). Similarly, the Mouse mammary tumour virus (MMTV) has been shown to frequently insert into both *N1* and *N4* genes (Dievart *et al.*, 1999; Uyttendaele *et al.*, 1996), where insertion results in expression of a truncated protein forms equivalent to $N1/4^{IC}$ (Callahan and Egan, 2004; Imatani and Callahan, 2000). Mouse models expressing either $N1^{IC}$ or $N4^{IC}$ frequently develop aggressive mammary adenocarcinomas (Gallahan *et al.*, 1996; Hu *et al.*, 2006; Jhappan *et al.*, 1992) and both proteins have been shown to induce the transformation of mouse mammary cells in culture (Soriano *et al.*, 2000).

Notch signalling is now also known to be frequently deregulated in human breast cancers (reviewed in Efstratiadis *et al.*, 2007) and N1 is required for maintenance of a transformed phenotype in these tumour cells (Stylianou *et al.*, 2006; Weijzen *et al.*, 2002a). Notch inhibition, therefore, induces cell cycle arrest in breast cancer cells (Rasul *et al.*, 2009), suggesting sustained Notch activity to be an essential feature of these tumours. In contrast to leukaemia, however, genetic alterations of the Notch receptors do not appear to be a common feature of breast, or any other solid tumours, and over-activation is therefore likely to occur through deregulated expression of the Notch ligands and/or receptors (Lee *et al.*, 2007b). As such, high grade (poorly differentiated) breast tumours demonstrate elevated levels of N1 protein (Parr *et al.*, 2004) and this correlates with both reduced patient survival (Parr *et al.*, 2004; Reedijk *et al.*, 2005) and decreased time to disease recurrence (Farnie *et al.*, 2007). It has also been shown that expression of the Notch ligand Jag1 is increased in breast tumour tissues and correlates strongly with increased invasion and a poorer patient prognosis (Leong *et al.*, 2007; Reedijk *et al.*, 2005). Alternatively, the expression of *Numb* is lost in 50% of human mammary carcinomas and demonstrates a strong negative correlation with tumour grade (Pece *et al.*, 2004). This highlights the fact that, although Notch signalling is predominantly altered at the receptor-ligand level, important regulatory changes may also occur downstream.

Over-expression of Notch receptors and/or their ligands has now been identified in a multitude of other solid cancers, including ovarian (Hopfer *et al.*, 2005), prostate (Santagata *et al.*, 2004), melanoma (Balint *et al.*, 2005; Massi *et al.*, 2006; Nickoloff *et al.*, 2003), brain (Fan *et al.*, 2004), pancreas (Kimura *et al.*, 2007; Miyamoto *et al.*, 2003), cervix (Gray *et al.*, 1999; Zagouras *et al.*,

1995) and sarcoma (Curry *et al.*, 2005; Li *et al.*, 2004). Surprisingly, the functional contribution of Notch signalling in the majority of these cancers remains largely unknown, although several tumour promoting roles have now been proposed, including stem cell overgrowth (Bolos *et al.*, 2009), promotion of cell cycle progression (Carlesso *et al.*, 1999), inhibition of apoptosis (Miele and Osborne, 1999), angiogenesis (Mailhos *et al.*, 2001; Patel *et al.*, 2005), and tumour invasion (Leong *et al.*, 2007). Therefore, how Notch contributes to the tumorigenic process is perhaps one of the least well known aspects of the Notch pathway; however, this is likely to be of paramount importance since the relative simplicity of the Notch pathway makes it an extremely attractive therapeutic target for neoplasia in many tissues, including the colon (Nam *et al.*, 2002; Purow *et al.*, 2005; van Es *et al.*, 2005).

1.6.4 A PREDICTABLE BUT UNDEFINED ROLE FOR NOTCH IN COLORECTAL CANCER

Increasing evidence has now begun to suggest aberrant roles for Notch activation in the formation of CRC, although at present this remains extremely poorly defined and has largely been derived from analysis in animal models. As described above, various Notch pathway components are expressed in the proliferative progenitor compartment of the intestinal crypt (*SECTION 1.5.1, p37*), where both N1 and Hes1 appear to be the principal receptor and effector expressed. Furthermore, it is clear that active Notch signalling is essential for proliferation in these progenitor cells and that N1 pathway over-activation is sufficient to induce proliferative expansion of this progenitor pool (*SECTION 1.5.9, p45*). This suggests that the function of Notch in these tissues is highly comparable to that of Wnt (*SECTION 1.5.10, p46*) and, since alterations to the Wnt pathway are a central feature of almost all colorectal tumours (reviewed in Segditsas and Tomlinson, 2006), this alludes to a similarly important role for aberrant Notch signalling in this highly prevalent human disease.

In support of this hypothesis, one of the earliest markers of intestinal tumorigenesis is known to be deregulation of the mucosal environment, where goblet cell number decreases early in tumour progression and is significantly reduced in both ACF and the vast majority of colorectal tumours (Augenlicht *et al.*, 1999; Femia *et al.*, 2008; Leow *et al.*, 2004). Furthermore, Mucin-2 (encoded by *Muc2*) is the principal mucin found in the intestine and *Muc2*$^{-/-}$ mice lack identifiable goblet cells, but also demonstrate increased epithelial proliferation and develop multiple intestinal adenomas (Velcich *et al.*, 2002). This suggests loss of the secretory phenotype to be one of the most important and fundamental features of intestinal tumorigenesis, although the underlying molecular basis for this is not yet entirely clear. Importantly, active Notch signalling is

classically known to suppress secretory differentiation through Hes-mediated repression of *Math1* (*SECTION 1.5.7, p43*). Accordingly, a significant reduction in the expression of both Math1 mRNA and protein is consistently observed in many human colorectal tumours, except those known to be mucinous (Leow *et al.*, 2004; Park *et al.*, 2006; Tsuchiya *et al.*, 2007). In aggregate, these findings, therefore, strongly suggest that Notch pathway activation suppresses secretory differentiation from the earliest stages of intestinal tumorigenesis.

Furthermore, intestinal tumours are known to closely resemble progenitor cells of the normal epithelium (*SECTION 1.1.8, p9*) and, like Wnt (van de Wetering *et al.*, 2002), the expression of Notch pathway components in tumours from $Apc^{+/Min}$ mice closely resembles that of the proliferative crypt compartment (van Es *et al.*, 2005). *Hes1* in particular was found to be uniformly expressed throughout these tumours and the treatment with Notch inhibitors results in a significant loss of proliferation, as well as the induction of secretory cell differentiation (van Es *et al.*, 2005). Study in these animal models, therefore, strongly suggests an essential role for Notch signalling in intestinal tumours. These animal models are highly analogous to the human scenario, however, a number of important differences are known to exist in the context of tumorigenesis (Yamada and Mori, 2007) and very little evidence has so far been obtained in human colorectal tumour tissues.

It has previously been shown that activating *Notch* mutations are not a common feature of human colorectal tumours (Lee *et al.*, 2005b) and it is likely, therefore, that pathway deregulation during colorectal carcinogenesis instead occurs through altered expression of the various Notch receptors, namely N1. As described above, this has been demonstrated in a number of other solid tumours (reviewed in: Koch and Radtke, 2007; Leong and Karsan, 2006); however, at present, very little is known of potential changes in the expression and/or functional contribution of N1 in human CRC tissues, which is now essential to improve our understanding of the molecular basis of this disease.

1.7 HYPOTHESIS AND GENERAL AIMS

The hypothesis guiding this research is that aberrant activation or misappropriation of the Notch1 pathway promotes tumorigenesis and/or progression to malignancy in the human colorectal epithelium. Consequently, the major aim of this study was; firstly to define the expression of Notch1 in human colorectal tissues *in vivo* and to investigate potential changes of expression in different states of disease and stages of tumour progression. Secondly, to validate these findings *in vitro* using colorectal tumour cell lines in order to study the functional contribution of Notch1 in the human CRC phenotype. More detailed, specific aims are also highlighted within the context of each chapter.

CHAPTER 2

MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES

2.1.1 EXPERIMENTAL CELL LINES

A number of established *in vitro* colorectal cell lines were used in this study and include cells derived from both human colorectal adenoma and adenocarcinoma tissues (*TABLE 2-1*).

2.1.1.1 Colorectal cell lines

Cell line	Growth Medium	Origin	Reference
Caco2	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Fogh et al., 1977
HCA7/C29	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Kirkland, 1985
HCT15	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Dexter et al., 1979
HCT116	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Brattain et al., 1981
HT29	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Fogh et al., 1977
JW2	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Paraskeva et al., 1984
LS174T	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Tom et al., 1977
SW1222	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Leibovitz et al., 1976
SW480	*10% FBS/DMEM*	*Human colorectal adenocarcinoma*	Leibovitz et al., 1976
SW620	*10% FBS/DMEM*	*Lymph node metastasis counter-part of SW480*	Leibovitz et al., 1976
AA/C1	*20% FBS/DMEM*	*Human colorectal adenoma*	Paraskeva et al., 1984
AN/C1	*20% FBS/DMEM*	*Human colorectal adenoma*	Paraskeva et al., 1989
BH/C1	*3T3 conditioned*	*Human colorectal adenoma*	Paraskeva et al., 1989
RG/C2	*20% FBS/DMEM*	*Human colorectal adenoma*	Paraskeva et al., 1989

Table 2-1| *Colorectal cell lines used during this study*

Protein expression analysis was performed on all of the colorectal cell lines, while SW480, HCT116, Caco2 and HT29 adenocarcinoma-derived cells, along with AA/C1 cells (adenoma-derived), were selected for further functional studies and are described in greater detail below. The cell lines were originally obtained from The American Type Tissue Culture Collection (ATCC), except for HCA7/C29 [*a kind gift from S. Kirkland*], JW2 and all adenoma-derived lines [*a kind gift from C. Paraskeva*].

2.1.1.1.1 SW480 cells

SW480 cells were established from a colonic adenocarcinoma (Leibovitz *et al.*, 1976) and express truncated APC (Nishisho *et al.*, 1991), with loss of heterozygosity (LOH) at the remaining *APC* locus (Rowan *et al.*, 2000). This truncated APC form also lacks the β-catenin nuclear export signal. SW480 express elevated levels of mutant p53 protein (Rodrigues *et al.*, 1990) and are positive for expression of both *c-Myc* and *KRAS* oncogenes (Trainer *et al.*, 1988).

2.1.1.1.2 HCT116 cells

HCT116 cells were established from a human colonic adenocarcinoma (Brattain *et al.*, 1981). HCT116 cells possess wild-type *APC* but instead harbour mutant β-catenin (*CTNNB1*) and mutant *KRAS* (Gayet *et al.*, 2001; Ilyas *et al.*, 1997; Rosin-Arbesfeld *et al.*, 2003; Rowan *et al.*, 2000). HCT116 cells are amenable to transient transfection and are commonly used in many colorectal cancer functional studies, particularly in conjunction with SW480 cells.

2.1.1.1.3 Caco2 cells

The Caco2 cell line was originally derived from a human colonic adenocarcinoma (Fogh *et al.*, 1964) and are widely used for various *in vitro* assays, namely functional differentiation studies (reviewed in Sambuy *et al.*, 2005). Caco2 cells are relatively well differentiated, but express increasing differentiation markers in post-confluent growth (Mariadason *et al.*, 2002).

2.1.1.1.4 HT29 cells

HT29 cells were first established from a sporadic colonic adenocarcinoma (Fogh *et al.*, 1964). HT29 cells harbour mutant *APC* (Browne *et al.*, 1994; Liu and Bodmer, 2006), mutant p53 (Rodrigues *et al.*, 1990) and are positive for c-*Myc* expression (Trainer *et al.*, 1988). HT29 cells have wild-type *KRAS* but harbour mutant *BRAF* (Davies *et al.*, 2002; Huang *et al.*, 1994). HT29 cells have a characteristic well differentiated phenotype (Kucerova *et al.*, 1999).

2.1.1.1.5 Adenoma-derived cell lines

The adenoma-derived cell lines used in this study retain the properties of the benign tumours from which they are derived and are both anchorage-dependent and non-tumorigenic in athymic nude mice (Paraskeva *et al.*, 1984; Paraskeva *et al.*, 1989). The AA/C1 cells were used for functional analysis of post-confluent differentiation, as demonstrated previously (Guy *et al.*, 2001; Qualtrough *et al.*, 2002).

2.1.1.2 Non-colorectal cell lines

A number of established non-colorectal cell lines were used during initial screens and antibody validation experiments as established positive controls for many of the Notch pathway components (*TABLE 2-2*).

Cell line	Growth Medium	Origin	Reference
Molt4	*10% FBS/RPMI*	*Human acute T-cell lymphoblastic leukaemia*	Minowada *et al*., 1972
HeLa	*10% FBS/DMEM*	*Human cervical carcinoma*	Gey *et al*., 1953
A549	*10% FBS/DMEM*	*Human lung carcinoma*	Giard *et al*., 1973
MCF7	*10% FBS/DMEM*	*Human breast carcinoma*	Sugarman *et al*., 1985
HUVEC	*10% FBS/DMEM*	*Human vascular endothelial*	Hoshi and McKeehan, 1984

Table 2-2| *Non-colorectal cell lines used during this study*

2.1.2 STANDARD TISSUE CULTURE PROCEDURE

2.1.2.1 Standard tissue culture maintenance

All colorectal cell lines were cultured as adherent monolayers in 12.5 cm^2 (T12.5) or 25cm^2 (T25) tissue culture flasks (*Corning Costar, UK*) and maintained at 37^oC and 5% CO_2 in dry incubators (*NuAire, USA*). The growth media was changed twice weekly and cells were passaged at approximately 80-90% confluency. The colorectal adenocarcinoma-derived cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM; *Gibco Life Technologies, UK*) supplemented with 10% foetal bovine serum (FBS; *Autogen Bioclear, UK*), glutamine (2mM) and penicillin/streptomycin (100 units.ml^{-1}) (*Autogen Bioclear, UK*). The colorectal adenoma lines were maintained under the same conditions in DMEM supplemented with 20% FBS, glutamine (2mM), penicillin/streptomycin (100 units.ml^{-1}) and human insulin (0.2 units.ml^{-1}) (*Novo Nordisk*). The BH/C1 adenoma line was maintained in 20%FBS/DMEM pre-conditioned by 24 hour exposure to cultured fibroblast cells.

2.1.2.2 Subculture of cells

For passaging, cells were washed with Phosphate Buffered Saline (PBS) before incubation at 37^oC in PBS containing 0.1% (w/v) Trypsin (*Difco, UK*) and 0.1% (w/v) ethylenediaminetetraacetic acid (EDTA; *Sigma, UK*). Trypsinised cells were removed in normal growth medium, collected by centrifugation at 1000*g* for 3 minutes (Precision 100 centrifuge; *Durafuge*), re-suspended in fresh growth medium and re-seeded accordingly.

2.1.2.3 Seeding of experimental cell lines

For experiments, sub-confluent cells were trypsinised and re-suspended in a known volume of normal growth medium. Cell number was determined by use of a Neubauer counting chamber (*BDH, UK*) and cells were centrifuged at 1000g for 3 minutes, then re-suspended in an appropriate volume of fresh growth medium. Depending on experimental requirement, cells were seeded at appropriate densities into tissue culture flasks (T12.5, T25 or T75) or tissue culture plates (6 well, 12 well or 24 well) (*Corning Costar, UK*). For standard experiments, SW480 and HCT116 cells were seeded at a density equivalent to $4x10^4$ cells.cm^2 ($1x10^6$ cells per T25 flask), although densities ranging from $1x10^4$ to $24x10^4$ cells.cm^2 were used for specific experiments (*as detailed in figure legends*).

2.1.3 NITROGEN STORAGE AND RETRIEVAL OF CELLS

To ensure consistency between experiments, cell use was confined within a narrow range of passage numbers and replenished from stocks maintained in liquid nitrogen. For freezing, confluent cells were trypsinised, re-suspended in growth medium and centrifuged at 1000*g* for 3 minutes. Cells were then re-suspended in growth medium containing 10% (v/v) DMSO cryoprotectant (*Sigma*) and dispensed as 1ml aliquots into cryovials (*Nunc, UK*). Cells were frozen slowly in a cryo-1°C-freezing chamber (*Nalgene*) overnight to -70°C and transferred to liquid nitrogen. Cells were recovered by thawing at 37°C and the DMSO/medium was removed by centrifugation. Cells were re-suspended and seeded in normal growth medium and were allowed to recover for two passages before experimental use.

2.1.4 DETERMINATION OF CELL SURVIVAL AND CELL DEATH

Following treatment, adherent living cells were trypsinised and counted as described above (*SECTION 2.1.2.3*, *p56*). The number of detached, floating cells has previously been shown to be indicative of apoptosis level (Hague *et al.*, 1993) and was determined by collection of the growth medium containing floating cells. This was centrifuged at 1000*g* for 3 minutes and cells were re-suspended in 1ml of PBS, with the cellular concentration being determined by use of a Neubauer counting chamber.

2.1.5 TISSUE CULTURE TREATMENTS

2.1.5.1 Butyrate

Sodium butyrate (referred to hereafter as butyrate) is a short-chain fatty acid, which is present at millimolar concentrations in the lumen of the large intestine (Cummings *et al.*, 1987) and has been used previously on various colorectal cell lines (reviewed in Augenlicht *et al.*, 1995). Sub-

confluent SW480, HCT116 and Caco2 cells were treated with 3mM butyrate (*Sigma*) in 10%FBS/DMEM for 48 hours, a physiologically relevant (and non-toxic) dose that induces growth arrest in CRC cell lines (Butt *et al.*, 1997; Qualtrough *et al.*, 2002).

2.1.5.2 MG-132

MG-132 is a widely used broad-spectrum (peptide aldehyde) protease inhibitor, which has previously been shown to act as a potent inhibitor of the γ-secretase complex (De Strooper *et al.*, 1999; Higaki *et al.*, 1995; Klafki *et al.*, 1995; Skovronsky *et al.*, 2000; Steinhilb *et al.*, 2001; Zhang *et al.*, 1999). In line with previous studies (Tsuchiya *et al.*, 2007), sub-confluent SW480 and HCT116 cells were treated for 24 hours with 10μM MG-132 (*Calbiochem*) or DMSO vehicle control (*Sigma*) in 10%FBS/DMEM.

2.1.5.3 Colcemid

Colcemid is a well defined metaphase inhibitor (reviewed in: Deysson, 1968; Rieder and Palazzo, 1992) that prevents spindle fibre formation (Taylor, 1965) and is frequently used in cell cycle analysis and cytogenetic techniques (Gray and Darzynkiewicz, 1987). Flow cytometry studies demonstrate that Colcemid-treated cells accumulate in the G2/M phase of the cell cycle (Dosik *et al.*, 1981). Previous studies have shown that 12 hours of treatment (0.03μg.ml^{-1}) is sufficient to induce a G2/M enrichment of rapidly dividing *in vitro* cell lines, including colorectal adenocarcinoma cells (Barlogie and Drewinko, 1980; Bezrookove *et al.*, 2003; Jha *et al.*, 1994). Sub-confluent SW480 and HCT116 cells were treated over a time course of 6, 12 and 24 hours with Colcemid (0.03μg.ml^{-1}; *Gibco*) diluted in 10%FBS/DMEM without antibiotics. G2/M enrichment was confirmed by cell cycle analysis (*SECTION 0, p70*).

2.1.5.4 Hydroxyurea

Hydroxyurea is a well established and potent cell cycle modulator that causes cells to arrest at G1/S by inhibiting DNA synthesis (Maurer-Schultze *et al.*, 1988; Yarbro, 1992). Hydroxyurea is used clinically as an anticancer agent in various tissues (Bolin *et al.*, 1982; Donehower, 1992; Kennedy, 1992) including the colon (Engstrom *et al.*, 1984). Sub-confluent SW480 and HCT116 cells were treated for 6, 12 and 24 hours with 1mM and 2mM Hydroxyurea respectively (*Sigma*) diluted in 10%FBS/DMEM (without antibiotics). The induction of a G1/S arrest was confirmed by cell cycle analysis (*SECTION 0, p70*).

2.1.6 CELL CYCLE SYNCHRONISATION

Hydroxyurea treatment is also known to induce cell cycle synchronization when applied transiently (Yarbro, 1992) and has previously been used *in vitro* to enrich cyclic cells in

progressive phases of the cell cycle (Gupta and Singh, 1994; Sato *et al.*, 1995; Wang *et al.*, 2002). Analysis was performed in line with these previous studies and doses/timings adapted to specifically match the SW480 and HCT116 cells used. A reversible accumulation of cells in G1/S was attained by treating sub-confluent SW480 and HCT116 cells for 8 hours with Hydroxyurea (1mM and 2mM respectively) in 10%FBS/DMEM (without antibiotics). Untreated controls were also included. Cells were then trypsinised, counted and washed in PBS, before being re-suspended at $2x10^6$ cells.ml^{-1} in 10% FBS/DMEM (without antibiotics) and split equally between two separate tubes. Cells were then diluted to a final concentration of $1x10^6$ cells.ml^{-1} with 10%FBS/DMEM (without antibiotics) ± 6mM Hydroxyurea (3mM final dose) and 2ml of each was seeded into T12.5 tissue culture flasks. Sufficient remaining cells were retained, along with the untreated controls, for cell cycle analysis to confirm treatment efficacy. Removal of Hydroxyurea results in an immediate resumption of cell cycling, leading to synchronous cyclic enrichment. Two parallel flasks of 'cytostatic' (+Hydroxyurea) and 'released' (-Hydroxyurea) cells were harvested at 2 hour intervals for up to 14 hours, with 1 flask being trypsinised for cell cycle analysis and 1 flask undergoing whole cell protein extraction for western blotting analysis (*SECTION 2.5, p65 AND SECTION 0, p70*).

2.2 PLASMID PREPARATION AND SUBCLONING

A range of expression and reporter plasmids were kindly donated by various laboratories and contain the coding sequences or promoter regions of a number of different Notch components and target genes (*TABLE 2-3, TABLE 2-4*).

2.2.1 EXOGENOUS EXPRESSION CONSTRUCTS

Construct	Function	Reference
pcDNA3	*Empty vector control*	Invitrogen
$N1^{IC}$-pcDNA3	*Expression of const. activated N1 ($N1^{IC}$)*	Aster *et al*., 2000
$N1^{FULL}$-pcDNA3	*Expression of full length N1 ($N1^{FULL}$)*	Aster *et al*., 2000
Hes1-pSV2-CMV	*Expression of Hes1*	Akazawa *et al*., 1995
caCSL-pCMX-N	*Expression of const. activated CSL (caCSL)*	Kato et al., 1997
dnTCF4-pcDNA3	*Expression of dominant negative TCF4*	Kolligs *et al*., 1999
pIRES-Neo2	*Empty vector control*	ClonTech
$N1^{IC}$-pIRES-Neo2	*Expression of activated N1*	Cloned from pcDNA3.1

Table 2-3| *Expression constructs used during this study*

2.2.2 PROMOTER REPORTER CONSTRUCTS

Construct	Function	Reference
pGL2	*Empty vector control*	Invitrogen
Hes1-luc (2.5kb)	*Full length Hes1 promoter reporter*	Nishimura *et al*., 1998
Hes1-luc (-467~46)	*N/CSL responsive Hes1 promoter*	Nishimura *et al*., 1998
Hes1-luc (RBP-J^{NEG})	*N/CSL un-responsive Hes1 promoter*	Nishimura *et al*., 1998
Hes2-luc	*Full length Hes2 promoter reporter*	Nishimura *et al*., 1998
Hes3-luc	*Full length Hes3 promoter reporter*	Nishimura *et al*., 1998
Hes5-luc	*Full length Hes5 promoter reporter*	Nishimura *et al*., 1998
TOPFLASH	*LEF/TCF responsive reporter*	Korinek *et al*., 1998
FOPFLASH	*TOPFLASH control reporter*	Korinek *et al*., 1998
CDH1-luc	*E-cadherin promoter reporter*	Batlle *et al*., 2000

Table 2-4| *Reporter constructs used during this study*

2.2.3 PLASMID PREPARATION

DH5α ACW *E. coli* stocks (*Promega*) were used to make competent bugs for transformation and plasmid production. DH5α ACW were grown to log phase in 500ml of Maitland LB broth and used to produce Glycerol stocks of either Chemocompetent or Electrocompetent bacteria, which were maintained at -70°C until required for transformation. Chemocompetent *E. coli* were transformed by resting on ice for 30 minutes in the presence of 5µl of plasmid DNA, then heat shocking for 90 seconds at 42°C and returning to ice for a further 15 minutes. Electrocompetent *E. coli* were transformed with 2µl of plasmid DNA and electroporated using a BioRad Gene Pulser module (*BioRad, USA*). Transformed *E. coli* were then supplemented with 400µl of SOC (Super Optimal broth with Catabolite repression) medium and incubated at 37°C with shaking for 1 hour. *E. coli* were plated out on nutrient agar plates (*Maitland, UK*) containing 50µg.ml^{-1} Ampicillin and incubated at 37°C overnight. A single colony was used to inoculate 10ml of LB broth containing 50µg.ml^{-1} of Ampicillin and incubated with shaking at 37°C overnight. 5ml of the resultant inoculum was then spun down at 6000*g* for 15mins and plasmid purified using Qiagen miniprep kit (*Qiagen*). 1ml of inoculum was used to inoculate 500ml of LB broth, which was incubated overnight at 37°C with shaking. The plasmids were extracted using Qiagen maxiprep kit (*Qiagen*) and re-suspended in TE buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20 (v/v), pH9.0). The final concentration and purity were determined by dsDNA analysis in an Eppendorf BioPhotometer (*Eppendorf, UK*) and purified plasmids in TE buffer were maintained at 4°C.

2.2.4 RESTRICTION DIGESTS AND AGAROSE GEL ANALYSIS

To ensure correct vector and insert size, the inserted region of each plasmid was excised using appropriate restriction enzymes (*New England Biolabs, UK*). DNA was then separated by size on 0.7% (w/v) agarose Tris-acetate-EDTA (TAE) gel containing 0.005% (v/v) Ethidium Bromide (*Sigma*) and run at 100V for 50 minutes. Bands were visualised using UV light (302nm).

2.2.5 SUB-CLONING PROTOCOL

An expression plasmid encoding the active $N1^{IC}$ protein was obtained from J. Aster (Aster *et al.*, 2000) and used for preliminary analysis. The *$N1^{IC}$* sequence was originally inserted into pcDNA3 vector (*FIGURE 1-1A*) using *Bam*HI (5') and *Eco*RI (3') restriction enzymes (*J. Aster – Personal correspondence*). The pcDNA3 vector is now known to interfere with several *in vitro* assays and the pIRES-Neo2 plasmid is preferred by recent studies. Therefore, the $N1^{IC}$ encoding region was sub-cloned from pcDNA3.1 into pIRES-Neo2 for use in all promoter reporter assays performed during this study. The *$N1^{IC}$* insert was restricted from the original vector using *Bam*HI and *Not*I and isolated by 0.7% (w/v) Agarose TAE gel electrophoresis. After excising the correct band, the insert was extracted using QIAquick gel extraction kit (*Qiagen*). The insert was purified using QIAquick PCR purification kit (*Qiagen*) and dephosphorylated using shrimp alkaline phosphatase (*Promega*). A compatible version of pIRES-Neo2 was created by *Bam*HI and *Not*I restriction of the multiple cloning site (*FIGURE 1-1B*) and the insert was ligated using a T4 ligation kit (*Promega*). The resultant plasmid was transformed into electrocompetent bugs and single colonies were picked for plasmid extraction by Miniprep (Promega). Correct banding pattern was determined by *Bam*HI and *Not*I restriction digest before running on a 0.7% (w/v) Agarose TAE gel. The insert was sequenced by Dundee University sequencing service to ensure correct coding and orientation.

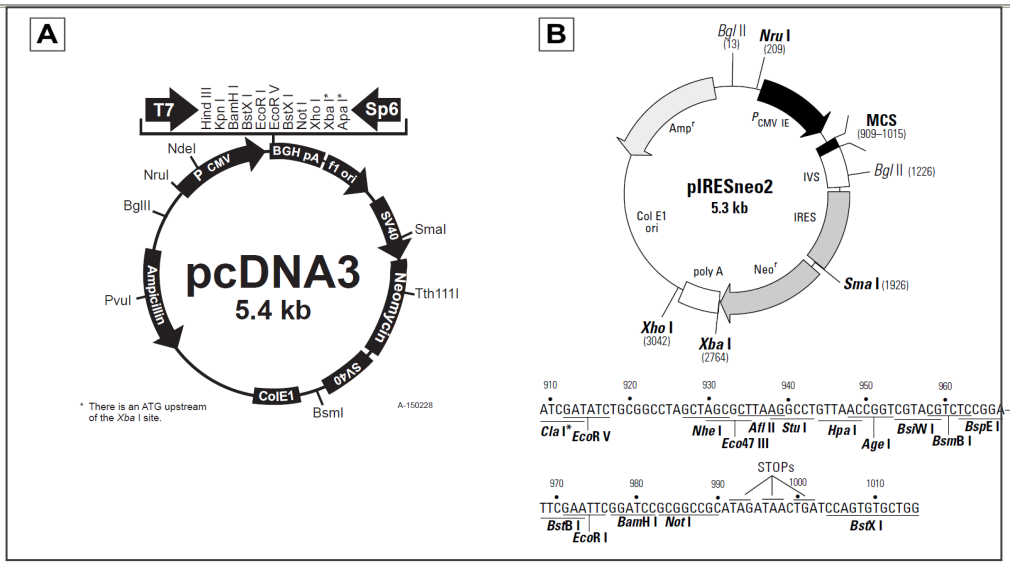


Figure 2-1| *Restriction map and multiple cloning sites of pcDNA3 and pIRESneo2.* The insert encoding $N1^{IC}$ was previously inserted into the pcDNA3 (*Invitrogen*) [**A**] vector using *Bam*HI (5') and *Eco*RI (3') (Aster *et al.*, 2000). For the purposes of this study, $N1^{IC}$ was excised using *Bam*HI/*Not*I and inserted into the corresponding multiple cloning site of the pIRESneo2 vector (*Clontech*) [**B**]. Images were obtained from the manufacturers' documentation.

2.3 STANDARD TRANSFECTION PROCEDURES

2.3.1 TRANSIENT TRANSFECTION

For all cell lines, cells were taken from sub-confluent flasks and seeded for 48 hours prior to transfection. Cells were transiently transfected with 5µg of total DNA per T25 tissue culture flask (or equivalent) in the presence of the associated transfection reagent. Optimal SW480 transfection was achieved using Superfect transfection reagent (*Qiagen*), while Lipofectamine (*Invitrogen*) was used for HCT116, all carried out according to the manufacturers' instructions. Details of the expression and reporter constructs used can be found above (*SECTION 2.2.1, p58/SECTION 0, p59*).

2.3.1.1 Superfect reagent protocol

For standard transfection of SW480 cells in a T25 tissue culture flask, 5µg of total DNA was diluted in 150µl of serum free medium (SFM; Optimem - *Gibco*) with 30µl Superfect, thoroughly mixed and incubated for 10 minutes at room temperature. The cells were washed once with PBS and incubated with the DNA-reagent complex mixed in 1ml of standard growth medium (10% FBS/DMEM). Following 3 hours of incubation, the transfection mix was replaced with fresh growth medium and cells were grown under standard conditions for a further 24-48 hours before harvest. Scaled measures were used for cells growing in different tissue culture wells/flasks.

2.3.1.2 Lipofectamine reagent protocol

For standard transfection of HCT116 cells grown in a T25 tissue culture flask, 5µg of total DNA was diluted in 250µl of serum free medium (SFM; Optimem - *Gibco*). 10µl of lipofectamine transfection reagent (*Invitrogen*) was mixed in another 250µl of Optimem and incubated for 5 minutes at room temperature. The two complexes were combined and incubated for a further 20 minutes. Cells were washed in PBS and incubated for 6-12 hours with the DNA-reagent complex mixed in a further 1.5mls of Optimem. The transfection mix was replaced with fresh growth medium (10% FBS/DMEM) and cells were grown under standard conditions for a further 24-48 hours before harvest. Scaled measures were used for cells growing in different wells/flasks.

2.3.2 STABLE TRANSFECTION

Stable transfectants were produced by transfection of sub-confluent flasks of SW480, HCT116, RG/C2, HT29 and AA/C1 using Lipofectamine transfection reagent (*Invitrogen*), with 48 hours under normal conditions prior to selection. The minimum inhibitory dose of G418 (*Sigma*) for each cell line was pre-determined as 600µg.ml^{-1} for SW480, 500µg.ml^{-1} for HCT116, 250µg.ml^{-1} for HT29, 400µg.ml^{-1} for RG/C2 and 800µg.ml^{-1} for AA/C1. Normal growth medium containing an appropriate G418 concentration was applied to transfected cells for 2-5 weeks and single resistant colonies were isolated and reseeded separately. Clonal stable transfectants were then maintained under normal growth conditions but in the continued presence of G418.

2.3.3 RNA INTERFERENCE

To avoid the pleiotropic effects of Notch pathway inhibitors (i.e. γ-secretase inhibitors), specific pathway inhibition was instead achieved by treatment with small interfering RNA (siRNA). siGENOME SMARTpool siRNA (*Dharmacon*) was obtained, targeting either *N1* or the common Notch co-factor *CSL*; leading to N1 or pan-Notch inhibition respectively. A scrambled control (*Dharmacon*) was also obtained for comparison. These oligonucleotides were re-suspended to 10µM under RNase free conditions and stored at -20^{o}C, as per the manufacturer's instructions.

All siRNA treatments were performed under RNase free conditions and transient transfection was performed on cells in suspension. Different siRNA doses and treatment times were tested and it was found that 72 hours with a 50nM treatment achieved optimal protein knockdown and pathway inhibition in the HCT116 cell line. No knockdown was achievable in SW480 cells. To maximise transfection efficiency, HCT116 cells were also medium changed with standard growth medium (10% FBS/DMEM) without antibiotics 24 hours prior to transfection. For a T25 tissue culture flask, 12µl of lipofectamine (*Invitrogen*) was mixed with 500µl of Optimem (*Gibco*) and

left to incubate for 5 minutes at room temperature. 20µl of siRNA oligonucleotide (10µM stock) was simultaneously mixed with another 500µl of Optimem. At the end of 5 minutes, the two solutions were combined and incubated for a further 20 minutes. Meanwhile, cells were trypsinised, counted and washed in PBS. Cells were re-suspended in Optimem to a concentration of $1x10^6$ cells.$1.5ml^{-1}$ and 3ml ($2x10^6$ cells) was added to the reaction mixture before being transferred to a flask. After 6 hours, the transfection mix was removed and replaced with normal growth medium (10% FBS/DMEM) and cells were harvested after 72 hours.

2.3.4 DUAL LUCIFERASE REPORTER ASSAY

2.3.4.1 Cell seeding

For analysis of promoter activity, the various promoter reporter constructs (*TABLE 2-4*) were transiently transfected into cells. In general this was performed on cells grown in 24 well plates (*Corning Costar, UK*) at $8x10^4$ cells.$well^{-1}$ (equivalent to $1x10^6$ cells in a T25 tissue culture flask). Cells were grown under standard growth conditions for 48 hours prior to transfection. SW480 cells were transfected using Superfect transfection reagent (*Qiagen*), while Lipofectamine (*Invitrogen*) was used for HCT116.

2.3.4.2 Superfect reagent protocol

For reporter and expression construct co-transfection, 1.08µg of each expression and reporter construct was mixed with 0.24µg *Renilla* (expression control plasmid; *Promega*) in 180µl of Optimem (2.4µg DNA in total) (*per triplicate wells*). Following the addition of 15µl of Superfect reagent, the solution was thoroughly mixed and incubated for 10 minutes at room temperature. The cells were washed with PBS and this was thoroughly removed to avoid dilution effects. The DNA-reagent complex was further diluted in 1050µl of standard growth medium (10% FBS/DMEM) and applied to the cells. After 3 hours, the transfection mix was replaced with fresh growth medium and the cells were maintained under standard conditions until harvested, normally after 24-48 hours.

2.3.4.3 Lipofectamine reagent protocol

For reporter and expression construct co-transfection experiments (per triplicate wells), 1.08µg each of expression and reporter construct were mixed with 0.24µg *Renilla* construct in 150µl of Optimem (2.4µg DNA in total). 6µl of Lipofectamine reagent was mixed in a separate 150µl of Optimem and incubated at room temperature for 5 minutes. The two reaction mixtures were combined with 1200µl of Optimem and incubated for a further 20 minutes. The cells were washed with PBS, which was thoroughly removed to avoid dilution effects, and 500µl of the

reaction mixture was added to each well. After 6 hours, the transfection mix was replaced with fresh growth medium (10% FBS/DMEM) and the cells were maintained under standard conditions until harvested, normally after 24-48 hours.

2.3.4.4 Passive cell lysis and luminescent analysis

Irrespective of transfection procedure, cells were lysed in 100µl.well^{-1} of 1X Passive Lysis Buffer (PLB) (*Promega, USA*). After 15 minutes, lysates were transferred to an Eppendorf tube for analysis or storage at -70^{o}C. Cell lysates were assayed using Promega Dual Luciferase Reporter assay system (*Promega*) on a Jade luminometer (*Labtech, UK*). Samples were corrected for background emission against the average of five readings from an untransfected control. Firefly luciferase activity was then calculated relative to the *Renilla* control and average readings were determined from three separate experiments performed in triplicate.

2.4 PROMOTER ANALYSIS

2.4.1 DETERMINATION OF THE HES1 ENHANCER SEQUENCE

The *Hes1* gene is located at *3q28-29* (*193,853,934-193,856,396*) in human and *16b2* (*300,654,43-30067882*) in the mouse (Feder *et al.*, 1993). The Hes1 5' regulatory region (enhancer and promoter region) contains a transcriptional start site 31 nucleotides downstream of a TATA box and transcriptional initiation occurs ~250 nucleotides upstream of the translation initiation codon (Takebayashi *et al.*, 1994). In *Drosophila Hes1,* all transcriptional regulation is thought to occur in the first 400-500bp upstream of the transcriptional start site (Cooper *et al.*, 2000). Therefore, the genomic sequence 1000 base pairs upstream of the *Hes1* (human and murine) transcriptional (5') start site (ATG) was identified using NCBI Map Viewer (Feolo *et al.*, 2000) [*http://www.ncbi.nlm.nih.gov/projects/mapview*] and saved in Microsoft Office Word format (*Microsoft*) for analysis.

2.4.2 PROMOTER AND TRANSCRIPTION FACTOR BINDING SITE ANALYSIS

The human and murine regulatory region was analysed for various putative transcription factor binding sites (TFBS) using on-line PROMO3.0 software (*Alggen*) (Farre *et al.*, 2003; Messeguer *et al.*, 2002) [*http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo*]. All PROMO3.0 searches are based on highly accurate sequence data from the TRANSFAC binding site database. CSL recognises the general DNA sequence CGTGGGAA (Tun *et al.*, 1994) which has previously been identified in the *Hes1* promoter region (Rodilla *et al.*, 2009; Takebayashi *et al.*, 1994; Wettstein *et al.*, 1997). The primary aim was to identify sequences related to Wnt pathway factors in the *Hes1*

promoter/enhancer region. The consensus sequences of the four vertebrate Lef/Tcf proteins and *Drosophila* Tcf are all highly conserved and resemble the general consensus AGATCAAAGG (Korinek *et al.*, 1997; van de Wetering *et al.*, 1997); however, subtle variations do exist between each one for specific binding (Hecht and Stemmler, 2003). To determine the degree of consensus sequence conservation, both the human and murine sequences were also aligned using the on-line EMBOSS Pairwise Alignment Algorithms (Rice *et al.*, 2000; Sarachu and Colet, 2005) [*http://www.ebi.ac.uk/Tools/emboss/align/index.html*].

2.5 WESTERN BLOTTING ANALYSIS

2.5.1 SAMPLE PREPARATION FROM TRYPSINISED CELLS

In experiments where cells were harvested by trypsinisation and counted (*SECTION 2.1.4*, *p56*), aliquots were frequently retained for analysis by western blotting. Counted cells were re-suspended in PBS to a concentration of 2 x 10^6 cells.ml^{-1} and 1ml aliquots were then transferred to 1.5ml Eppendorf tubes. Cell pellets were obtained by centrifugation at 1000*g* for 3 minutes and the PBS was aspirated. Cell pellets were boiled in 80µl of sample lysis buffer (*TABLE 2-5*) for 5 minutes and then stored at -20^{o}C until required.

Buffer	Composition
Sample Lysis Buffer	*62mM Tris-HCl pH6.8* *10% (v/v) Glycerol* *5% (v/v) 2-mercaptoethanol* *4% (w/v) SDS* *0.01% Bromophenol Blue*
TG Lysis Buffer	*20mM Tris-HCL (pH7.5)* *150mM NaCl* *1mM EDTA* *1% (v/v) Triton X-100* *2.5M Sodium Pyrophosphate* *1mM β-glycerophosphate* *1mM Na_2VO_4* *Protease Inhibitor Cocktail Mini (Roche Diagnostics)*
5X Laemmli Sample Buffer	*312mM Tris-HCl (pH6.8)* *50% (v/v) Glycerol* *25% (v/v) 2-mercaptoethanol* *10% (w/v) SDS* *0.05% (w/v) bromophenol blue*

Table 2-5| *Sample buffer composition*

2.5.2 WHOLE CELL PROTEIN EXTRACTION

To avoid significant cleavage of cellular proteins, cell samples were mostly prepared by lysing the cells *in situ* using TG lysis buffer (*TABLE 2-5*) containing protease inhibitors (*Roche, UK*). The

growth medium was aspirated, the cells were washed twice in ice cold PBS and an appropriate amount of TG lysis buffer was added. Cells were incubated on ice for 15 minutes before scraping and then transferred to a 1.5ml Eppendorf tube. Lysates were centrifuged at 18500*g* for 10 minutes at 4°C and, if not used immediately, were stored at -70°C. Protein concentration was quantified using Bio-Rad D_c protein assay kit (*BioRad, USA*) and samples were made to a protein concentration of 500 or 1000µg.µl^{-1} with Elgastat water (ddH_20) plus 5X Laemmli buffer (⅕ total volume) (*TABLE 2-5*). Samples were then boiled for 5 minutes and stored at -20°C.

2.5.3 SDS-PAGE TECHNIQUE

Proteins from cell lysates (trypsinised or whole cell protein extracts) were separated by protein size using Sodium Dodecyl Sulphate Polyacylamide Gel Electrophoresis (SDS-PAGE) and western blotting analysis. The Mini-Protean 3 electrophoresis system (*BioRad, USA*) was used to cast 7.5-13% acrylamide resolving gels (1.5mm thick) (*TABLE 2-6*), depending on the protein size of interest. A 4.5% acrylamide stacking gel was also poured around a 10 or 15 well comb for sample loading. Two formed gels were loaded into each electrophoresis cell and submerged in running buffer (*TABLE 2-7*). Equal and appropriate volumes of samples were loaded into each lane, usually equalling 100µg protein. 10µl of Precision Weight Marker (*BioRad, USA*) was loaded into a spare well and samples were electrophoresed at 200V for approximately 1 hour until no stain front remained visible.

Gel composition	**Resolving gels (x2)**			**Stack gel (x2)**
	7.5%	**10%**	**13%**	**4.5%**
Acrylamide / Bis-Acrylamide 30% solution (Severn Biotech, UK)	4.4ml	5.8ml	7.6ml	0.6ml
1.5M Tris pH 8.8, 0.4% SDS	4.4ml	4.4ml	4.4ml	-
0.5M Tris pH 6.8, 0.4% SDS	-	-	-	1.1ml
ddH_20	8.7ml	7.3ml	5.5ml	2.2ml
50% (w/v) Ammonium Persulfate (APS)	110µl	110µl	110µl	29µl
N,N,N',N'-Tetramethylethylenediamine (TEMED; BioRad, UK)	3.6µl	3.6µl	3.6µl	0.9µl

Table 2-6| *SDS-PAGE acrylamide gel composition*

Buffer	Composition
Running Buffer	*192mM Glycine* *25mM Tris* *0.1% (w/v) SDS*
Transfer Buffer	*192mM Glycine* *25mM Tris* *20% (v/v) Methanol* *(Methanol replaced with water for large proteins)*
Milk Block Buffer	*10mM Tris-HCL pH7.4* *150mM NaCl* *4% (w/v) skimmed milk powder*
Tween Wash Buffer	10mM Tris-HCL pH7.4 150mM NaCl 0.2% (v/v) Tween-20
Stripping Buffer	62.5mM Tris HCl, pH 6.7 100mM 2-mercaptoethanol

Table 2-7| *Western blotting buffer composition*

2.5.4 PROBING FOR PROTEINS OF INTEREST

Following SDS-PAGE, proteins were transferred onto an Immobilon-P PVDF (polyvinylidine difluoride) membrane (*Millipore, USA*) by electroblotting in a Transblot Cell (*BioRad, USA*). Proteins were transferred in cold transfer buffer (*TABLE 2-7*) at 120V for approximately 1.5 hours. The protein-loaded membrane was then blocked in milk block buffer (*TABLE 2-7*) for 1 hour at room temperature (or overnight at 4^{o}C) prior to antibody application. Primary antibodies (*TABLE 2-8*) were diluted to an appropriate concentration in milk block buffer and incubated with the membrane overnight at 4^{o}C. The membrane was then washed with successive rounds of milk block buffer and tween wash buffer (*TABLE 2-7*) before being incubated for 1 hour at room temperature with an appropriate Horse Radish Peroxidase (HRP) conjugated secondary antibody (diluted to 1:1000 in milk block buffer; *Sigma, USA*). Further milk and tween washes were performed and the protein bands were visualised by enhanced chemiluminesence (ECL) (*LumiGLO, USA*) on light sensitive films (*Kodak, UK*). Loading accuracy was subsequently assessed by analysis using primary antibodies raised against either β-actin or α-Tubulin (*Sigma*).

2.5.5 STRIPPING AND RE-PROBING

In order to re-probe membranes, stripping of previous antibodies was performed by submerging in stripping buffer (*TABLE 2-7*) and incubating at 50^{o}C for 30 minutes. The membrane was then washed in tween wash buffer and milk block buffer before application of new primary antibody.

2.5.6 DENSITOMETRIC ANALYSIS

In order to quantify differences in protein expression observed by western blotting analysis, the films were scanned (600dpi) using Photoshop software (*Adobe*) and densitometric analysis was performed using Scion Image analysis software (*ScionCorp, USA*). All densitometric analysis was normalised to a common band on each film and for protein screening experiments, averages are representative of data from three separate experiments.

1° Antibody	Source	Dilution	Origin
Notch 1 (bTan20)	*DSHB (USA)*	1:200	Rat
Notch 1 (C20-R)	*SantaCruz (sc6014r; USA)*	1:200	Rabbit
Hes1	*SantaCruz (USA)*	1:200	Rabbit
Hes1	*A kind gift of T. Sudo (Japan)*	1:1000	Rabbit
Jagged1	*SantaCruz (USA)*	1:200	Rabbit
pan-Delta	*SantaCruz (USA)*	1:200	Goat
E-cadherin	*Transduction labs (USA)*	1:8,000	Mouse
Slug	*SantaCruz (USA)*	1:200	Rabbit
Snail	*SantaCruz (USA)*	1:200	Rabbit
Twist	*SantaCruz (USA)*	1:200	Rabbit
Tcf-4	*Sigma (USA)*	1:200	Mouse
c-Myc	*Oncogene (USA)*	1:100	Mouse
p21	*Oncogene (USA)*	1:100	Mouse
CDK2	*Santa Cruz (USA)*	1:1000	Rabbit
CDK4	*Santa Cruz (USA)*	1:1000	Rabbit
β-actin	*Sigma (USA)*	1:10,000	Rabbit
α-Tubulin	*Sigma (USA)*	1:10,000	Mouse

Table 2-8| *Primary antibody concentrations for immunoblotting*

2.6 IMMUNOCYTOCHEMISTRY

2.6.1 IMMUNOCYTOCHEMICAL ANALYSIS

SW480, HCT116 and HT29 cells were seeded onto sterile 19mm glass coverslips (*VWR international*) at 1.2 x 10^5 cells.well^{-1} in 12 well plates (*Corning Costar, UK*). Cells were grown under standard growth conditions for 48 hours prior to transient transfection with pcDNA3.1-N1^{IC} or pcDNA3.1-N1^{FULL}, vs. untransfected control (*SECTION 2.3.1, p61*). Cells were about 60% confluent at the end of the experiment. For confluency-specific experiments, SW480 and HCT116 cells were seeded in the same way but also at low (0.3x10^5 cells.well^{-1}) or high (2.4x10^5 cells.well^{-1}) density and grown for 72-96 hours under standard growth conditions. At the end of the experiment the growth medium was removed and cells were washed once with PBS before being fixed for 15 minutes in 4% (w/v) paraformaldehyde (PFA) containing 0.1% (v/v) Triton X-100

(*BDH, UK*). Cells were blocked for 20 minutes in PBS containing 2% (w/v) BSA (Bovine serum albumin; *Sigma*) and then incubated for 1 hour at room temperature with anti-N1 primary antibody (1:100; C20/sc6014 goat or rabbit polyclonal; *Santa Cruz, USA*) diluted in 0.5% (w/v) BSA/PBS. After three 5 minute washes in PBS, cells were then incubated for 1 hour in the dark with donkey-anti-goat or goat-anti-rabbit AlexaFluor488 (*GREEN*) conjugated secondary antibody (1:100; *Molecular Probes, USA*). For some experiments, cells were also co-stained using anti-E-cadherin primary (1:100; *Transduction labs, USA*) and goat-anti-mouse AlexaFluor594 (*RED*) conjugated secondary antibody (1:100; *Molecular Probes, USA*). Following antibody adhesion, cells were washed for a further three times and then incubated for 5 minutes in 1:10,000 DAPI nuclear counterstain (*Sigma*). Cells were then mounted on glass slides using Mowiol (*Calbiochem*) containing 25mg.ml^{-1} DABCO (1,4-diazobicyclo(2,2,2)-octane) stain preserver (*Sigma*). Positively stained cells were visualised using the inverted Leica TCS-NT confocal laser-scanning microscope and images were acquired/compiled using Leica Confocal Software (*Leica, Germany*).

2.7 FLOW CYTOMETRY

Flow assisted cell cytometry was used to analyse protein expression and cell cycle distribution following various treatments. As outlined above, these included Notch activation (CTR vs pcDNA3.1-N1^{IC}), Notch inhibition (siRNA: *siCTR*, *siN1*, *siCSL*), or treatment with butyrate, colcemid or hydroxyurea.

2.7.1 IMMUNOFLUORESCENT STAINING

Following appropriate treatment, cells were trypsinised and counted before centrifugation at 1000*g* for 3 minutes. Cells were then washed in 5ml PBS with 0.1% (v/v) Triton X-100 (PBS/Triton), re-centrifuged and re-suspended at 2x10^6 cells.ml^{-1} in 2% (w/v) BSA in PBS/Triton. Cells were maintained on ice for 20 minutes to block, then gently mixed and 2ml was distributed to each FACS tube (*BD Biosciences, UK*). Cells were spun for 5 minutes at 600*g* and pellets re-suspended in 100µl of primary anti-N1 antibody for incubation at 4^{o}C for 1 hour with constant agitation (1:100 in 0.5% (w/v) BSA in PBS/Triton; C20-R/sc6014 rabbit polyclonal; *Santa Cruz, USA*). Cells were then washed in 4ml of cold FACS wash buffer (PBS/Triton supplemented with 5% foetal calf serum and 0.1% (w/v) sodium azide) and spun for 5 minutes at 600*g*, re-washed and re-spun. Due to constraints of overlapping emission spectrums, all cells (except controls) were stained for either N1 and E-cadherin or N1 and Propidium iodide (PI). Cells were re-suspended in 100µl of 0.5% (w/v) BSA in PBS/Triton containing goat-anti-rabbit AlexaFluor488 (*GREEN*) conjugated secondary antibody (1:100; *Molecular Probes, USA*) ± directly conjugated mouse anti-

E-cadherin antibody (AlexaFluor647 (*RED*); *BD Biosciences, UK*) and incubated at 4°C for 1 hour with constant agitation. After washing twice in cold FACS wash buffer, cells were then fixed for 30 minutes at 4°C in 70% ethanol. Fixed cells were washed twice in cold FACS wash buffer, spun for 5 minutes at 600*g* and finally re-suspended in 1ml FACS wash buffer. Cells not dual labelled for E-cadherin were stained using PI (*SECTION 2.7.2, p70*) and all cells were either analysed immediately or maintained at 4°C in the dark.

2.7.2 PROPIDIUM IODIDE STAINING

PI is routinely used for cell cycle analysis of DNA content and enables determination of the percentage cells in each phase of the cell cycle (Crissman and Steinkamp, 1973). Just prior to analysis, re-suspended cells (1ml) were incubated for 5 minutes at RT with 10µg.ml^{-1} RNase-A (*Sigma*) to remove endogenous RNA. Following RNase-A treatment, 400µl of PI was added (from stock of 50 µg.ml^{-1} in PBS; *Sigma*) and cells were incubated for 5 minutes before analysis.

2.7.3 FLOW CYTOMETRIC TECHNIQUE

Flow analysis was performed using a BD FACSCanto flow cytometer and BD FACSDiva 6.0 analysis software (*BD Biosciences, UK*). Non-apoptotic cells of an appropriate size were selected on the basis of forward scatter (FSC-A) vs. side scatter (SSC-A). Single cells were then gated on the basis of DNA dot-plot distribution and their cell cycle position was visualised by DNA histogram. A total of 10,000 events for each sample were measured and all experiments were repeated a minimum of three times. The specificity of N1 immunoreactivity in each case was confirmed by comparison with unstained and secondary (2°) only staining controls. The mean increase in N1 fluorescence compared to 2° only controls was 232% in SW480 and 304% in HCT116. The mean increase in E-cadherin fluorescence compared to unstained controls (directly labelled) was 1140% in SW480 and 1228% in HCT116. Computational analysis of cellular immunofluoresence was performed using the BD FACSDiva 6.0 (*BD Biosciences, UK*) and FlowJo (*Tree Star, USA*) software, while detailed cell cycle analysis was performed using ModFit software (*Verity Software House, USA*). Comparisons between cell cycle analysis and immunofluoresence were performed using FlowJo (*Tree Star, USA*) software.

2.8 MIGRATION ASSAY

2.8.1 TRANSWELL FILTER ASSAY

Cell migration assays were carried out using a transwell filter migration assay as previously described (Efstathiou *et al.*, 1999; Qualtrough *et al.*, 2007). Insert filters, 8µm in pore size (*BD*

Biosciences, UK), were coated with 500µl of 10µg/ml Vitrogen type I collagen (*Cohesion, USA*). 48 hours prior to assay, HCT116 cells were transiently transfected in suspension with siRNA targeting *N1* or *CSL* vs. scrambled control (*SECTION 2.3.3, p62*). 24 hours prior to assay, additional flasks of adherent HCT116 cells were transfected with either pcDNA3.1, $N1^{IC}$ or caCSL for comparison with untransfected control (*SECTION 2.3.1, p61*). Following treatment cells were prepared for motility assays, while a sample was also simultaneously prepared for western blotting to confirm the efficacy of transfection. Pre-treated cells were lifted using versene (0.1% w/v EDTA in PBS), counted and re-suspended to a final concentration of $1x10^5$ cells.ml^{-1} in calcium free-DMEM/2% FBS. The lower chamber was filled with calcium free-DMEM/5%FBS to act as an attractant and 2ml of cell solution ($2x10^5$ cells) was added to each filter. After 24 hours of incubation at 37°C, cells attached to the filter were fixed for 10 minutes in 100% methanol and stained using Mayer's haematoxylin (*BDH, UK*). Cells were removed from the upper filter surface with a cotton swab, while those on the lower filter surface were considered migratory and counted in 10 separate fields at x20 magnification.

2.9 SEQUENTIAL CRYPT FRACTIONATION

2.9.1 ISOLATION OF SEQUENTIAL MURINE CRYPT SAMPLES

The sequential isolation (*Weiser fractionation*) of intestinal epithelial cells along the crypt (-villus) axis has previously been described and validated in a number of species (Ferraris *et al.*, 1992; Mariadason *et al.*, 2005; Smartt *et al.*, 2007; Weiser, 1973a; Weiser, 1973b). Much of this previous work was carried out by Augenlicht and colleagues and analysis performed here was guided and assisted by H. Smartt [*HS*], formerly of the Augenlicht laboratory. In line with previous studies (Mariadason *et al.*, 2005), this experiment was performed in triplicate using untreated, freshly dissected, wild type adult C57BL/6 mice (2-3 months old).

The small and large intestines were separately dissected [*HS*] and flushed thoroughly with PBS/Ca^{2+}/Mg^{2+}/DTT (*TABLE 2-9*). One end of both tissues was tied off for everting and filling to distension with plain PBS. Tissues were incubated for 5 minutes in pre-warmed buffer A (*TABLE 2-9*) at 37°C before being transferred to 20ml buffer B (*TABLE 2-9*) for sequential fractionation. Tissues were then incubated with shaking at 37°C before being gently and completely eluted at the appropriate times (*TABLE 2-10*) and stored on ice. To avoid contamination between fractionation samples, an additional 5ml wash was also performed and added to the fractionated sample. A fresh 20ml aliquot of buffer B was then added to the tissue. All resulting samples were spun down for 8 minutes at 4°C/600*g* and re-suspended in 1ml PBS/DTT/PMSF for transfer to a

pre-cooled eppendorf tube. After centrifugation for 5 minutes at 4°C/600*g,* the supernatant was removed and frozen at -70°C. The protein concentration was determined and protein expression was analysed by western blotting (*SECTION 2.5, p65*).

Solution	Constituent	Quantity
PBS/Ca2+/Mg2+/DTT	PBS	*500ml*
(*pH7.4*)	$MgCl_2$	*0.05g*
	$CaCl_2$	*0.05g*
	DTT	*1mM - 500µl (1M stock)*
Buffer A	PBS	*500ml*
(*pH7.4*)	KCl	*1.5mM (3.75ml of 2M stock)*
	NaCl	*96mM (2.81g)*
	Sodium Citrate	*27mM (27ml of 0.5M stock)*
	KH2PO4	*8mM (0.54g)*
	Na2HPO4	*5.6mM (0.75g)*
	DTT	*1mM (500µl of 1M stock)*
Buffer B	PBS	*500ml*
(*pH7.4*)	EDTA	*1.5mM (1.5ml of 0.5M stock)*
	DTT	*0.5mM (250µl of 1M stock)*
PBS/DTT/PMSF	PBS	*50ml*
(*pH7.4*)	DTT	*1mM - 50µl (1M stock)*
	PMSF	*0.2mM - 50µl (20mM stock)*

Table 2-9| ***Buffer compositions for sequential fractionation lysis***

Fraction N°	SI - time (mins)	LI - time (mins)
1	*10*	*30*
2	*10*	*30*
3	*6*	*30*
4	*5*	*30*
5	*5*	/
6	*9*	/
7	*10*	/
8	*15*	/
9	*25*	/
10	*30*	/

Table 2-10| ***Sequential large and small intestinal crypt fraction times***

2.10 IMMUNOHISTOCHEMICAL ANALYSIS

2.10.1 HUMAN TISSUES AND ETHICAL APPROVAL

Surgically resected human tissues (paraffin-embedded) were selected and anonymised by a qualified histopathologist from tissue archives at the Bristol Royal Infirmary, Department of Histopathology. All tissues were obtained under ethical approval from the North Somerset & South Bristol Research Ethics Committee (*REC Ref # 05/Q2003/54 "The role of Notch signalling in*

colorectal cancer and gastrointestinal disease"). Tissues were selected to represent the various stages of human colorectal carcinogenesis, from non-neoplastic mucosa, through adenoma to adenocarcinoma. Representative 'normal' human colorectal epithelium was provided by analysis of 17 tumour resection margins (TRMs), taken at the time of tumour surgery but from the distant margins of resection; plus 12 diverticulitis resection margins (DRMs), a non-neoplastic inflammatory condition which frequently requires surgical intervention. A total of 57 benign colorectal tumours were selected, including 13 tubular, 38 tubulovillous and 6 villous adenoma. Malignant colorectal tissues (*n=49*) included 13 well, 14 moderately and 22 poorly differentiated cases of colorectal adenocarcinoma. Tissues from inflammatory bowel disease (IBD) patients were also included and comprised 29 cases of ulcerative colitis (UC) and 4 cases Crohn's disease (CD). 3 cases of cervical (Veeraraghavalu *et al.*, 2004; Zagouras *et al.*, 1995) and lung (sc-6014 data sheet, *Santa Cruz, USA*) cancer were also included for positive control purposes during protocol development.

2.10.2 IMMUNOHISTOCHEMICAL TECHNIQUES

2.10.2.1 Tissue sectioning and rehydration

Tissues were formalin fixed and paraffin embedded. The embedded tissues were sectioned at a thickness of 5µM and left overnight at 35^{o}C to anneal to glass Polylysine coated slides (*VWR*). Tissues were de-paraffinised and rehydrated using Histoclear (*National Diagnostics*) (2 x 2 minutes) and decreasing alcohol (methylated spirit) concentrations (1 minute in each: 1 x 100%, 2 x 70%, 1 x Tap water). Endogenous peroxidases were then quenched with 3% (v/v) H_2O_2 (*BDH, UK*) and either subjected to antigen retrieval or rested in a solution of PBS with 0.1% (v/v) Triton X-100 (referred to hereafter as PBS/Triton) for 2 minutes. Where required, antigen retrieval was performed by means of microwaving in 0.1M citrate buffer (pH 6.0; 20 mins) (Shi *et al.*, 1995) before transfer to PBS/Triton for two minutes. Antigen retrieval is often required, depending on antigen and antibody, to remove formalin cross links and other fixation artefacts that may mask the antigenic target (Cregger *et al.*, 2006). Serial tissue sections were used to stain for both Notch1 and Hes1 by immunohistochemistry, as well as H&E (Haematoxylin and Eosin) detail of tissue structure. All immunohistochemical protocol development was carried under the guidance of experienced histopathologists [*M. Pignatelli (MP) and N. Banu (NB)*] and a histopathology technician [*J. Baker - JB*].

2.10.2.2 Notch1 (C20-goat) immunohistochemistry

Analysis using C20-goat (C20G) anti Notch1 primary antibody (sc6014 goat polyclonal; *Santa Cruz, USA*) was carried out in line with previous studies (Ando *et al.*, 2003; Baldi *et al.*, 2004; Veeraraghavalu *et al.*, 2004). No antigen retrieval was required and tissues were blocked for 10 minutes in 5% rabbit serum (*Dako, USA*) made up in PBS/Triton. Tissues were incubated with primary antibody (1:50) made up in 5% serum for 3 hours at room temperature, then 16h overnight at 4°C.

2.10.2.3 Notch1 (C20-rabbit) immunohistochemistry

At the time of this study, no previous studies had been performed using the more recent C20-rabbit (C20R) variant (sc6014r rabbit polyclonal; *Santa Cruz, USA*) in human tissues. Several rounds of protocol development were performed and antibody specificity was confirmed using blocking peptide controls (*SECTION 2.10.3*, *p75*). Following antigen retrieval, tissues were blocked for 10 minutes in 5% goat serum (*Dako, USA*) made up in PBS/Triton. Tissues were then incubated for 2 hours at room temperature with primary antibody (1:50) made up in 5% serum.

2.10.2.4 Hes1 immunohistochemistry

Hes1 analysis was carried out in line with previous studies (Ito *et al.*, 2000; Kayahara *et al.*, 2003). Following antigen retrieval, tissues were blocked for 10 minutes in 5% goat serum (*Dako, USA*) made up in PBS/Triton. Tissues were then incubated for 1.5 hours at room temperature with rabbit anti-Hes1 primary antibody [*A kind gift of T. Sudo, Japan*] (1:100) made up in 5% serum.

2.10.2.5 Protein visualisation

Secondary only control sections were also included in each staining batch to ensure specificity of any immunoreactivity. Tissue sections were washed three times for 5 minutes in PBS/Triton and bound anti-N1 and anti-Hes1 primary antibodies were detected by 30 minute incubation at room temperature with appropriate anti-goat or anti-rabbit biotinylated secondary antibodies (1:100 in PBS/Triton; *Dako, USA*). Following a further round of three washes in PBS/Triton, sections were incubated for 30 minutes with 0.004% (v/v) Streptavidin in PBS (*Dako, USA*) and washed again. Positive immunoreactivity was then visualised by use of 3-3'-Diaminobenzidine (DAB) in chromogen solution (*Dako, USA*) (*brown colour in the photomicrographs*) and unstained nuclei highlighted using Meyer's haemotoxylin (*blue colour in the photomicrographs*). Tissue sections were washed in Scott's tap water (3.5% (w/v) Sodium bicarbonate and 2% (w/v) Magnesium sulphate in dH_20) and then 1% acid alcohol (70% methylated spirits plus 1% (v/v) Hydrochloric

acid), before dehydrating (1 minute in each: 1 x 70%, 3 x 100% methylated spirit). Tissues were then cleared (2 x 1 minute washes in xylene; *VWR*) and mounted (DPX mountant; *BDH, UK*).

2.10.3 DETERMINATION OF PROTEIN SPECIFICITY

2.10.3.1 Blocking peptide

In line with manufacturer's specification (*and personal correspondence with Santa Cruz*), both the C20-goat and C20-rabbit anti-Notch1 antibodies (sc6014; *Santa Cruz, USA*) were pre-incubated with mixing at room temperature for 3 hours in the presence or absence of 100x N1 blocking peptide (sc6014p, *Santa Cruz, USA*). Antibody conjugates were then applied to representative tissue sections and processed as above. Any stain remaining in the presence of the blocking peptide indicates un-specific immunoreactivity of the primary antibody.

2.10.3.2 Protein knockdown by siRNA targeting in cell lines

HCT116 cells were treated with siRNA targeting *N1 vs.* scrambled control, as described above (*SECTION 2.3.3*, *p61*). After 72 or 96 hours cells were washed twice in PBS and fixed in 4ml of formalin for 1 hour (*Hayman Ltd.*). Following fixation, cells were washed twice in PBS and scraped in a further 4ml of PBS. Cell pellets were formed in 30ml centrifuge tubes (*Falcon*) by centrifugation at 1500*g* for 10 minutes and the PBS was thoroughly removed. The cell pellet was submerged in 5ml of molten 33% agar (60^{o}C) and left at 4^{o}C overnight to harden. Excess agar was trimmed and the embedded cell pellet was then processed as paraffin-embedded tissue.

2.10.4 HAEMATOXYLIN AND EOSIN

Tissue histopathology was confirmed by H&E staining of serial sections, detailing tissue structure with blue nuclei and pink highlighted tissue components. Deparaffinised and rehydrated tissues were stained with Mayer's haematoxylin (*BDH, UK*) for 1 minute and washed in running tap water for 10 minutes. After being counterstained with Eosin (*BDH, UK*) for 1 minute, tissues were washed in water, dehydrated, cleared and mounted as outlined above (*SECTION 2.10.2.5, p74*).

2.10.5 SCORING AND ANALYSIS

Tissues stained for N1 were scored by three independent observers, including one trained histopathologist [*NB, DQ and PR*]. Assessment of N1 immunoreactivity was made on the basis of the proportion and relative intensity of the positive tumour epithelium. Staining proportion was scored using an established three category graded system - i) *0%,* ii) *1-50%* or iii) *over 50% of positive tumour epithelium*. This method has been preferentially adopted by more recent immunohistochemical studies (Zlobec *et al.*, 2006), compared to a more historical two-tier system (*Positive vs. Negative*).

Staining intensity in the tumour epithelium was scored by a standard four-tier system, as described previously in both colorectal (Zlobec *et al.*, 2007) and N1 (Miyamoto *et al.*, 2003; Ramdass *et al.*, 2007) immunohistochemical studies. In order to improve accuracy and minimise variation, assessment of N1 staining intensity was determined relative to the internal positive control tissue, the smooth muscle. Therefore tissues were scored as either i) *negative*, ii) *mild (less than muscle),* ii) *moderate (equal to muscle)* or iv) *strong (more than muscle)*.

Many tissues were accompanied by significant background epithelium, which is largely histologically normal when distant from the tumour site. In contrast, the transitional epithelium directly bordering the colorectal tumours frequently demonstrates altered morphology in response to its proximity to the tumour. This non-neoplastic tissue is considered to be a 'tumour-like lesion', composing of a thickened epithelium and elongated, branching crypts (Jass and Sobin, 1989). For the scoring of these tissues, distinction was made between the distant background epithelium (~30 crypts away from the neoplastic edge) and the transitional epithelium (first 10-15 crypts). Changes in the expression of N1 were assessed by scoring the localisation of N1 immunoreactivity within the crypt-axis, as described previously (Boman *et al.*, 2004). Similar assessment was also made in the non-neoplastic epithelium of the tumour and diverticulitis resection margins for comparison.

IBD tissues are associated with varying degrees of inflammation, epithelial damage and associated severe failure to regenerate the damaged epithelium (reviewed in Podolsky, 2002). Positive IBD tissues were also scored in terms of epithelial crypt localisation. Due to epithelial ulceration, many IBD tissues frequently present areas of restitutive epithelium and granulation tissue. When present, these tissues were scored on the basis of heightened N1 immunoreactivity.

2.10.6 TISSUE IMAGING

All photomicrographs of stained resected tissue presented in this investigation were acquired using an Ultraphot III-B microscope (*Carl Zeiss*) combined with a DS-5 digital camera (*Nikon*) and NIS elements F2.20 imaging software (*Nikon*).

2.11 STATISTICAL ANALYSIS

2.11.1 DETERMINATION OF SIGNIFICANT DIFFERENCE

Statistical tests were carried out using the two tailed Student's t-test performed on Excel (*Microsoft*). Significance was determined and expressed as *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$ or NS: not significant.

2.11.2 DETERMINATION OF SIGNIFICANT CORRELATION

Analysis of statistical correlations in the N1 immunohistochemistry was performed using Kendall's tau B analysis (SPSS 15.0 for Microsoft Windows; *IBM*). By this method, N1 immunoreactivity was compared directly with various aspects of tissue structure and pathology, including extent of tumour progression (normal, benign or malignant), adenoma histological type (tubular, tubulovillous or villous), adenocarcinoma grade (well, poorly or moderately differentiated) or non-neoplastic tissue type (TRM, DRM, background or transitional mucosa).

CHAPTER 3

NOTCH1 IS INCREASED WITH COLORECTAL MALIGNANCY

3 NOTCH1 IS INCREASED WITH COLORECTAL MALIGNANCY

3.1 INTRODUCTION

3.1.1 HISTOPATHOLOGY IS ESSENTIAL FOR PREDICTING DISEASE PROGNOSIS

As described previously, the histological changes associated with colorectal cancer (CRC) are well characterised and remain a paradigm for a multi-stage theory to carcinogenesis in general (Luebeck and Moolgavkar, 2002). Adenocarcinomas, arising from the colorectal epithelium, account for the vast majority (90-95%) of colorectal cancers and progress from benign adenomas through a well defined sequence of histological changes (Ponz de Leon and Di Gregorio, 2001). Surgical intervention remains the primary mode of treatment for colorectal tumours (~92% of cases) and predicted patient outcome is largely based on subsequent histopathological assessment of resected, formalin fixed and paraffin-embedded tissues (Compton, 2003). The central techniques for examining the cell types and histological patterns in these tissues are staining and immunohistochemistry (IHC). The most widely used histological staining process is Haematoxylin and Eosin (H&E), which highlights both the nuclei of the cell and the various protein structures of the cellular matrix (Jass and Sobin, 1989). In contrast, IHC enables the immunological detection and visualisation of specific proteins of interest, providing an indispensible tool for both clinical assessment and *in vivo* based research (Cregger *et al.*, 2006).

For histopathological categorisation, a number of standard clinical definitions and systems are used that enable the degree of severity and therefore patient prognosis to be determined. For example, colorectal adenomas are further classified on the basis of their structural morphology and are histologically typed as being either tubular, villous or tubulovillous (Jass and Sobin, 1989; Ponz de Leon and Di Gregorio, 2001). Tubular adenomas constitute the majority of colorectal adenomas and consist of at least 80% branching neoplastic tubules, resembling normal intestinal crypts (Morson, 1974). Villous adenomas comprise of more finger-like processes of the lamina propria, reminiscent of villi in the small intestine, while intermediate tubulovillous adenomas comprise of at least 20% of each morphological type. An increasing degree of villous morphology is believed to associate with an increased risk of malignant progression and poorer patient prognosis (Morson, 1974), as is an increasing degree of dysplasia (Jen *et al.*, 1994).

Colorectal tumours are considered to be malignant and therefore cancerous once cells pass through the muscularis mucosae and infiltrate the submucosa. The resulting colorectal adenocarcinoma tissues may be further graded on percentage gland formation, ranging from well

(>95%) and moderately differentiated (50-95%) (*Low grade*) to poorly (5-50%) and un-differentiated (0-5%) (*High grade*) (Compton *et al.*, 2000; Hamilton and Aaltonen, 2000). Since higher grade adenocarcinomas are associated with a more invasive phenotype and poorer prognosis, classification of heterogeneous tissues is based on the highest presented grade (Purdie and Piris, 2000).

It is clear that tumour typing and grading has strong prognostic links, however, the most significant indicator of patient outcome in colorectal tumours continues to be a distinction between the benign and malignant phenotype, with further definition based on the degree of invasive growth (Tumour stage). As such, a number of clinical staging systems have been devised, of which the TNM (Tumour, Node, Metastasis) (Sobin, 2003; Wittekind *et al.*, 2002) and Dukes staging systems (Rudy and Zdon, 2000) are typically applied to colorectal cancer. Both systems rely heavily on determining the extent of tumour invasion into the nearby tissue, lymph nodes, vasculature and metastatic spread into other organs, therefore highlighting the paramount importance of benign to malignant tumour progression in patient survival.

3.1.2 THE PROGNOSTIC SIGNIFICANCE OF TUMOUR INVASION AND METASTASIS

Tissue invasion and metastasis is one of the major hallmarks of any cancer (Hanahan and Weinberg, 2000) and is the defining feature of the benign to malignant transition. Metastasis is the principal cause of cancer-related deaths and approximately 50% of patients presenting with colorectal adenocarcinomas will die from subsequent metastatic disease (Sporn, 1996; Young and Rea, 2000). The route for colorectal adenocarcinoma metastasis is primarily to the liver but also the lung, bone and brain (Tanaka, 2009). When considered in terms of patient survival, colorectal tumour presentation prior to malignancy is associated with a greater than 90% 5-year survival with surgical intervention, but a gradual decline is observed with greater invasive extent, reducing to only 3% in the later stages of malignancy (Markowitz *et al.*, 2002). However, at diagnosis, approximately 50% and 33% of CRC patients will already present with lymphatic metastases and haematogenous metastases respectively (Tanaka, 2009). CRC therefore continues to be one of the most frequent and life threatening diseases throughout the world, especially in well developed and industrialised countries (Boyle and Leon, 2002). Despite the unquestionable clinical significance of CRC invasion and metastasis, very little is currently known about the cellular-molecular basis governing the malignant phenotype.

Malignant progression requires colorectal adenoma cells to undergo considerable and defining cellular changes, namely in acquiring the ability to i) erode the basement membrane, lamina

propria and muscularis mucosae; ii) disrupt normal cell junctions; iii) up-regulate cell motility and iv) survive in the changing environments of the submucosa, blood/lymphatic systems and secondary tumour site. Recent analysis has shown that it takes approximately 17 years for a large adenoma to progress to malignancy, but less than 2 years for adenocarcinoma cells to acquire the ability to metastasise (Jones *et al.*, 2008). Given the highly distinctive adenoma-adenocarcinoma route of CRC formation and the sufficiency of time available to intervene, a greater understanding of the molecular-genetic changes governing malignancy should enable pharmacological interference at a stage preceding tumour invasion or metastasis, therefore greatly enhancing patient survival.

3.1.3 SIMILARITIES BETWEEN INTESTINAL TUMORIGENESIS AND REPAIR

Cancer has long been known to share many similarities with active tissue regeneration and is thought to resemble overactive repair or a wound that never heals, with malignant tumours often developing at sites of chronic injury (Dvorak, 1986; Haddow, 1972). Therefore, highly analogous changes in cellular morphology and behaviour to those observed in CRC are also found in non-neoplastic pathophysiological conditions of the colorectal epithelium. For example, inflammatory bowel disease (IBD) is a debilitating and incurable condition that is characterised by varying degrees of abnormal inflammation, epithelial damage and a severe failure to regenerate, leading to chronic epithelial ulceration (reviewed in Podolsky, 2002). IBD is associated with both epithelial dysplasia and an increased risk of colorectal tumorigenesis (reviewed in Zisman and Rubin, 2008). The symptoms of IBD have been appreciated for over 200 years but, like CRC, are increasingly associated with a modern, westernised lifestyle (reviewed in Kirsner, 2001). The major clinical forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Although UC is largely confined to the colonic mucosa, CD may occur throughout the entire GI tract and extend through the muscularis mucosae (reviewed in Xavier and Podolsky, 2007).

In IBD it is known that epithelial repair can be completed rapidly after the removal of tissue inflammation, leading to remission of the disease (Okamoto and Watanabe, 2005). Epithelial repair proceeds through several tightly controlled processes (reviewed in Okamoto and Watanabe, 2005), of which restitution is the first and most rapid response to intestinal injury and is characterised by de-differentiation and migration of the surrounding epithelium to cover the exposed region of sub-epithelial tissue (Taupin and Podolsky, 2003). Following restitution, increased proliferation in the neighbouring tissue is evident as crypt elongation and increased fission (Thompson *et al.*, 2000), reminiscent of aberrant changes in early intestinal tumorigenesis

(Renehan *et al.*, 2002). An abundance of inflammatory cells is also a common feature of many cancers and it is known that exposure to chronic inflammation accelerates colorectal tumorigenesis (Rescigno, 2008) and malignant progression (Balkwill *et al.*, 2005; de Visser *et al.*, 2006). Furthermore, a significant reduction in the expression of E-cadherin, a key epithelial adhesion protein and differentiation marker, is observed in cells at the edge of ulceration and is believed to promote the restitutive process (Hanby *et al.*, 1996; Karayiannakis *et al.*, 1998). Changes in E-cadherin expression are also a key feature of colorectal cancer (Nigam *et al.*, 1993) and are believed to promote the adoption of a more mesenchymal phenotype and increasing malignant behaviour (Brabletz *et al.*, 2005a). Therefore, intestinal repair shares evident similarities with colorectal cancer from the earliest stages of initiation through to malignant progression.

3.1.4 PATHOGENIC REACTIVATION OF DEVELOPMENTAL SIGNALLING PATHWAYS

As described previously, developmental signalling pathways form an essential basis for both normal intestinal homeostasis and colorectal carcinogenesis (*SECTION 1.3, p21*) (Sancho *et al.*, 2004; van den Brink and Offerhaus, 2007). Wound healing is also known to require potent reactivation of latent developmental signalling pathways, with localised secretion of various growth factors to promote the restoration of tissue integrity, protective inflammation and longer term regeneration (Schafer and Werner, 2008). Increased Wnt pathway activation is known to play a fundamental role in the vast majority of colorectal tumours (Fodde, 2002) and also appears to be a consistent and essential feature of general tissue repair (reviewed in Zhao *et al.*, 2009), with Wnt-related genes being highly expressed in IBD tissues (Wehkamp *et al.*, 2005).

A predictable role for the Notch pathway in wound healing is only just beginning to emerge (Chigurupati *et al.*, 2007), although Notch signalling is now known to play an essential role in an increasing number of human malignancies (*SECTION 1.6, p46*) (reviewed in: Koch and Radtke, 2007; Leong and Karsan, 2006). As described previously, Notch1 (N1) was originally discovered through its effects as a potent initiator of human neoplasia (Reynolds *et al.*, 1987) and continues to be the Notch receptor most frequently associated with carcinogenesis in human and animal tissues. Since Notch-associated mutations are not a common feature of solid tumours (Lee *et al.*, 2007b), the study of N1 in many tissues has instead relied on establishing correlative associations between aberrant expression and disease severity.

From analysis in animal models, it is believed that N1 is also the principal Notch receptor expressed in the intestinal epithelium, where the majority of its function can be attributed to

transcriptional regulation of the immediate target gene *Hes1* (*SECTION 1.5, p37*). The normal colorectal epithelium has previously been shown to be positive for N1 expression by IHC, as part of a whole body tissue microarray, although only details of relative intensity and not of specific location were provided (Baldi *et al.*, 2004). Furthermore, it has been shown by IHC that N1 protein expression is increased in human colorectal adenocarcinoma tissues compared to the normal epithelium but this was limited to a single case and was performed as part of a study addressing N1 changes related to cervical pathology (Zagouras *et al.*, 1995). As such, a paucity of evidence currently exists for N1 protein expression in the normal human colon as well as in colorectal cancer. Despite the undeniable prognostic significance of tumour invasion in the colorectal epithelium, comparison between benign and malignant tumour tissues is commonly overlooked in colorectal cancer research and, as discussed in a very recent review, the role of Notch signalling in pre-cancerous lesions, such as adenomas or IBD patients, has scarcely been addressed (Qiao and Wong, 2009).

3.1.5 HYPOTHESIS AND SPECIFIC AIMS

The hypothesis guiding this chapter was that N1 is expressed in the normal human colorectal epithelium and may be altered in various stages of pre-malignant and malignant colorectal carcinogenesis. Furthermore, N1 may be an essential feature of intestinal repair and potential tumorigenic alterations in N1 would also be mirrored by analogous changes in non-neoplastic colorectal lesions, such as tissues from patients with IBD.

The specific aims of this chapter were therefore;

1) To develop and validate an immunohistochemical technique to analyse N1 protein expression in the normal human colorectal epithelium.
2) To investigate changes in N1 protein expression during colorectal tumorigenesis and to assess a potential correlation with markers of malignant potential, such as adenoma type.
3) To investigate changes in N1 protein expression during malignant colorectal tumour progression and to assess potential correlation with markers of disease prognosis, such as adenocarcinoma grade.
4) To assess changes in N1 expression in analogous non-neoplastic lesions of the colorectal epithelium, including intestinal repair, as demonstrated in tissues from IBD patients.
5) To validate *in vivo* findings with expression analysis using *in vitro* colorectal tumour cell lines, in order to establish a potential model for the functional study of Notch in CRC.

3.2 RESULTS

3.2.1 AN IMMUNOHISTOCHEMICAL TECHNIQUE FOR THE DETECTION OF NOTCH1

For *in vivo* analysis of N1 in human CRC, this study had access to a large selection of archival resected human colorectal tissues representing various pathological backgrounds (formalin-fixed and paraffin-embedded). As N1 protein expression in the human colorectal epithelium had not been thoroughly investigated prior to this study, a suitable IHC protocol was established and thoroughly validated for these tissues. Changes in N1 protein expression had previously been addressed in solid tissues by IHC, namely in the cervical epithelium (Veeraraghavalu *et al.*, 2004; Zagouras *et al.*, 1995). As described above, one of these studies also demonstrated increased N1 protein expression in a single case of malignant colorectal cancer compared to the normal epithelium (which was negative), using the bTan20 rat anti-N1 antibody (*DSHB, USA*) (Zagouras *et al.*, 1995). More recently, various tissues, including the colorectal epithelium, were shown to be positive for N1 by IHC using an alternative anti-N1 antibody raised in goat (C20G/sc-6014*; Santa Cruz, USA*) (Baldi *et al.*, 2004). Both antibodies are raised against a similar portion of the C-terminal (intracellular) domain of the N1 protein and are therefore capable of detecting all protein isoforms; full length ($N1^{FULL}$), membrane receptor ($N1^{TM}$) and intracellular ($N^{\Delta E}$/$N1^{IC}$).

In addition to a preliminary cohort of colorectal adenocarcinoma tissues, three cases of resected human cervical carcinoma (Veeraraghavalu *et al.*, 2004; Zagouras *et al.*, 1995) and lung carcinoma (sc-6014 datasheet, *Santa Cruz*, *USA*) tissues were also selected as positive controls. Tissues were primarily stained using the bTan20 rat anti-N1 antibody (*DSHB, USA*) but, despite extensive attempts, sufficiently strong immunoreactivity was not achieved in any of the tissues using this antibody. In contrast, analysis using the C20G goat anti-N1 antibody (*Santa Cruz, USA*) resulted in strongly positive and cytoplasmic immunoreactivity in the tumour epithelium of all positive control (*FIGURE 3-1A-B*) and colorectal adenocarcinoma (*FIGURE 3-2A*) tissues tested. These findings are consistent with the preferential use of the C20G antibody over bTan20 in the majority of more recent N1 immunohistochemical publications, including studies which continue to use bTan20 for other techniques (Veeraraghavalu *et al.*, 2004; Veeraraghavalu *et al.*, 2005). In further confirmation, these findings were mirrored using a more recent C20-antibody variant, raised against the same epitope, but in rabbit (C20R/sc-6014*; Santa Cruz, USA*) (*FIGURE 3-1C-D, FIGURE 3-2D*). Importantly, this antibody requires a completely different immunohistochemical technique to C20G and thus, the highly analogous staining pattern observed suggests a high degree of N1-specific immunoreactivity by both methods.

Analysis using C20G was carried out in line with previously published methods (Ando *et al.*, 2003; Baldi *et al.*, 2004; Veeraraghavalu *et al.*, 2004) (*SECTION 2.10.2, p73*), but no previous studies had used the newer C20R antibody for IHC analysis and neither antibody had been used for IHC detection of N1 in neoplastic colorectal tissue before. A considerable number of validation steps were therefore performed in an initial cohort of human colorectal tissues of differing pathology before any large scale analysis was undertaken. In addition to standard secondary only (2^o only) negative controls, the specific nature of all N1 positive stain using C20G and C20R was also assessed by the inclusion of blocking peptide controls (*SECTION 2.10.3, p75*), which rely on antibody neutralisation in the presence of their specific antigen (sc6014p; *Santa Cruz, USA*). In confirmation of N1-specific immunoreactivity in these colorectal tissues, no stain was observed in the secondary only or blocking peptide controls for either antibody (*FIGURE 3-2B-C,E-F*).

Hes1 is believed to be the principal Notch target in the intestinal epithelium and has been used in a number of studies as a readout of active Notch signalling (Jensen *et al.*, 2000; van Es *et al.*, 2005). In support of correct N1 staining here, a corresponding pattern of Hes1 immunoreactivity was observed in nearly all tissues tested using an established rabbit anti-Hes1 antibody (*T. Sudo, Japan*) (*FIGURE 3-2G,H*), as used previously for analysis in murine intestinal tissues (Jensen *et al.*, 2000). Interestingly, although the C20G antibody has now been used in a number of *in vivo* N1 studies, expansion of the preliminary cohort of colorectal tissues revealed an additional punctate staining pattern in a small number of tissue samples (*FIGURE 3-2I*). Blocking peptide and secondary only controls confirmed this punctate distribution to be mediated by the primary antibody but to result from un-specific cross-reactivity (*FIGURE 3-2J,K*). It should be noted that all other stain was lost in these regions following blocking peptide neutralisation, confirming the cytoplasmic distribution to be specific. Furthermore, this alternative staining pattern was not observed in any tissue analysed using the C20R primary antibody and no immunoreactivity was ever observed in secondary only or blocking peptide controls corresponding to this antibody. Therefore, continued analysis of N1 expression in a large cohort of resected human colorectal tissues was performed using the C20R rather than the C20G primary antibody. Nevertheless, immunoreactivity between the C20R and C20G antibodies was highly comparable and the un-specific stain observed using the C20G antibody was both highly distinctive and limited to only small regions of some tissues. Therefore, concurrent stain was also performed on serial sections of many tissues using the C20G antibody to further validate findings presented here for C20R.

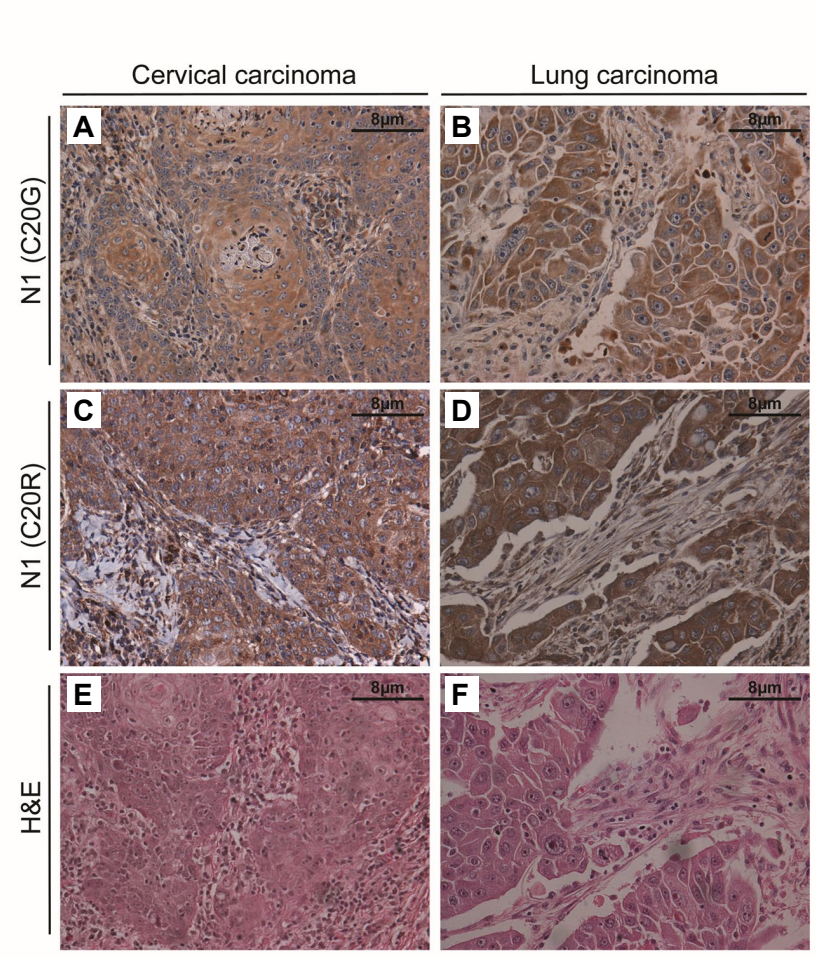


Figure 3-1| ***Validation of Notch1 immunohistochemistry using positive control tissues*****.** Immunohistochemical analysis of Notch1 (N1) protein expression in resected human cervical and lung cancer tissues. Results shown are representative of three different cases of each tissue using the goat (C20G) [A-B] and rabbit (C20R) [C-D] anti-N1 antibody variants (sc6014; Santa Cruz). Serial sections were also stained with Haematoxylin and Eosin (H&E) for greater detail of tissue structure [E-F]. (Magnification x20)

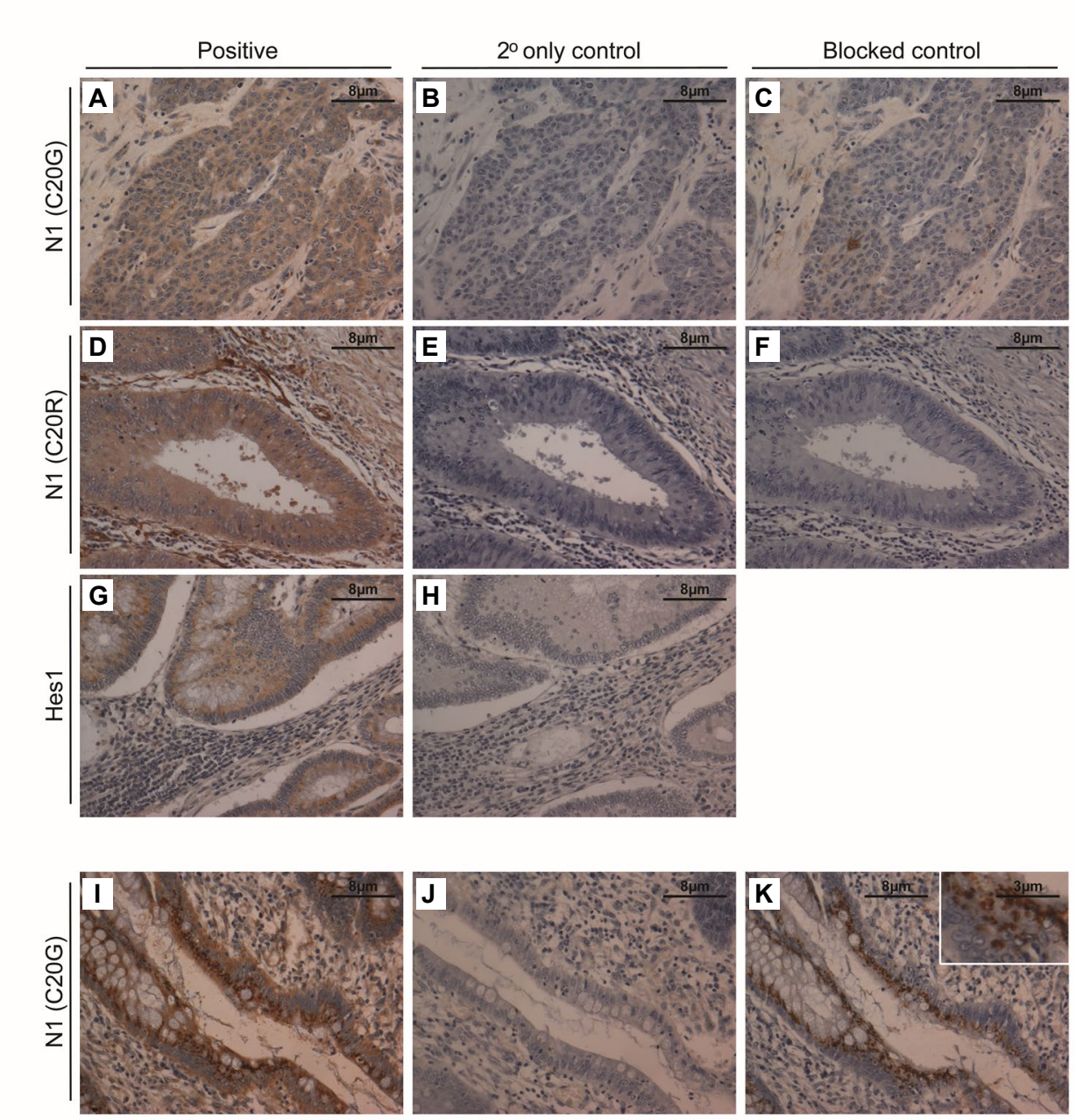


Figure 3-2| ***Notch1 and Hes1 are expressed by human colorectal adenocarcinoma tissues.*** Immunohistochemical analysis of Notch1 (N1) and Hes1 protein expression in resected human colorectal adenocarcinoma tissues. N1 immunoreactivity was demonstrated using both goat (C20G) [**A**] and rabbit (C20R) [**D**] anti-N1 primary antibodies (*sc6014 Santa Cruz*), while specific immunoreactivity was confirmed by the inclusion of a secondary only control (2º only; *absence of primary antibody*) [**B,E**] and by blocking peptide control (*specific antigen neutralised*) [**C,F**] in each case (*serial sections*). Hes1 expression was analysed using an established rabbit anti-Hes1 antibody (*T Sudo, Japan*) [**G**] and compared with 2º only controls [**H**]. Non-specific N1 immunoreactivity was identifiable in some colorectal adenocarcinoma tissues using the C20G but not the C20R anti-N1 primary antibodies, as determined by blocking peptide but not 2º only controls [**I-K**]. (*Magnification x20*)

To further confirm the specificity of the C20R antibody, N1 protein expression was knocked-down in the HCT116 colorectal adenocarcinoma cell line *in vitro* by *N1* siRNA treatment (*Dharmacon*) (*SECTION 2.3.2, p62*). Reduction in N1 protein expression was confirmed by western blotting analysis (*FIGURE 3-3A*) (*SECTION 2.5, p65*) and cells were formalin fixed and paraffin embedded for sectioning and IHC staining by the same methods as *in vivo* tissues (*SECTION 2.10.3, p75*). A substantial reduction in N1 immunoreactivity was observed in cells treated with *N1* siRNA compared to si-scrambled control treated cells by IHC (*FIGURE 3-3B-D*). This result further substantiates the use of the C20R antibody to specifically detect N1 by IHC but also confirms that differences in N1 concentration are readily detectable by this technique.

Nearly all resected colorectal epithelial tissues were accompanied by underlying connective tissues comprising the mucosa, submucosa, various muscle layers, lymph vessels and blood vasculature. In agreement with previous tissue microarray analysis (Baldi *et al.*, 2004), smooth muscle cells of the enteric muscle layers and surrounding many of the blood vessels were found to be consistently and strongly positive for N1 in almost all tissue sections irrespective of relative changes in epithelial N1 immunoreactivity (*FIGURE 3-4A,B*). Blocking peptide analysis was used to confirm N1 specificity in these tissues (*FIGURE 3-4C,D*) and an indistinguishable pattern of positive stain was also observed using the C20G antibody (*FIGURE 3-4E,F*). These tissues therefore provided an internal positive control, present in nearly every tissue, enabling accurate comparative analysis between different colorectal tissue sections.

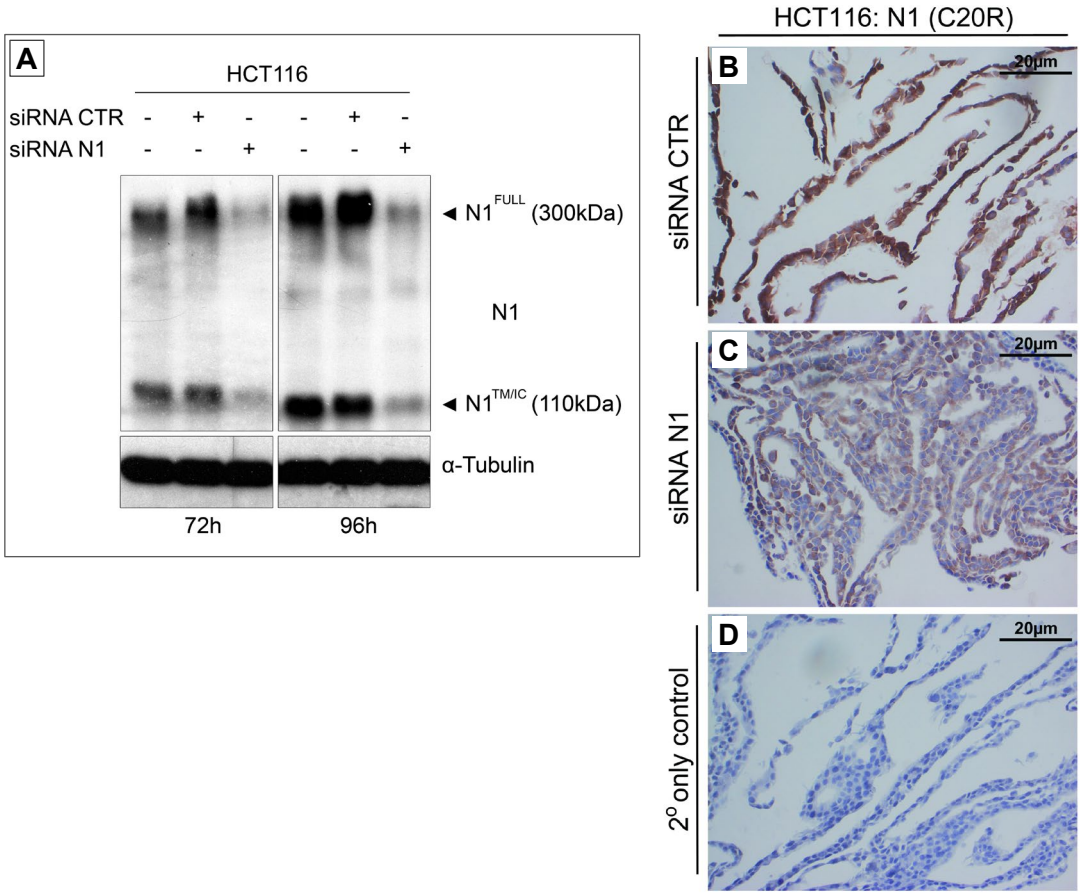


Figure 3-3| *Specific colorectal Notch1 immunoreactivity is confirmed by siRNA treatment.* Analysis of protein expression in the HCT116 (human colorectal adenocarcinoma) cell line following siRNA-mediated knockdown of Notch1 (N1). HCT116 cells were treated with either siRNA targeting *N1* (siRNA N1) or scrambled control (siRNA CTR). Reduced expression of the full length ($N1^{FULL}$) and cleaved ($N1^{TM/IC}$) N1 protein isoforms was confirmed by western blotting analysis at 72 and 96 hours post-transfection (α-Tubulin is included as a loading control) [**A**]. Cells treated for 72 hours were formalin fixed and paraffin embedded for analysis by immunohistochemistry. N1 protein expression was determined using the rabbit anti-N1 primary antibody (C20R; *Santa Cruz, USA*) [**B-C**] and compared with secondary (2^o) only staining control (siRNA CTR treated cells) sections [**D**]. (*Magnification x10*)

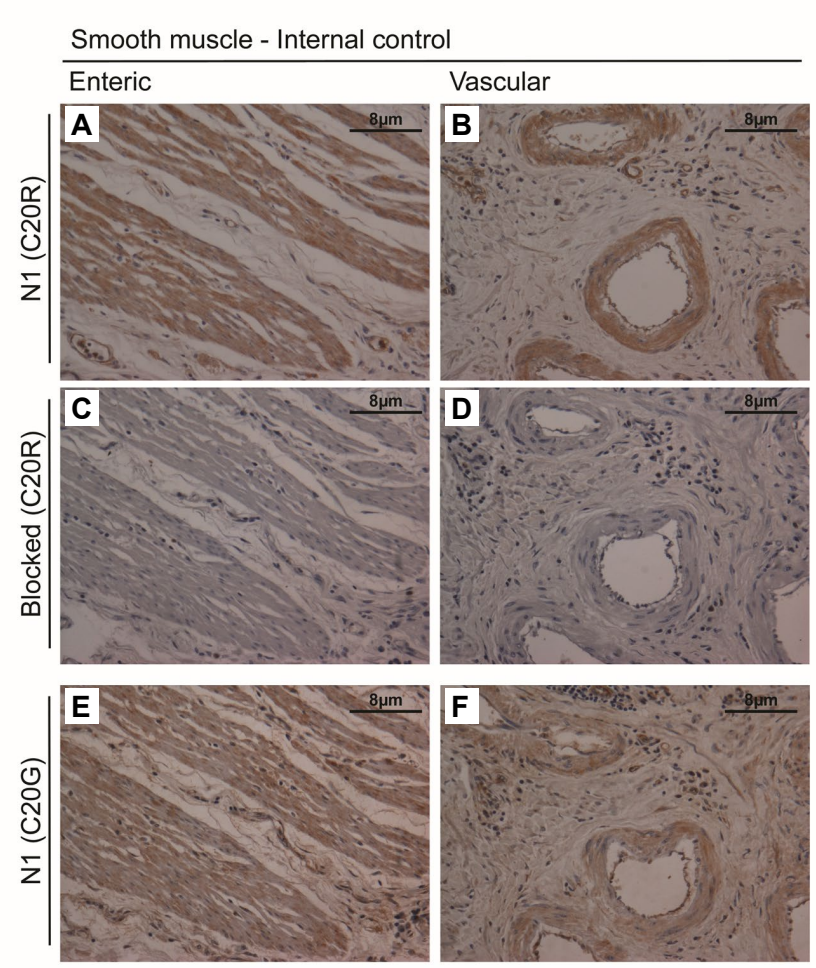


Figure 3-4| ***Smooth muscle provides an internal positive control for Notch1 expression.*** Positive Notch1 (N1) immunoreactivity was consistently observed within the smooth muscle of the enteric muscularis layers and surrounding many blood vessels in the underlying colorectal mucosa. N1 expression was detected using the C20R rabbit anti-N1 primary antibody (sc6014; *Santa Cruz*) [**A-B**] and confirmed by the inclusion of blocking peptide controls [**C-D**]. Staining using the C20G antibody variant (also sc6014; *Santa Cruz*) [**E-F**] on serial sections was also included for confirmation. (*Magnification x20*)

3.2.2 NOTCH1 IS EXPRESSED IN THE NORMAL COLORECTAL EPITHELIUM

Much of the previous evidence for Notch expression in the intestinal epithelium has been derived from mRNA analysis in the small intestine (SI) of animal models (Sander and Powell, 2004; Schroder and Gossler, 2002). These studies suggest a Notch expression profile consistent with localisation in the proliferative region towards the base of the crypt. However, no studies had previously provided evidence for N1 protein expression in the normal human intestinal epithelium. Therefore, as a prelude to investigating N1 in human colorectal neoplasia, the pattern of N1 protein expression in the normal human colorectal mucosa was assessed by immunohistochemistry.

Given that truly normal human colorectal tissues are not surgically resected, histologically normal mucosal samples are instead provided by the distant margins of tumour resection (tumour resection margins; TRM). A margin of 2cm is considered adequate to prevent local recurrence (Compton, 2003) and therefore considerable non-neoplastic background tissue is often removed and represents the best source of histologically normal colorectal mucosa. Surprisingly, it was found from IHC analysis in these tissues (*n=17*) using the C20R anti-N1 antibody that the N1 protein was predominantly and consistently expressed towards the top of the colonic crypt, away from the proliferative crypt zone (*FIGURE 3-5A,E*). It should be noted that scattered N1 positive cells were observed in the bottom half of the colonic crypt, although staining was much more pronounced and widespread towards the lumenal surface. Furthermore, a highly analogous pattern of expression was also observed using the C20G anti-N1 antibody (*FIGURE 3-5B,F*) and following analysis the immediate target, Hes1 (*FIGURE 3-5C*). This distribution of N1 immunoreactivity in the normal human colorectal mucosa was subsequently shown to be specific by comparison with both secondary only and blocking peptide controls (*FIGURE 3-6A*).

In addition to these TRMs, an alternative source of normal human colorectal mucosa was also provided by tissue margins from diverticulitis resection (diverticulitis resection margins, DRM). Diverticulitis is a non-neoplastic inflammatory condition, in which small abnormal epithelial pouches (diverticula) are believed to arise from insufficient intake of dietary fibre (Stollman and Raskin, 2004). Analysis of N1 and Hes1 protein expression in these tissues (*n=12*) was indistinguishable from observations made using colorectal TRMs (*data not shown*). In order to quantify these findings, both TRM and DRM tissues were scored for the expression of N1 along the colonic crypt axis (Boman *et al.*, 2004), where N1 expression was found to be predominantly expressed at the top of the colonic crypt in 10/17 TRM and 12/12 DRM tissues (*FIGURE 3-6B*). In all

TRM tissues not exhibiting N1 at the top of the crypt, staining was observed uniformly along the entire crypt axis, showing that detection of N1 protein at the base of the crypt is possible by this technique. Taken together these findings confirm that in the normal human colorectal epithelium, N1 protein expression is present in cells distributed along the crypt axis but, in nearly all cases, N1 is predominantly located in the upper crypt towards the lumenal surface.

3.2.3 NOTCH1 IS EXPRESSED IN THE HUMAN AND MURINE SMALL INTESTINE

These findings differ from mRNA analysis in the small intestine of animal models (Sander and Powell, 2004; Schroder and Gossler, 2002). In order to assess potential differences between N1 protein expression in the small and large intestine, IHC analysis was performed on resected normal human small intestine (SI; *TRM*). In agreement with observations made in the colonic crypt, N1 was found to be strongly expressed in differentiated cells along the length of the villi but was almost completely absent from the proliferative intervillus crypt region (*shorter than the colonic crypt*) (*FIGURE 3-7A*). To account for possible species variation, normal wild-type murine SI was also analysed for N1 protein expression by IHC and an identical pattern of epithelial N1 expression was observed (*FIGURE 3-7B*), confirming this pattern of N1 expression to be highly conserved between the small and large intestine, as well as human and murine tissues.

Alternatively, discrepancies between mRNA and protein expression are known to occur in tissues demonstrating rapid cellular turnover and migration, such as the intestinal epithelium (Boller *et al.*, 1988). In agreement with analysis by *in situ* hybridisation (Sander and Powell, 2004; Schroder and Gossler, 2002) it was recently shown by sequential fractionation that *N1* mRNA is most highly expressed in fractions of cells derived from the crypt base (Guilmeau *et al.*, 2008). Therefore, to address potential differences between *N1* mRNA and protein expression, sequential fractions of epithelial cells were obtained from the normal small and large intestine of adult wild-type mice (2-3 months old) by methods described previously (Mariadason *et al.*, 2005). In the former study, this technique was extensively validated and has since been used by several recent studies (Flandez *et al.*, 2008; Guilmeau *et al.*, 2008; Smartt *et al.*, 2007). Although it is known that a small percentage of cryptal lymphocytes are present in these fractions, this technique has been shown to isolate mainly epithelial cells, excluding most other cell types (Mariadason *et al.*, 2005), as confirmed here by a lack of α-smooth muscle actin detection (α-SMA) (*data not shown*). Correct cellular distribution was confirmed by western blotting analysis of the proliferation markers CDK2 and CDK4, alongside the key intestinal differentiation marker E-cadherin (*FIGURE 3-7C*).

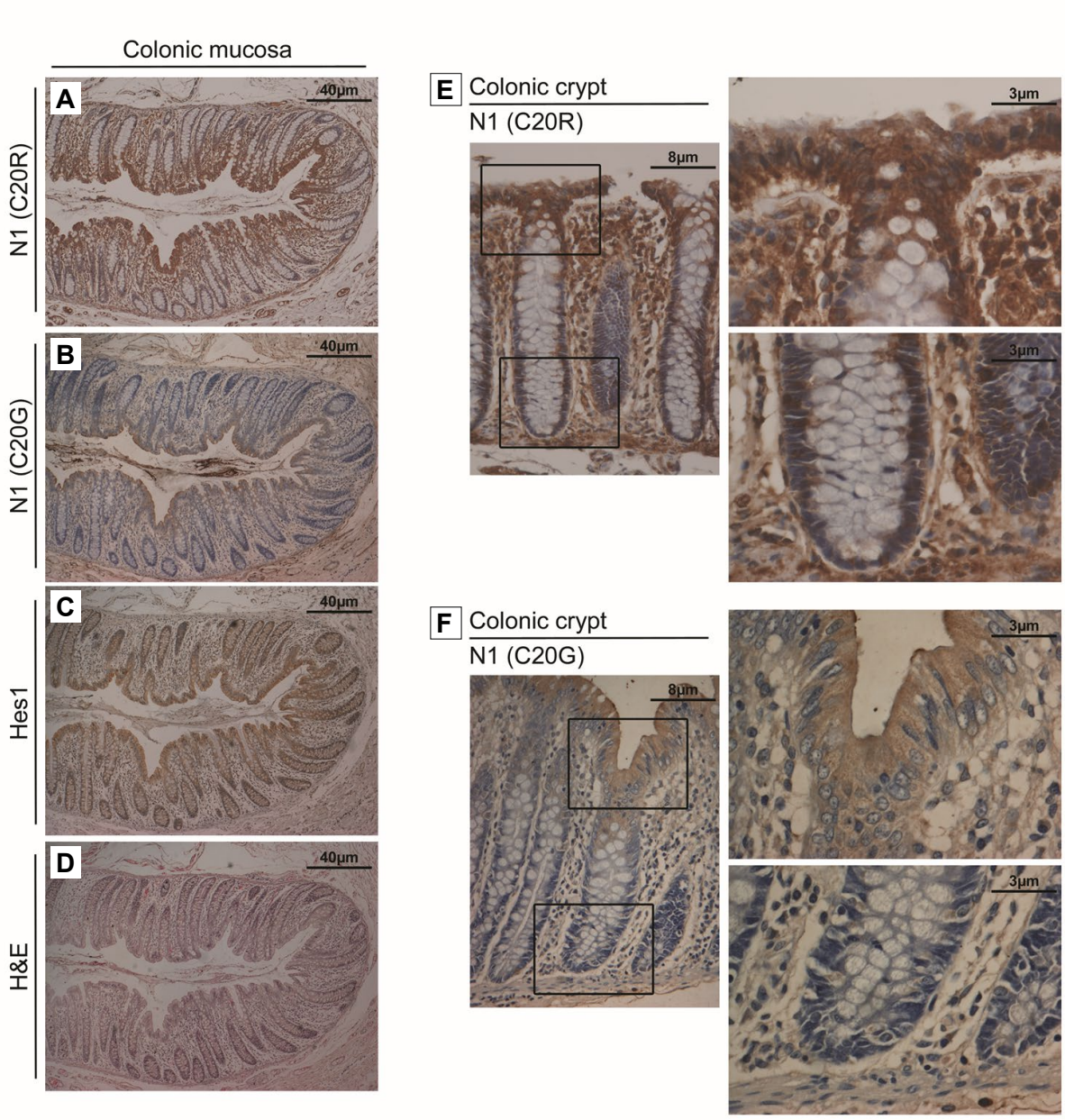


Figure 3-5| ***Notch1 and Hes1 are expressed in the normal colorectal epithelium.*** Immunohistochemical analysis of Notch1 (N1) and Hes1 protein expression in the 'normal' human colorectal epithelium derived from the margins of tumour resection. N1 protein was detected using both the rabbit (C20R) [**A**] and goat (C20G) [**B**] anti-N1 primary antibodies (sc6014; *Santa Cruz*). Serial sections were stained for the immediate Notch target Hes1 [**C**] and detail of tissue structure was provided by Haematoxylin and Eosin (H&E) [**D**]. N1 immunoreactivity in the colorectal epithelium localises predominantly in the upper third of the crypt at the lumenal interface, as demonstrated in both C20R [**E**] and C20G [**F**] stained tissues. (*Magnification x5, x20 & x40*)

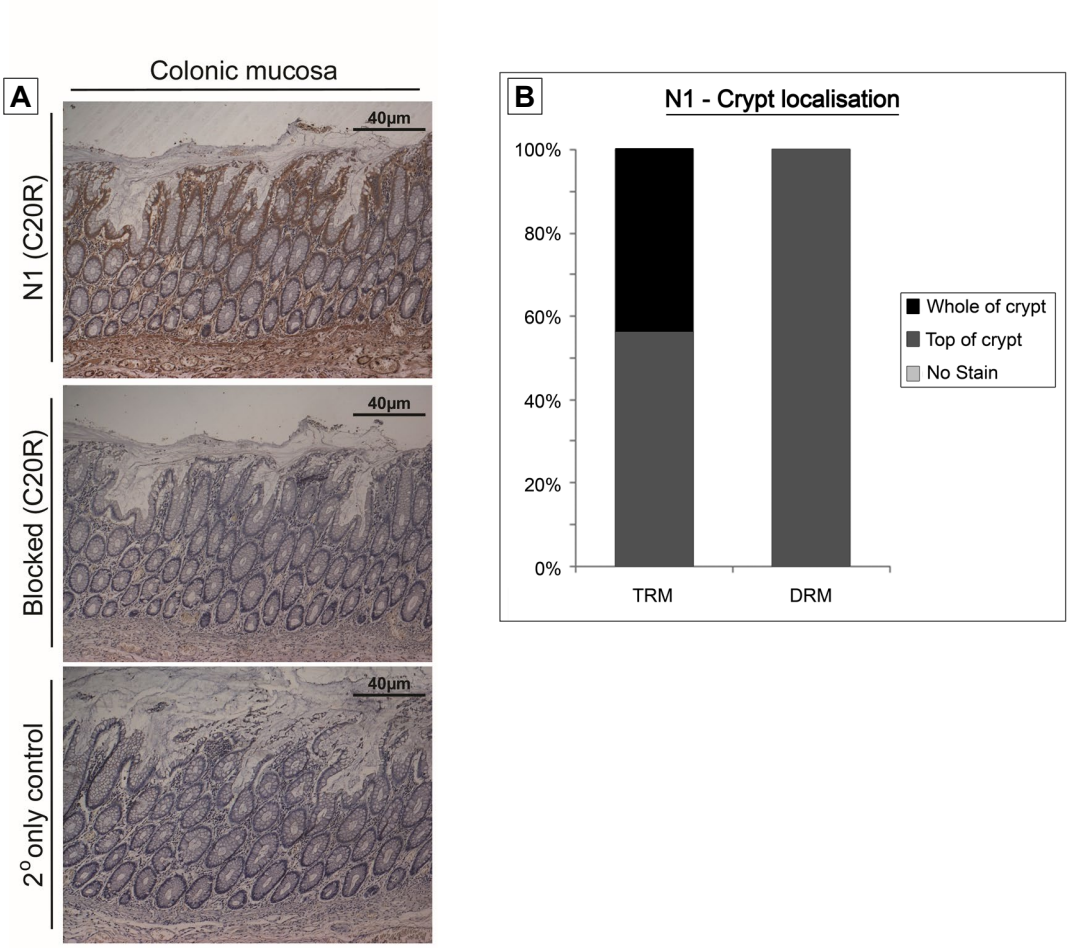


Figure 3-6| *Notch1 is expressed in the upper half of the crypt in the normal colonic epithelium.* [**A**] Notch1 immunoreactivity in the 'normal' colorectal mucosa (using C20R rabbit anti-N1 antibody; *Santa Cruz*) was shown to be specific by comparison with blocking peptide and secondary (2^o) only controls. (*Magnification x5*). [**B**] All normal tissues were scored by three independent observers for N1 localisation along the crypt axis and figures are representative of 17 tumour resection margins (TRM) and 12 diverticulitis resection margins (DRM).

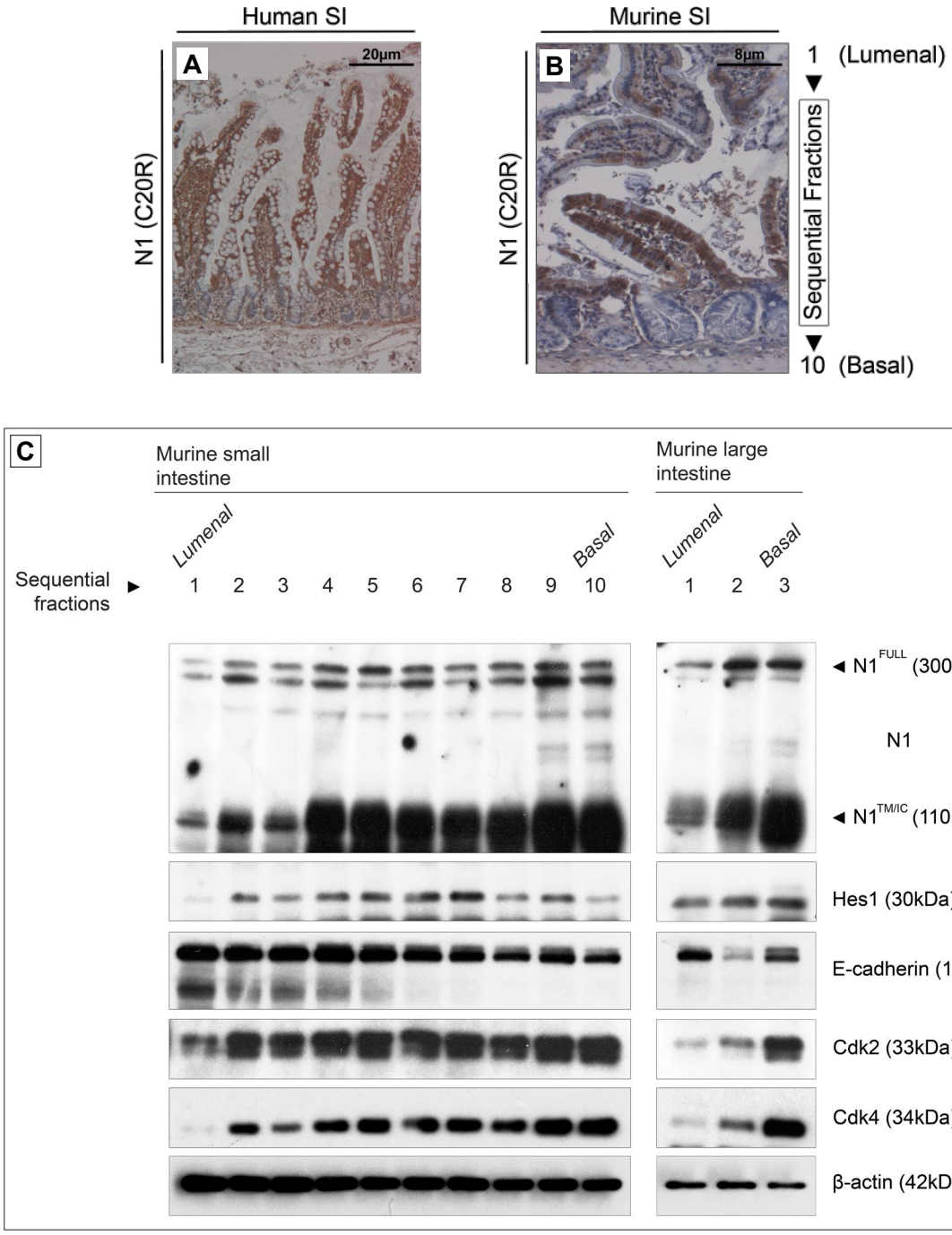


Figure 3-7| A *corresponding pattern of Notch1 expression in the human and murine small intestine.* Immunohistochemical analysis of N1 expression in normal human [**A**] and murine [**B**] small intestine using the C20-R rabbit anti-N1 primary antibodies (*Santa Cruz, USA*) (*Magnification x10/x20*). Sequential epithelial fractions were obtained from freshly resected murine small and large intestine according to their position along the crypt axis. Numbered fractions represent a cell gradient from the lumenal surface to crypt base in both tissues and relative protein expression was determined by western blotting analysis [**C**]. Cell lysates were analysed for the expression of both N1 and Hes1. A lumenal-basal gradient was confirmed by decreasing expression of E-cadherin (*marker of epithelial differentiation*) and increasing expression of CDK2/CDK4 (*markers of proliferation*) towards the base of the crypt. β-actin was used as a loading control and these data are representative of three independent experiments.

Cell fractions were also analysed for N1 and Hes1 protein expression (*FIGURE 3-7*). Both proteins were found to be readily detectable in nearly all fractions along the entire length of the crypt. Although the cleaved N1 ($N1^{TM/IC}$) protein was differentially expressed towards the crypt base, the expression of $N1^{FULL}$, which is indicative of translation levels, demonstrated only moderate variation between fractions in either the SI or LI. The Hes1 protein was also found to be uniformly expressed by virtually all cells along the crypt axis in both tissues (*9/10 in SI and 3/3 in LI*), strongly suggesting that N1 expression and activation is steadily maintained along the entire length of the crypt. Therefore, the determination of N1 and Hes1 localisation by this method does not directly correlate with IHC data of the present study, nor is it entirely consistent with previous *in situ* hybridisation analysis (Sander and Powell, 2004; Schroder and Gossler, 2002), suggesting that Notch pathway distribution and activation may be more complex and widespread than previously appreciated. This also begins to suggest that Notch signalling may function both in the base of the crypt as well as the lumenal surface, consistent with a potential multifaceted role in this tissue.

3.2.4 NOTCH1 IS UP-REGULATED IN TRANSITIONAL AND INFLAMATORY LESIONS

In addition to TRM and DRM samples, non-neoplastic human colorectal epithelium was also frequently attached to many of the colorectal tumour tissues used in this study. Like TRMs, this 'background' epithelium is histologically normal when sufficiently distant (*>30-40 crypts*) from the main tumour bulk not to be affected by the tumour microenvironment (*FIGURE 3-8A*). In contrast, the transitional epithelium, found directly bordering many colorectal tumours (*first 10-15 crypts*), is frequently abnormal and composes of a thickened epithelium, plus elongated and branching crypts (Mori *et al.*, 1990; Robey-Cafferty *et al.*, 1990). The transitional mucosa has therefore been defined as a 'tumour-like lesion' and, although normally found bordering neoplastic regions, may also arise in otherwise normal epithelium (Jass and Sobin, 1989). IHC analysis of N1 in distant background epithelial tissues demonstrated that, like tumour and diverticulitis resection margins, N1 protein expression is predominantly located in the upper colonic crypt towards the lumenal surface (*FIGURE 3-8B*). In contrast, N1 immunoreactivity was often altered in the transitional epithelium and was frequently found to occupy the entire length of the crypt (*FIGURE 3-8C*). In agreement, a corresponding change in Hes1 protein expression was also frequently observed in these tissues, switching from predominantly lumenal to whole crypt expression (*FIGURE 3-8D,E*).

In order to quantify these findings, both the 'distant' and 'transitional' regions of the background epithelium were scored for N1 localisation along the crypt axis (Boman *et al.*, 2004). Not all tumours had associated background epithelium and in some cases only the transitional epithelium was present. 34 cases of distant epithelium and 42 cases of transitional epithelium were found associated with colorectal adenomas (*n=57*), while 26 cases of distant epithelium and 29 cases of transitional epithelium were found associated with colorectal adenocarcinomas (*n=49*). N1 localisation along the entire length of the crypt was found to be significantly increased between the distant and transitional background epithelium ($p=5.12x10^{-10}$), although separate analysis revealed a more significant trend in tissues adjacent to colorectal adenocarcinomas ($p=1.13x10^{-14}$) than those adjacent to colorectal adenomas ($p=5x10^{-3}$). Therefore, full length crypt N1 localisation was also found to significantly increase between the transitional epithelium adjacent to adenomas and adenocarcinomas ($p=5.26x10^{-6}$) and full length crypt expression in this tissue was shown to directly correlate with malignant tumour progression ($Tau_b=0.524; p<0.001$). However, no significant difference was observed in the distant epithelium between adenoma and adenocarcinoma sections ($p=0.193$), suggesting substantial differences in the effects of benign and malignant tumour microenvironment on N1 expression in colorectal epithelial cells.

Active alterations of the mucosal architecture also arise in other non-neoplastic conditions, such as the inflammatory mucosa associated with IBD. As described previously, the abnormal and chronic inflammation associated with IBD patients gives rise to continual cycles of intestinal damage and regeneration (Podolsky, 2002). The intestinal repair process is known to share many analogies to colorectal tumour initiation and progression (*SECTION 3.1.3, p81*) and may also be considered to be a 'tumour-like' lesion of the colorectal epithelium. In particular, these tissues are associated with increased crypt branching and abundant inflammatory cells, plus epithelial de-differentiation and increased motility. Continual disease recurrence and increasing severity also entails an increased risk of epithelial dysplasia and malignant progression. Increased growth factor expression is known to be a prominent feature of these tissues (Wehkamp *et al.*, 2005); however, the expression of N1 had not previously been tested in the inflamed and regenerative epithelium derived from IBD patients.

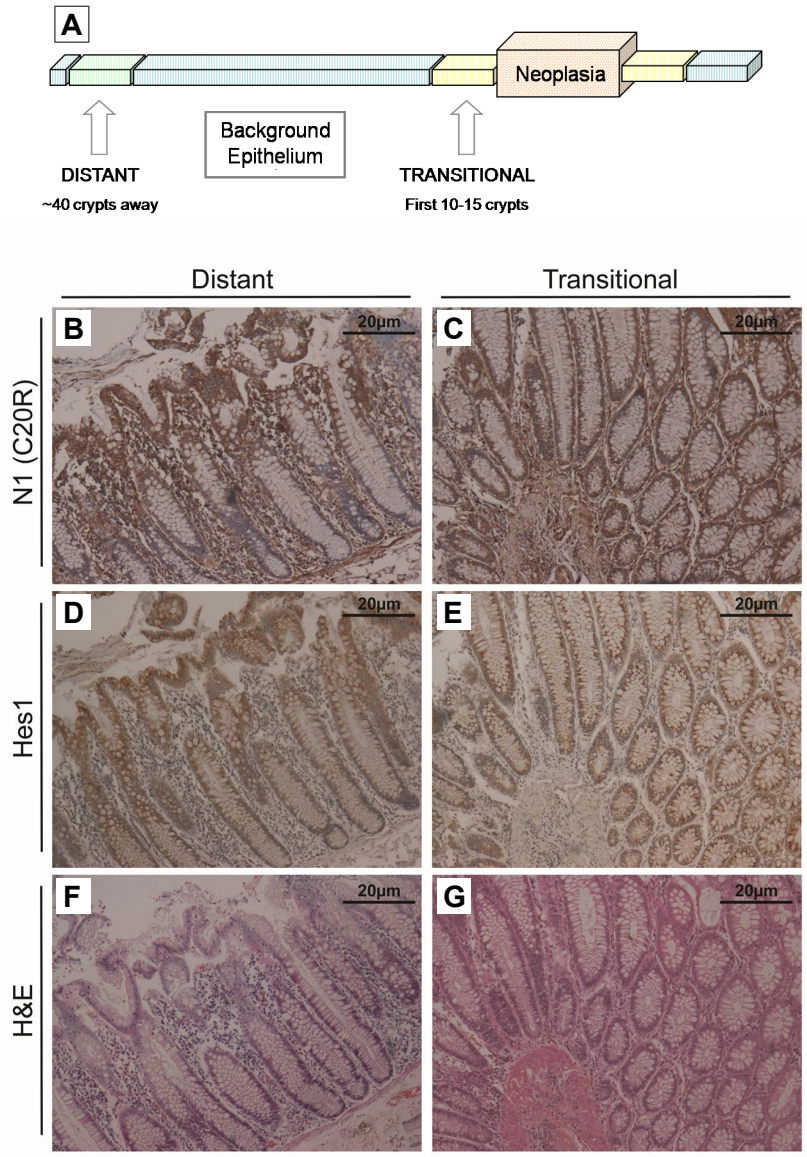
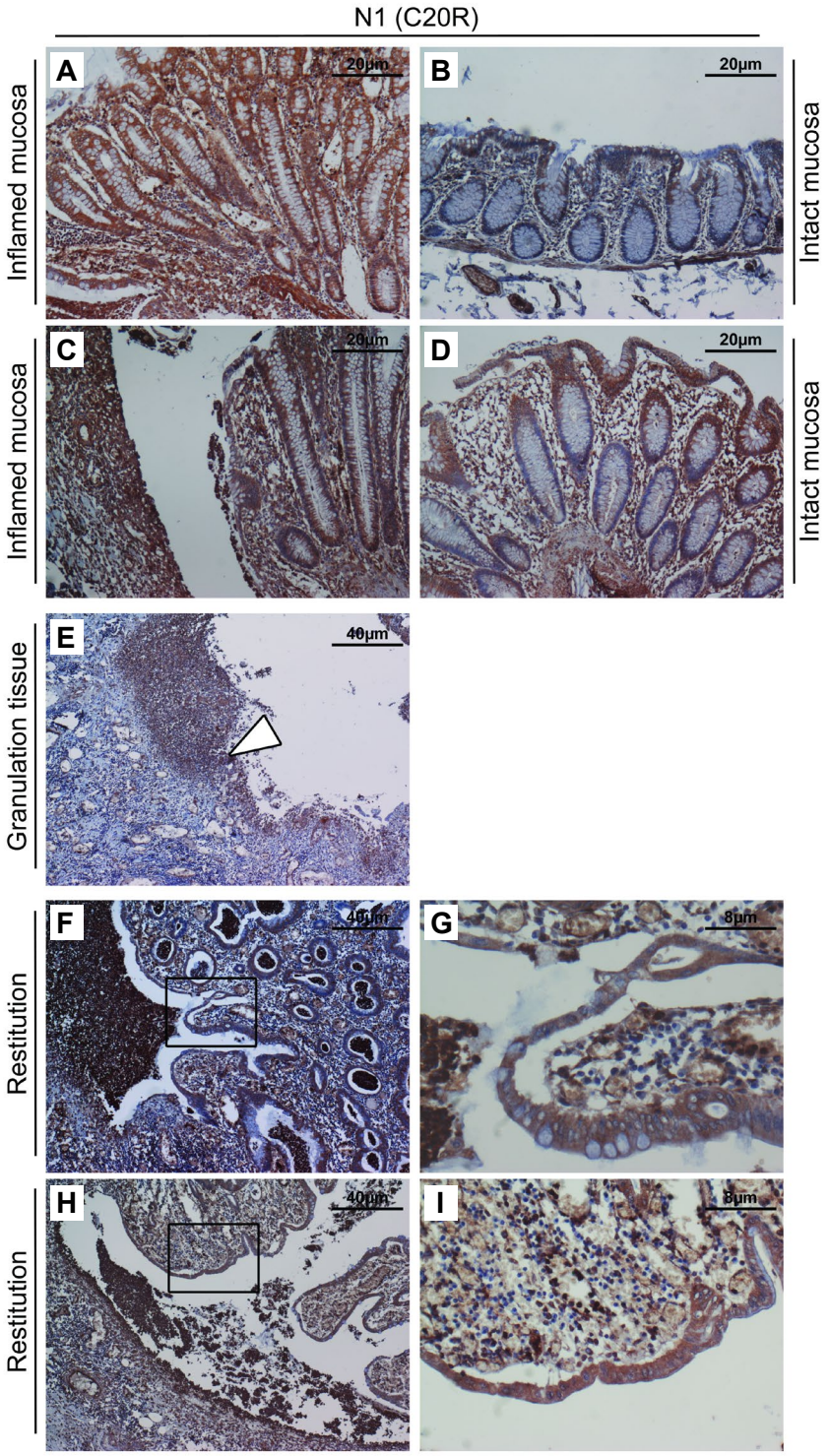


Figure 3-8| ***Notch1 and Hes1 are up-regulated in transitional colorectal epithelium.*** Schematic representation of the distant and transitional background epithelium associated with resected tumour samples [**A**]. The distant epithelium is histologically normal while the transitional epithelium is considered to represent 'tumour-like' changes. The distant background epithelium and transitional mucosa were immunohistochemically analysed for Notch1 [**B-C**] and Hes1 [**D-E**] protein expression. H&E stained sections are included for further detail of tissue structure [**F-G**]. Images are representative of 60 cases of distant background epithelium and 71 cases of transitional mucosa associated with both adenoma and adenocarcinoma tissue. (*Magnification x10*)

The expression of N1 was therefore assessed in a cohort of resected IBD cases, comprising of both ulcerative colitis (UC; *n=29*) and Crohn's disease (CD; *n=4*) tissues. Differing degrees of inflammation and epithelial damage (ulceration) were observed between cases but also heterogeneously within different areas of the same section. In support of findings from the transitional epithelium, the majority (*21/33*) of IBD cases (UC and CD) demonstrated N1 localisation along the full length of the crypt in at least some tissue regions (*FIGURE 3-9A*) and no significant difference was observed in crypt localisation between UC and CD sections (*p=0.466*). In cases showing little sign of active disease, N1 resembled that of the normal or distant colorectal epithelium, with expression predominantly localised towards the lumenal surface (*FIGURE 3-9B*). In tissues demonstrating heterogeneous regions of inflammation and crypt distortion, N1 expression in inflamed and ulcerated regions was shown to occupy the full crypt length (*FIGURE 3-9C*), while more histologically normal regions demonstrated N1 expression towards the lumenal surface (*FIGURE 3-9D*). Another common feature of the ulcerated mucosa from IBD patients is the presence of active tissue repair structures such as granulation tissue and epithelial restitution. Granulation tissue refers to the highly vascularised and cell-rich morphology that develops at the exposed wound surface, taking its name from the granular appearance of the numerous capillaries. In almost all cases where present (*9/10*), the granulation tissue was found to be strongly N1 positive (*FIGURE 3-9E; ARROW*). Similarly, the de-differentiated and flattened out epithelium undergoing restitution at the edge of ulceration was also found to be strongly positive for N1 expression in all cases where present (*12/12*) (*FIGURE 3-9F-I*).

Both the transitional mucosa and the damaged epithelium in IBD patients represent substantial non-neoplastic changes to the normal mucosal architecture and epithelial behaviour. From analysis of N1 expression and crypt localisation in these tissues (*FIGURE 3-10*) it is clear that increased N1 expression is a common feature of disease activation and 'tumour-like' changes in the colorectal epithelium, although changes in N1 expression in benign and malignant colorectal tumour growth remains to be established.

Figure 3-9| ***Notch1 expression is up-regulated in the damaged mucosa of IBD patients.*** Immunohistochemical analysis of Notch1 (N1) expression in resected tissue taken from inflammatory bowel disease (IBD) patients using the C20R rabbit anti-N1 antibody (*Santa Cruz*). Depending on the degree of epithelial damage, changes in N1 protein expression were observed between different cases of disease **[A-B]**, as well as between heterogeneous regions of the same section **[C-D]**. Strong N1 immunoreactivity was frequently observed in the granulation tissue **[E]** and restitutive epithelium **[F-I]** associated with ulceration. These data are representative of 29 cases of ulcerative colitis (UC) and 4 cases of Crohn's disease. (*Magnification x5, x10 & x20*) ▶


N1 (C20R)
A
20μm
B
20μm
Inflamed mucosa
Intact mucosa
C
20μm
D
20μm
Inflamed mucosa
Intact mucosa
E
40μm
Granulation tissue
F
40μm
G
8μm
Restitution
H
40μm
I
8μm
Restitution

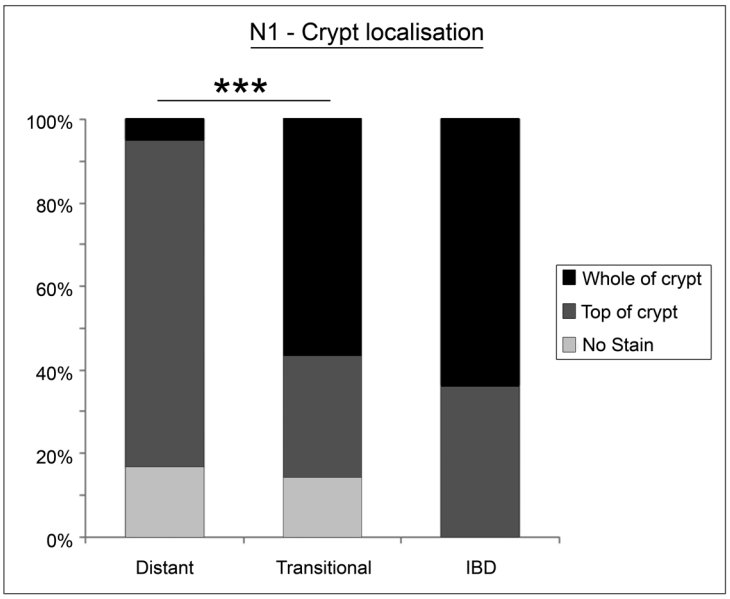


Figure 3-10| ***Notch1 crypt localisation is altered in the transitional mucosa and in IBD tissues.*** Non-neoplastic colorectal epithelium derived from tumour background and IBD patients were stained for N1 expression using C20R rabbit anti-N1 antibody (*Santa Cruz*). Tissues were scored by three independent observers for N1 localisation along the crypt axis and figures are representative of 60 cases of distant background epithelium, 71 cases of transitional epithelium and 33 cases of IBD.

3.2.5 NOTCH1 IS UP-REGULATED DURING HUMAN COLORECTAL TUMOUR PROGRESSION

In order to address possible changes in N1 protein expression during colorectal carcinogenesis, a range of benign (*n=57*) and malignant (*n=49*) colorectal tumours were selected, encompassing a range of histopathological subtypes. Colorectal adenoma tissues included 13 tubular (TA), 38 tubulovillous (TVA) and 6 villous (VA) tumours. Colorectal adenocarcinoma tissues composed of 13 well (WD), 14 moderately (MD) and 22 poorly (PD) differentiated cases. All tissue sections were stained using the C20-R anti-N1 primary antibody and positive staining was confirmed in each case using the smooth muscle as an internal control. Despite consistently positive and largely unchanging N1 immunoreactivity in the smooth muscle, N1 protein expression in the colorectal tumour epithelium was found to vary between different tissue samples and between different regions of the same section. Importantly, N1 protein immunoreactivity was found not to change substantially between the different adenoma types or adenocarcinoma grades; however, N1 expression was evidently increased between benign and malignant colorectal tissues (*FIGURE 3-11A-F, FIGURE 3-12A-F*).

In many immunohistochemical studies, positive immunoreactivity is classically evaluated on the basis of proportion and/or intensity, and a number of scoring methods have previously been developed. The proportion of N1 positive colorectal tumour cells was determined using an established three tier system of i) *0%,* ii) *1-50%* or iii) *greater than 50%* (Zlobec *et al.*, 2006). In addition, N1 immunoreactivity in the tumour epithelium was also scored for the intensity of N1 positivity (relative to the smooth muscle internal control), as being i) *negative*, ii) *mildly positive* (less than muscle), iii) *moderately positive* (equal to muscle) or iv) *strongly positive* (more than muscle) (Miyamoto *et al.*, 2003; Ramdass *et al.*, 2007; Zlobec *et al.*, 2007). All scoring was performed by three independent observers, including a trained histopathologist (*NB, DQ and PR*), and statistical analysis was performed on amalgamated data from the three sets (*TABLE 3-1*).

HISTOLOGY		PROPORTION			INTENSITY			
		0%	1-50%	51-100%	No stain	Mild	Moderate	Strong
Adenoma	TA	*2*	*5*	*6*	*2*	*5*	*4*	*2*
	TVA	*4*	*25*	*9*	*4*	*18*	*13*	*3*
	VA	*0*	*1*	*5*	*0*	*1*	*3*	*2*
	All	**6 (11%)**	**31 (54%)**	**20 (35%)**	**6 (11%)**	**24 (42%)**	**20 (35%)**	**7 (12%)**
Adenocarcinoma	WD	*0*	*0*	*13*	*0*	*1*	*8*	*4*
	MD	*0*	*0*	*14*	*0*	*3*	*4*	*7*
	PD	*0*	*1*	*21*	*0*	*6*	*10*	*6*
	All	**0 (0%)**	**1 (2%)**	**48 (98%)**	**0 (0%)**	**10 (20%)**	**22 (45%)**	**17 (35%)**

Table 3-1| ***The proportion and intensity of Notch1 expression is up-regulated in colorectal adenocarcinoma tissues.*** Immunohistochemical analysis of Notch1 (N1) protein expression in resected human colorectal adenoma (*n=57*) and adenocarcinoma (*n=49*) tissues. Adenoma tissues included 13 tubular (TA), 38 tubulovillous (TVA) and 6 villous (VA) histotypes. Adenocarcinoma tissues included 13 well (WD), 14 moderately (MD) and 23 poorly differentiated (PD) cases. Tissues were scored by three independent observers and data represents amalgamated scores.

Statistical analysis of N1 immunoreactivity primarily revealed that staining intensity and tumour proportion strongly correlate with each other (Tau_b*=0.619; p<0.001*) in these tumour tissues. Furthermore, analysis of both the intensity (Tau_b*=-0.136; p=0.264*) and proportion (Tau_b*=-0.101; p=0.422*) of N1 were shown to be independent of adenoma histology (TA *vs*. TVA *vs*. VA subtypes). Similarly, analysis in the adenocarcinoma tissues also revealed that both the intensity (Tau_b*=-1.26; p=0.331*) and proportion (Tau_b*=-0.140; p=0.306*) of N1 were independent of colorectal cancer grade (well, moderately or poorly differentiated). Although analysis of adenoma type or adenocarcinoma grade are frequently used to estimate patient prognosis (Morson, 1974; Purdie and Piris, 2000), as described previously, acquisition of a malignant phenotype and the extent of subsequent tumour invasion remain the most important prognostic indicators and impact severely on patient survival (Compton, 2003; Markowitz *et al.*, 2002; Sporn, 1996; Young and Rea, 2000). Importantly, a highly significant increase in N1 protein expression was observed between the grouped adenoma and adenocarcinoma tissues (*Proportion,* $p=2.76x10^{-12}$*/Intensity,* $p=5.84x10^{-5}$), where both proportion (Tau_b*=-0.643; p<0.001*) and intensity (Tau_b*=-0.366; <0.001*) were shown to demonstrate a highly significant correlation with malignant progression (*FIGURE 3-13A,B*). Furthermore, the degree of dysplasia is one of the most important markers of malignant potential in the colorectal adenoma tissues (Houlston, 2001); however, from analysis in the adenomas for which dysplasia data was available (*n=53*), it was shown that neither the intensity

(*Tau_b=-0.202; p=0.107*) nor the proportion (*Tau_b=-0.243; p=0.060*) of N1 immunoreactivity in these tissues significantly correlated with the degree of dysplasia. In support, N1 localisation in the transitional epithelium was also shown not to significantly correlate with adenoma dysplasia (*Tau_b=-0.132; p=0.412*), suggesting that other features governing the benign to malignant transition in these tissues may impact on or be regulated by the expression of N1.

Figure 3-11| ***Notch1 and Hes1 are expressed in human colorectal adenoma tissues.*** Immunohistochemical analysis of Notch1 (N1) (C20R; *Santa Cruz*) [**A-F**] and Hes1 [**G-L**] protein expression in resected human colorectal adenoma tissues. Representative images are shown of tubular (*n=13*), tubulovillous (*n=38*) and villous (*n=6*) adenoma subtypes, with corresponding high magnification detail of cellular localisation for both N1 [**D-F**] and Hes1 [**J-L**]. H&E stained sections highlight differences in tissue structure for each histological type [**J-K**]. Arrow demonstrates that N1 positive stain was confirmed by strong N1 immunoreactivity in the smooth muscle (vascular or enteric) present in underlying tissue. (*Magnification x10 & x50*) ▶

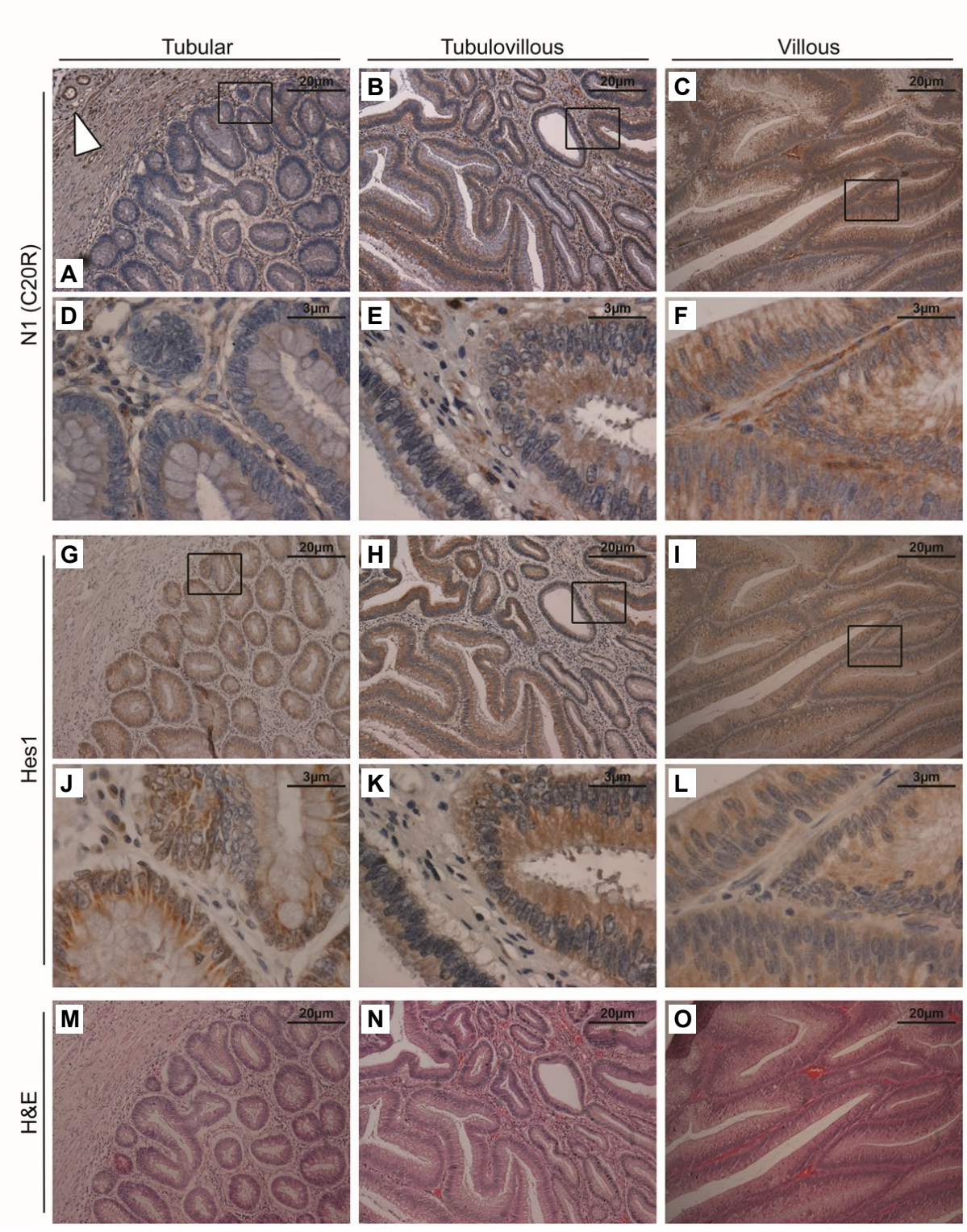
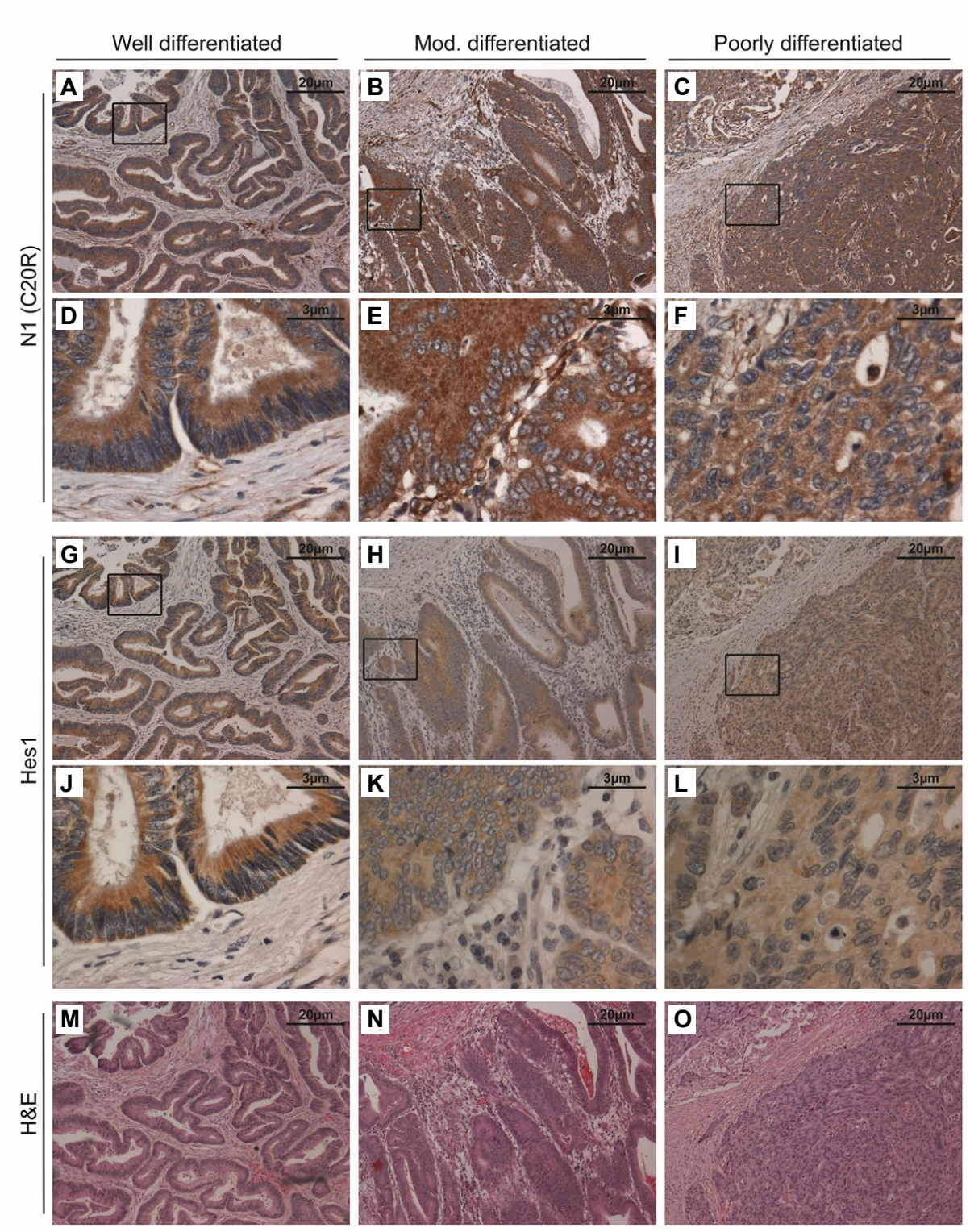

Tubular
Tubulovillous
Villous
N1 (C20R)
Hes1
H&E
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
20µm
3µm

Figure 3-12| ***Notch1 and Hes1 are expressed in human colorectal adenocarcinoma tissues.***
Immunohistochemical analysis of Notch1 (N1) (C20R; *Santa Cruz*) [**A-F**] and Hes1 [**G-L**] protein expression in resected human colorectal adenocarcinoma tissues. Representative images are shown of Well (*n=13*), Moderately (*n=14*) and Poorly differentiated (*n=22*) adenocarcinoma subtypes, with corresponding high magnification detail of cellular localisation for both N1 [**D-F**] and Hes1 [**J-L**]. H&E stained sections highlight differences in tissue structure for each histological type [**J-K**]. (*Magnification x10 & x50*) ▶


Well differentiated
Mod. differentiated
Poorly differentiated
N1 (C20R)
Hes1
H&E
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
20µm
3µm

3.2.6 NOTCH1 IS CONSISTENTLY OBSERVED IN THE CYTOPLASM

In agreement with previous studies (Baldi *et al.*, 2004; Veeraraghavalu *et al.*, 2004) and analysis in non-colorectal positive-control tissues (*FIGURE 3-1*), N1 sub-cellular localisation was shown to be consistently cytoplasmic in all colorectal epithelial tissues examined, irrespective of pathological status. This is highly suggestive of active $N1^{IC}$ detection in these tissues and in agreement, a highly overlapping pattern of expression for the immediate target Hes1 was also observed (*FIGURE 3-11G-L, FIGURE 3-12G-L*). Although the relative localisation of N1 and Hes1 proteins were highly analogous, the intensity of N1 and Hes1 stain did not appear to directly correspond, suggesting Hes1 expression in colorectal cells may be complex and may be regulated by factors other than $N1^{IC}$ concentration alone.

Following cleavage at S3, $N1^{IC}$ is known accumulate in the cytoplasm, but also rapidly translocates to the nucleus to promote transcriptional activation of Notch target genes (Jarriault *et al.*, 1995). The C20R primary antibody had not previously been used for IHC studies, but nuclear immunoreactivity had been demonstrated using the C20G variant in other tissues, where N1 positivity continued to be predominantly cytoplasmic (Ando *et al.*, 2003). Consistent with this, nuclear N1 immunoreactivity was also observed in colorectal adenocarcinoma tissues (*FIGURE 3-14*), confirming its potential for detection by this technique; however, evidence of nuclear sub-cellular localisation was limited to small areas of only two (2/106) of the colorectal tumour tissues used in this study. These findings are in direct agreement with the consensus that nuclear N1 localisation is difficult to detect by immunohistochemistry without the use of S3 cleavage site-specific antibodies (Efstratiadis *et al.*, 2007; Schroeter *et al.*, 1998), which instead are indicative N1 activation rather than N1 expression.

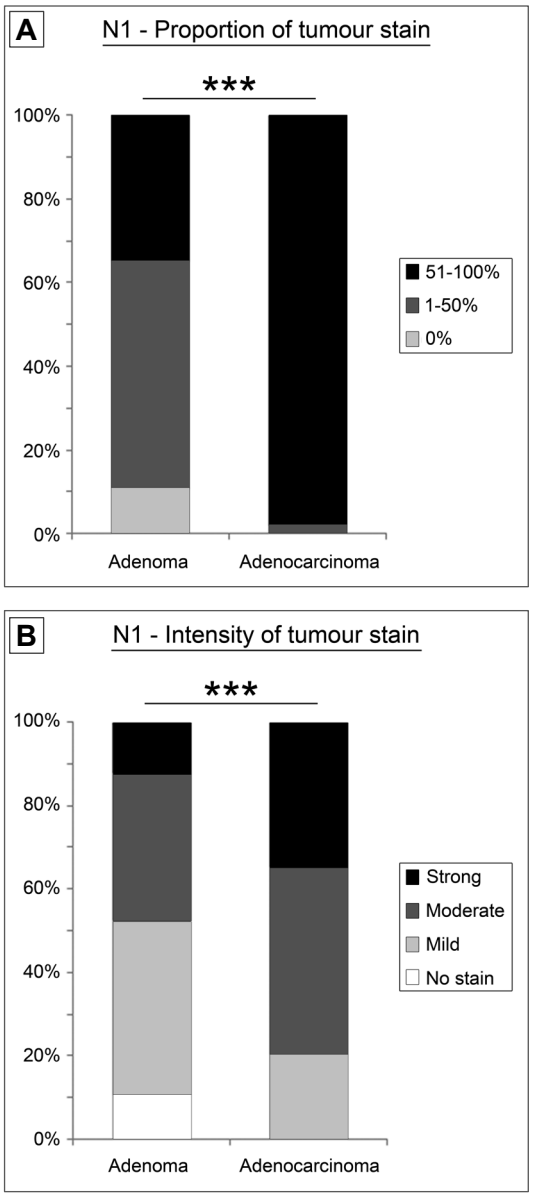


Figure 3-13| *Notch1 protein expression is significantly increased between colorectal adenoma and adenocarcinoma tissues.* Resected human colorectal adenoma (*n=57*) and adenocarcinoma (*n=49*) tissues were stained for Notch1 using the C20R rabbit anti-N1 antibody (*Santa Cruz*). Stained tissues were scored by three independent observers for the proportion [**A**] and intensity [**B**] of N1 immunoreactivity in the tumour epithelium. The proportion of positive tumour cells was defined as either none (0%), low (1-50%) or high (51-100%). The intensity was scored relative to the smooth muscle (internal positive control) as either no stain or mild, moderate or strongly positive. Scores are collated as percentages of each tissue.

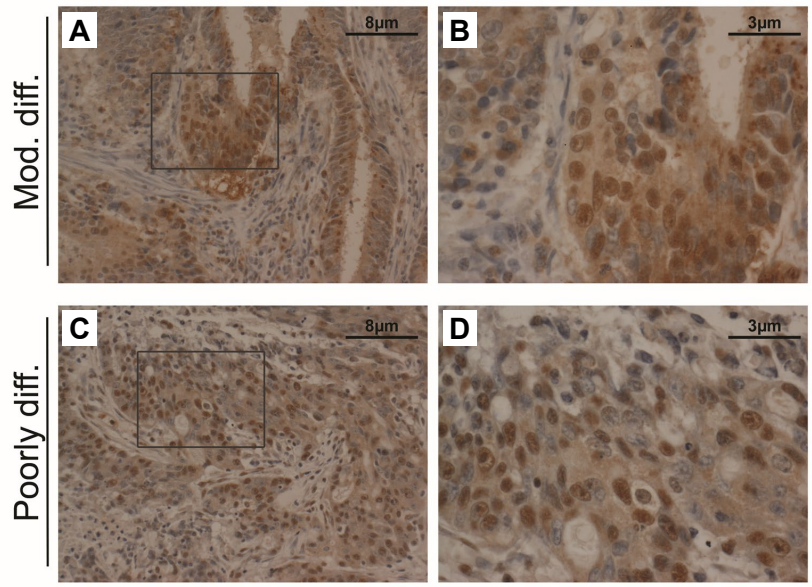


Figure 3-14| *Nuclear Notch1 is observed in colorectal adenocarcinoma tissues.* Immunohistochemical analysis of Notch1 (N1) in colorectal adenocarcinoma tissues demonstrated that, although sub-cellular localisation was predominantly cytoplasmic, localised nuclear N1 was detected in small regions of one moderately differentiated [**A-B**] and one poorly differentiated [**C-D**] adenocarcinoma case. (*Magnification x20 & x50*)

3.2.7 NOTCH1 IS INCREASED IN ADENOCARCINOMA-DERIVED CELL LINES

Although IHC analysis of N1 and Hes1 protein expression is highly indicative of *in vivo* function, one facet of the immunohistochemical technique is its reliance on fixed resected tissue. Having established that N1 protein expression is significantly increased in colorectal cancer *in vivo*, the expression of N1 and associated pathway components were also assessed in a range of established *in vitro* cell lines, with an aim to further validate *in vivo* findings and enable functional characterisation of the N1 pathway in human colorectal tumour cells. Initially, human cell lines were selected to represent a range of positive control tissues, comprising of MOLT4 (T-ALL) (Weng *et al.*, 2003), HeLa (cervical epithelial carcinoma) (Wang *et al.*, 2007), A549 (Lung epithelial carcinoma) (Zheng *et al.*, 2007), MCF7 (Breast epithelial carcinoma) (Jaime *et al.*, 2008) and HUVEC (Human umbilical vascular endothelial) (Taichman *et al.*, 2002) cells. The SW480 adenocarcinoma cell line was also included for preliminary *in vitro* analysis of CRC cells.

Lysates of sub-confluent cells were analysed for N1 protein expression by western blotting (*FIGURE 3-15*) (*SECTION 2.5, p65*) and both a ~300kDa and ~110kDa band were observed, consistent with the expression of full length N1 ($N1^{FULL}$) and cleaved N1 ($N1^{TM/IC}$) respectively (Aster *et al.*, 1997; Blaumueller *et al.*, 1997; Schroeter *et al.*, 1998). Full length protein expression was observed in the MOLT4 cell line only, although all cell lines expressed cleaved N1. The $N1^{TM}$ and $N1^{IC}$ isoforms are known to be closely related in size and difficult to distinguish by western blotting (*J Aster, personal correspondence*), although the presence of a doublet $N1^{TM/IC}$ band in the MOLT4 cell line is agreeable with PEST (proline, glutamate, serine, threonine rich) domain truncations in these cells (Weng *et al.*, 2004). Interestingly, the greatest expression of cleaved N1 in non-T-ALL cells was observed in the SW480 colorectal adenocarcinoma cell line.

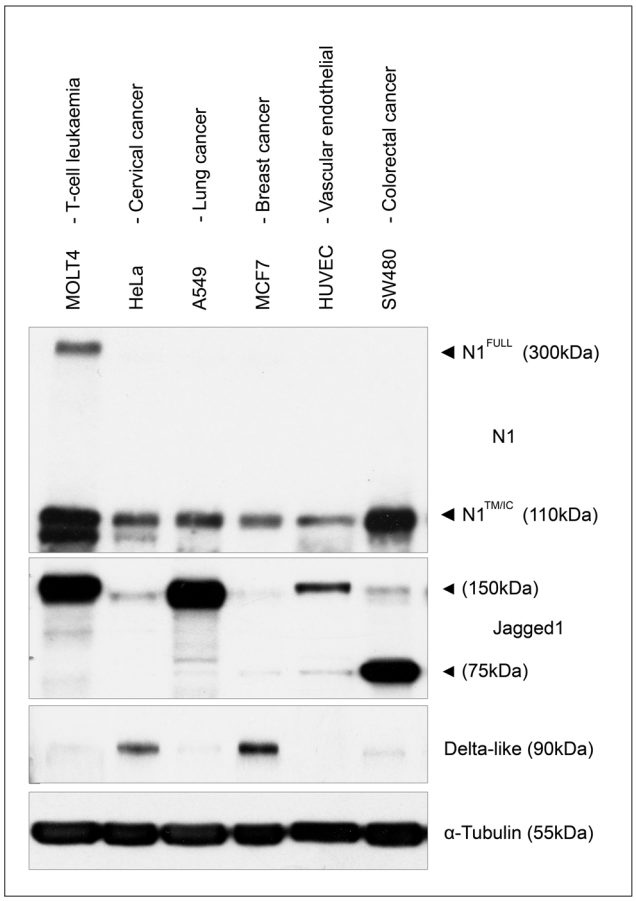


Figure 3-15| ***Notch1 pathway components are detected in positive control cell lines.*** Expression of Notch1 (N1) and associated ligands in cell lines derived from positive control tissues. Western blotting analysis of lysates derived from MOLT4 (T-cell acute lymphoblastic leukaemia), HeLa (cervical carcinoma), A549 (Lung carcinoma), MCF7 (Breast carcinoma), HUVEC (Human umbilical vascular endothelial) as well as lysate from the colorectal adenocarcinoma cell line, SW480. All samples were assessed for relative expression of N1, as well as the Notch ligands Jagged1 and Delta-like. Equal protein loading was confirmed by assessment of α-Tubulin.

Cell lysates were also probed for the Notch ligands Jagged1 (Jag1; *Santa Cruz, USA*) and Delta-like (Dll, *Santa Cruz, USA*) (*Figure 3-15*). High levels of Jag1 were demonstrated in the MOLT4 and A549 cell lines, while Dll was shown to be expressed by both HeLa and MCF7. Interestingly, instead of the established Jag1 150kDa band (Lindsell *et al.*, 1995), the SW480 cell line strongly expressed a ~75kDa isoform, possibly suggesting the existence of a truncating *Jag1* mutation in these cells (Boyer-Di Ponio *et al.*, 2007). Interestingly, Jag1 expressing cells were shown not to express Dll, and *vice versa,* suggesting that these ligands are mutually exclusive and Dll expression was virtually undetectable in the SW480 cell line. These results confirm that N1 pathway components are readily detectable in human cell lines using this technique and that relatively high expression level may be observed in colorectal adenocarcinoma cells.

A large number of well characterised colorectal adenoma and adenocarcinoma cell lines have previously been established and provide an excellent *in vitro* model for the analysis of N1 expression and function in human colorectal tissue (*SECTION 2.1.1, p53*). To determine the basal levels of N1 protein expression in these cell lines, western blotting analysis was performed on cell lysates taken from both adenoma- (*n=4*) and adenocarcinoma-derived lines (*n=10*). Due to the contact-dependent nature of the Notch signalling pathway (Bray, 2006), all cells were lysed when approximately 80% confluent to ensure a consistent degree of cell-cell contact. Serum treatment is also known to stimulate activation of the Notch pathway (Hirata *et al.*, 2002) and therefore the medium was changed 48 hours prior to harvesting. As seen in the positive control cell lines (*FIGURE 3-15*), two distinct protein bands were observed at approximately 300 and 110kDa, corresponding to full length ($N1^{FULL}$) and cleaved N1 ($N1^{TM/IC}$) respectively (*FIGURE 3-16A*). A high level of $N1^{FULL}$ expression was observed in the HCT116 adenocarcinoma cell line (Sureban *et al.*, 2008) but expression was either faint (2/14) or undetectable (11/14) in the remaining cell lines. In contrast, cleaved N1 expression was observed in the majority of colorectal tumour cell lines (13/14), although, reminiscent of *in vivo* findings, expression was highly dependent on differences in pathological type. The greatest expression was observed in the SW480 colorectal adenocarcinoma cell line, along with the SW620 metastasis-derived counterpart. Significantly, cleaved N1 expression in the adenoma-derived cell lines was much lower than that observed in the majority (9/10) of adenocarcinoma-derived cell lines.

Dll was found to be weakly expressed in a number of the colorectal tumour cell lines (8/14), while Jag1 was found to be moderately to highly expressed in almost all (13/14). These results imply that Jag1 is the predominant Notch ligand expressed in colorectal tumour cells, although this

remains to be formally addressed using *in vivo* colorectal tumour tissue. Furthermore, Hes1 was shown to be expressed by all cell lines tested here (14/14), suggesting that active Notch signalling is a consistent feature of colorectal tumours. Interestingly, the basal expression levels of either Hes1, Jag1 or Dll proteins in these cells did not appear to correlate with the expression of cleaved N1, alluding to complex regulatory mechanisms of Notch pathway expression and activation in these cells (Bray, 2006; Kadesch, 2004).

In order to quantify relative differences in N1 expression between the different colorectal tumour cell lines, the cleaved N1 protein band was analysed by densitometry (*SECTION 2.5.6, p68*), providing average expression levels from three independent experiments (*FIGURE 3-16B*). In support of the *in vivo* findings of this study, N1 protein expression was shown to be significantly increased between adenoma- and adenocarcinoma-derived cell types. It should be noted that the adenoma-derived cell lines differ from those of adenocarcinoma origin by retaining the benign phenotype and remaining both anchorage-dependent and non-tumorigenic (Paraskeva *et al.*, 1984; Paraskeva *et al.*, 1989). While a highly analogous pattern of N1 expression between the *in vivo* tissues and *in vitro* cell lines further underscores the importance of N1 expression in the malignant colorectal tumour progression, this also confirms these cells to be an excellent *in vitro* model for future functional analysis of Notch signalling in human colorectal tumours.

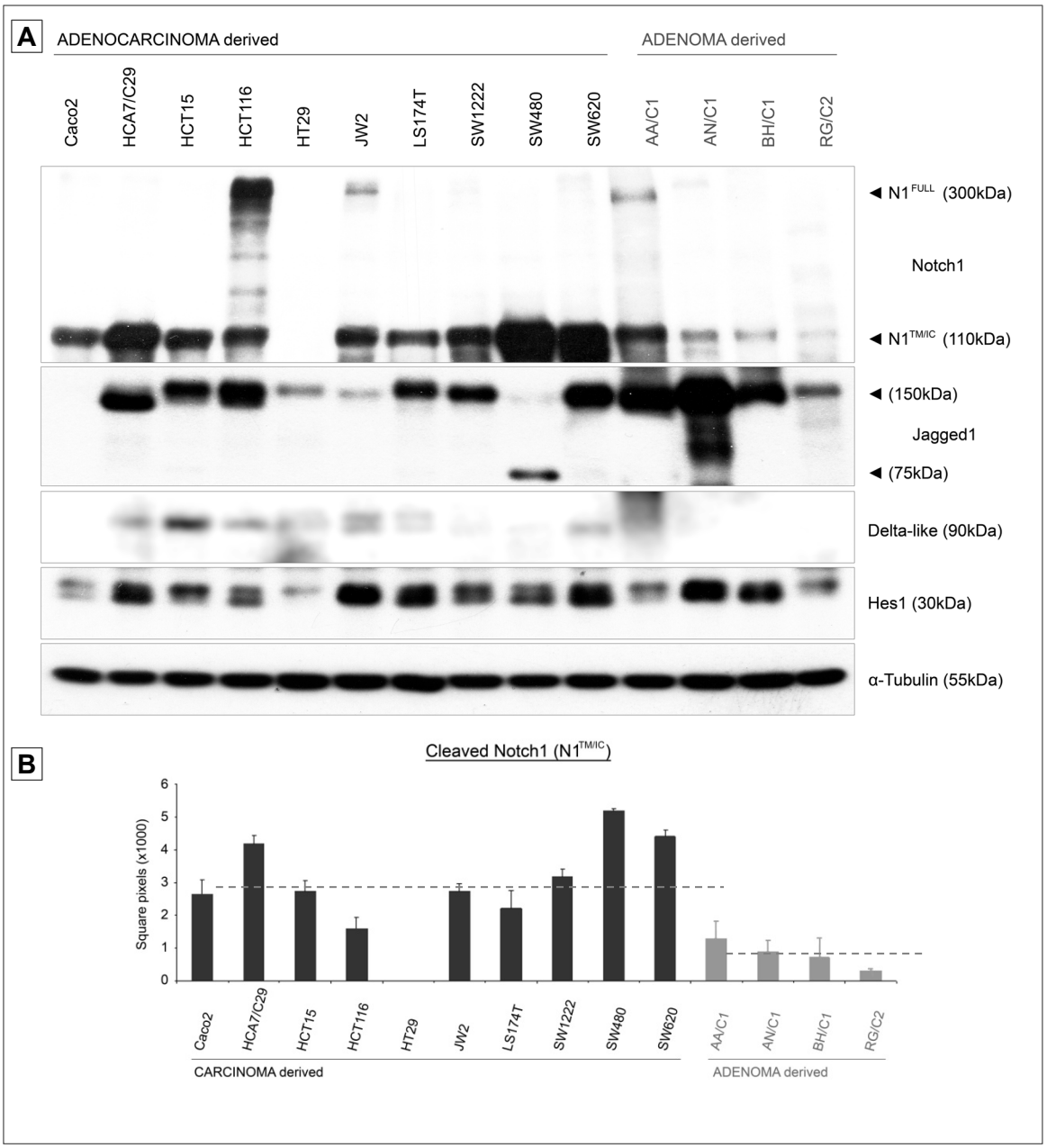


Figure 3-16| ***Notch1 is up-regulated between colorectal adenoma and adenocarcinoma cell lines.*** [**A**] Western blot analyses of four adenoma-derived and ten adenocarcinoma-derived *in vitro* colorectal cell lines for the expression of Notch1 (N1), the Notch ligands Jag1 and Dll, plus the immediate Notch target Hes1. α-Tubulin was used to ensure equal loading. [**B**] Densitometric analysis of cleaved N1 ($N1^{IC/TM}$) protein expression in colorectal tumour cell lines. Results represent normalised and averaged densitometric units taken from three separate experiments (*error bars = SEM*).

3.3 DISCUSSION

It is clear from studies performed in animal models that Notch pathway components are expressed in the intestinal epithelium and have an essential role in normal intestinal homeostasis. These studies also strongly suggest that active Notch signalling may be an integral feature of intestinal tumours; however, the expression and/or function of Notch signalling in the human colorectal epithelium and during colorectal carcinogenesis have remained largely unknown. The results of this chapter are therefore important in demonstrating that both N1 and Hes1 are expressed in the normal human colorectal epithelium, but also that their expression is significantly altered in colorectal tumour progression to malignancy. Moreover, these findings were also shown to be closely reflected by analogous changes in the non-neoplastic epithelium and by colorectal tumour cells in culture, further suggesting changes in Notch signalling to be an important contributor to human colorectal disease.

3.3.1 DIFFERENTIAL ROLES FOR NOTCH1 IN THE NORMAL EPITHELIUM

The observation that N1 protein expression predominantly localises to the upper part of the colonic crypt was interesting because it contradicts mRNA analysis in the SI of rodent models and initial expectations for analysis in the human epithelium (*FIGURE 3-5*, *Figure 3-6*). Nevertheless, this IHC technique was rigorously validated and was shown to be highly specific by two different methods and closely corresponded with the pattern of Hes1 expression. Furthermore, these differences in expression were shown not to relate to variation between the small and large intestine or between tissues of human and mouse origin, suggesting potential differences between N1 mRNA and protein localisation in this tissue (*FIGURE 3-7*). This is not unfeasible given the rapid upward cell movement in the intestinal crypt as has previously been shown for other intestinal proteins, such as villin (Boller *et al.*, 1988), which demonstrate protein translation at a higher crypt position than gene transcription. In support of similar mechanisms for Notch, analysis of sequential fractions from the murine small and large intestinal epithelium revealed that, rather than being limited to the base of the crypt, the N1 and Hes1 proteins are indeed widely distributed along the length of the crypt axis. Moreover, Hes1 was shown to be fairly uniformly expressed in both the SI and LI, suggesting that active signalling may be maintained throughout the crypt.

Interestingly, Okamoto *et al.* (2009) also recently demonstrated N1 expression in the normal human colonic crypt by IHC, but instead used an antibody specific to the $N1^{IC}$ S3 cleavage site, indicative of N1 activation rather than expression. By this method, active nuclear N1 was shown

to be expressed predominantly in the lower crypt region but was not strictly limited to the crypt base and was quite widespread, gradually becoming more diffuse towards the lumenal surface (Okamoto *et al.*, 2009). It is likely that differences in N1 expression between these two studies result almost entirely from differences in antibody specificity and it might be suggested that an alternative interpretation of N1 expression in the colonic crypt could be derived from consideration of both staining patterns, indicating both total and activated N1 in the crypt. Following receptor activation, an increasing number of intracellular regulatory mechanisms have now been identified that alter downstream N^{IC}-mediated transcription by various mechanisms, including altered nuclear localisation (Bray, 2006; Tien *et al.*, 2009) and analysis in *Drosophila* now also confirms altered nuclear translocation to be a major regulator of Notch activation (Mummery-Widmer *et al.*, 2009). It is conceivable that a decrease in N1 positive nuclei towards the lumenal surface (Okamoto *et al.*, 2009) may correlate with the increasing cytoplasmic accumulation observed here. Therefore, these two distributions may highlight the action of important but largely unappreciated mechanisms of Notch pathway regulation in this tissue.

Alternatively, expression of N1 in the upper crypt portion also suggests that N1 may promote terminal differentiation in the intestinal epithelium. Although genetic manipulation studies suggest a requirement for N1 in the proliferation and cell fate of the early progenitors (Fre *et al.*, 2005; van Es *et al.*, 2005), given the hierarchy of cellular progression along the crypt, it is clear that any phenotypic alterations in these cells would predominate over other potential secondary effects further up the crypt. In fact, inducible activation of N1 in these tissues is believed to target a cellular population higher up in the crypt axis, resulting in a completely opposing phenotype and instead acting to promote post-mitotic differentiation without affecting proliferation (Zecchini *et al.*, 2005). This begins to suggest contrasting and dichotomous roles for N1 in the colorectal epithelium, depending on the context of the cells in which it is activated and it appears that N1 may be important in both the proliferative and the differentiating compartments of the crypt. From analysis performed here it should be noted that, although N1 localises predominantly towards the lumenal surface in the normal human crypt, scattered N1 positive cells were also observed towards the base. Therefore, rather than discrediting previously hypothesised roles for N1 in the intestinal progenitor compartment, this study expands on the findings and suggests that N1 may also have additional and essential differentiating roles in this tissue.

This distinction between the proliferative and differentiating compartments in the intestinal epithelium is known to be maintained by opposing gradients of Ephrin-B ligands and EphB

receptors (*SECTION 1.3.7*). Active Wnt signalling is known to indirectly maintain the proliferative crypt phenotype by promoting the expression of EphB but inhibiting Ephrin-B. It has recently been shown that N1 regulates the expression of these proteins in an opposing fashion to Wnt, maintaining Ephrin-B expression in the differentiating crypt region and, therefore, indirectly promoting cellular differentiation in general (Koo *et al.*, 2009). Although Notch and Wnt are known to cooperate at the base of the crypt to promote proliferation, it is also known that the Notch effect on cell fate determination occurs independently of Wnt (Fre *et al.*, 2009) and begins to suggest both Wnt dependent and independent functions along the length of the crypt. In contrast, BMP and TGF-β are expressed towards the top of the colonic crypt and oppose Wnt activity by promoting differentiation and restricting the crypt progenitor population (*SECTION 1.3.4, p25 & 1.3.5, p26*). Notch and TGF-β signalling are well known to converge in the regulation of several differentiation events (Goumans *et al.*, 2002; Kim and Hebrok, 2001; Shah *et al.*, 1996) and active N1 signalling has been shown to be essential for growth arrest by this pathway in breast epithelium (Niimi *et al.*, 2007). Similar mechanisms may also function in the intestinal epithelium and it is tempting to hypothesise that opposing gradients of Wnt and TGF-β/BMP may cause a 'contextual shift' in Notch signalling depending on cellular position along the crypt axis, therefore switching from proliferation to differentiation promoting functions.

A differentiating role in the intestinal epithelium would not be entirely surprising since N1 has been shown to be a potent promoter of differentiation in a number of other tissues. This is most clearly described in epithelial keratinocytes, where the expression of Notch ligands and receptors is markedly increased in the differentiating layers of the epidermis and active N1 signalling has a potent tumour suppressing role (Nicolas *et al.*, 2003; Rangarajan *et al.*, 2001b). What is now also known from analysis in the epidermis is that under different cellular contexts, Notch may also promote growth in this tissue (Lee *et al.*, 2007a). These findings are likely to have significant consequences when considering changes in N1 expression in cancer, since it is clear that Notch may have oncogenic or tumour suppressor functions depending on the context of the tissue in which it is acting (reviewed in: Radtke and Raj, 2003; Roy *et al.*, 2007).

3.3.2 NOTCH1 EXPRESSION IS INCREASED WITH COLORECTAL TUMOUR MALIGNANCY

The progression of a benign tumour to malignancy is by definition the point of cancer formation and its significance is recognised at the most basic level of public understanding and clinical approach. This study was one of the first of its kind to address changes in the expression of N1 in human colorectal cancer and was unique in studying a possible change in N1 expression between

adenoma and adenocarcinoma samples, demonstrating N1 to be significantly altered in these tissues depending on the severity of disease. Analysis in *Apc*$^{+/Min}$ mice has previously suggested that active N1 signalling is an essential feature of benign intestinal tumours (van Es *et al.*, 2005); however, analysis in the present study strongly suggests that N1 expression is not increased early in intestinal tumorigenesis, but is instead a significant feature of the malignant progression in these tissues. While no significant difference in N1 expression was observed between the different adenoma or adenocarcinoma subtypes (probably due to uniform requirement), both the proportion and intensity of N1 increased significantly between adenoma and adenocarcinoma tissues, therefore correlating strongly with the malignancy. This is important because, although classification into the different tumour subtypes has important prognostic implications (Morson, 1974; Purdie and Piris, 2000), the transition to malignancy is, by far, the most important determinant of disease severity and patient survival (Compton, 2003).

Comparison between the benign and malignant phenotype is one of the most commonly overlooked facets in cancer research and one of the major strengths of the present study is the inclusion of both colorectal adenoma and adenocarcinoma tissues. It was also recently shown by another group that N1 mRNA and protein expression is increased in resected human colorectal cancer tissues compared to adjacent normal tissue by IHC (Chu *et al.*, 2009). While the normal colonic epithelium was stated to be positive for N1 protein expression, no description or photographs of staining distribution within these tissues were shown and this study was limited to adenocarcinomas only. However, in agreement with a role for N1 in tumour invasion, they confirm that expression correlates significantly with increasing adenocarcinoma stage. It was also shown in this study that N1 expression is increased in cases demonstrating a poorly differentiated phenotype but, crucially, this was shown not to be as significant as positive associations made between increasing N1 expression and tumour invasion.

It was also recently shown by *in situ* hybridisation and RT-PCR that increased expression of *N1*, *Jag1* and *Hes1* are consistent features of CRC (Fernandez-Majada *et al.*, 2007; Reedijk *et al.*, 2008) and in support of N1 analysis performed here, no significant change in *Hes1* expression was observed between well, moderately and poorly differentiated adenocarcinoma subtypes. This study also demonstrates that, while functional redundancy between N1 and N2 is an important feature of the normal murine intestine (Riccio *et al.*, 2008; Vooijs *et al.*, 2007), *N2* expression remains low in CRC and both *N3* and *N4* are undetectable (Reedijk *et al.*, 2008). Taken together with the present study, these findings confirm N1 to be the principal Notch ligand of interest in

human colorectal tumours and increasingly suggest that increased N1 may be a major determinant of malignant progression in these patients.

Unfortunately, tissue anonymisation constraints meant that survival data was not available for tissues used in the present study, but increased N1 expression in adenocarcinoma compared to adenoma tissues strongly implies that N1 is a marker of poorer prognosis. Although no direct correlation between N1 and survival has yet been made in CRC patients, increased expression of N1 or Jag1 have been linked to a poorer prognosis in breast cancer patients (Farnie *et al.*, 2007; Parr *et al.*, 2004; Reedijk *et al.*, 2005) and similar associations have been made in prostate (Santagata *et al.*, 2004) and lung (Westhoff *et al.*, 2009) cancer patients. Interestingly, *Hes1* expression was shown not to be a significant prognostic indicator in CRC patients (Reedijk *et al.*, 2008), although analysis was limited only to patients with adenocarcinomas. Alternatively, in the present study it was also shown that, although a highly overlapping distribution of Hes1 and N1 protein expression was observed in human colorectal tumours, increased N1 expression rarely corresponded to similar changes in Hes1, suggesting that other complex regulatory mechanisms may govern the expression of this protein.

A significant increase in N1 expression with colorectal tumour malignancy also contrasts with previously proposed roles for this pathway in early tumorigenesis (van Es *et al.*, 2005). This might, however, be easily explained since both Notch and Wnt are equally required for maintenance of the crypt progenitor phenotype in the normal intestine and this is also likely to be true of early intestinal tumours (Nakamura *et al.*, 2007). This may arise because many important genes and factors are known to be under the combinatorial control of both Notch and Wnt in these tissues (Fre *et al.*, 2009; Rodilla *et al.*, 2009) and both pathways are believed to maintain the progenitor phenotype through convergence on important differentiation factors, such as Atoh1 (Aragaki *et al.*, 2008; Tsuchiya *et al.*, 2007). Therefore, a proportion of active Notch signalling may be essential for the maintenance of intestinal tumours but it is possible that aberrant Wnt signalling, with physiologically normal N1 levels, is sufficient to promote the benign tumour phenotype. This model also explains how aberrant Wnt activation alone may also be sufficient for the down-regulation of secretory differentiation observed early in intestinal tumorigenesis.

A non-tumour initiating role for N1 in these tissues is further underscored by the fact that, unlike many other developmental signalling pathways, no congenital CRC syndromes have yet been attributed to alterations in the Notch pathway. Similarly, genetic models of N1 activation in the intestinal epithelium have existed for several years now and, like $Apc^{+/Min}$ mice, are known to

result in proliferative progenitor expansion (Fre *et al.*, 2005); however, these models have not yet shown that $N1^{IC}$ alone can initiate tumorigenesis in this tissue. Interestingly, it was instead recently shown that N1 activation greatly enhances the tumorigenicity of $Apc^{+/Min}$ mice (Fre *et al.*, 2009), leading to increased number and earlier presentation of intestinal tumours, although it is clear that tumour formation in these animals is still dependent on the presence of a concurrent *Apc* mutation. These results further confirm that, rather than a tumour initiating role for N1 in these tissues, increased N1 may act as a potent promoter of tumour progression, although a basis for this continues to remain poorly defined.

Alternatively, *Jag1* has also recently been defined as a prominent target of active Wnt signalling and increased Jag1 expression is observed in tumour tissue derived from both $Apc^{+/Min}$ mice and from human FAP patients (Guilmeau *et al.*, 2008; Rodilla *et al.*, 2009). Jag1 protein was also shown here to be expressed in almost all (13/14) colorectal cell lines tested, including the human colorectal adenoma-derived lines (*FIGURE 3-16*). Furthermore, Jag1 was more strongly expressed in adenoma cells known to harbour mutant *APC,* compared to RG/C2 cells that possess wild type alleles (Paraskeva *et al.*, 1984; Paraskeva *et al.*, 1989; Williams *et al.*, 1990). The limited expression of Delta-like ligands (Dll) in this colorectal cell line screen is in agreement with recent *in vivo* analysis demonstrating Dll to be only marginally expressed in CRC tissues (Reedijk *et al.*, 2008). Through an early increase in Jag1 expression, N1 activation may also be increased in colorectal adenoma tissues without a corresponding increase in N1 protein expression. Although changes in N1 activation may be accounted for by the lack of cytoplasmic staining in many adenoma tissues presented here, intracellular N1 is known to be rapidly degraded (Fior and Henrique, 2008) and it might also be interesting in future to analyse the degree of N1 activation in these colorectal adenoma and adenocarcinoma tissues using a $N1^{IC}$ specific antibody, as demonstrated recently in the normal colonic epithelium (Okamoto *et al.*, 2009).

N1 expression was shown here to be increased in the abnormal transitional epithelium adjacent to intestinal tumours and in the inflamed/reparative epithelium from IBD patients, where increased N1 expression was frequently observed along the entire length of the crypt axis (*FIGURE 3-8* - *Figure 3-10*). This result has now been supported by analysis of N1 activation in IBD tissues (Gersemann *et al.*, 2009; Okamoto *et al.*, 2009), suggesting that both increased expression and pathway activity is a consistent feature of intestinal inflammation and repair. Both the transitional and damaged epithelium demonstrate histological changes consistent with intestinal tumorigenesis (Mori *et al.*, 1990; Podolsky, 2002; Robey-Cafferty *et al.*, 1990) and, in possible

explanation of increased N1, both are exposed to abnormal micro-environmental cues and increased levels of diffusible growth factors (Mori *et al.*, 1996; Schafer and Werner, 2008). Crucially, a highly significant increase in N1 expression was observed in the transitional epithelium adjacent to adenocarcinoma tissues, compared to adenomas, confirming that N1 expression in both the tumour and transitional epithelium positively correlates with the malignant phenotype. Furthermore, expression of N1 along the entire length of the crypt in these tissues demonstrates that N1 expression is readily detectable in the crypt base when present and confirms the staining pattern in the normal epithelium to be correct.

3.3.3 A CONTRIBUTORY ROLE FOR NOTCH1 IN COLORECTAL CANCER

The functional significance of increasing N1 expression in colorectal tumour progression is yet to be realised but, reminiscent of a multifaceted role in the normal epithelium, it is possible that Notch may be responsible for different context-dependent modes of action at different stages of colorectal carcinogenesis. In a similar fashion, aberrant Wnt activity and nuclear β-catenin continue to increase between colorectal adenomas and adenocarcinomas (Amos-Landgraf *et al.*, 2007; Anderson *et al.*, 2002; Blaker *et al.*, 2003), then undergo yet further transient increases during tumour de-differentiation at the invasive front (Brabletz *et al.*, 1998). Wnt signalling is therefore believed to promote different stages of intestinal tumorigenesis in a dose-dependent manner, where the tumour initiating properties of this pathway appear to be distinct from those promoting tumour progression (Fodde and Brabletz, 2007). Wnt signalling primarily promotes colorectal carcinogenesis through an increase in proliferative potential and proliferation is known to increase between adenoma and adenocarcinoma tissues (Jackson *et al.*, 1995; Shpitz *et al.*, 1997; Yang *et al.*, 1996). N1 activation is also known to promote proliferation in the normal intestinal epithelium (Fre *et al.*, 2005) and other tissues (Carlesso *et al.*, 1999) but the potential proliferative role for N1 in colorectal carcinogenesis remains to be established.

Alternatively, transient changes in Notch activation (Hes1 protein expression) have been shown to be a consistent feature in colorectal tumour metastasis, being increased in primary colorectal tumours but decreasing again at secondary tumour sites (Veenendaal *et al.*, 2008). This is in agreement with dose-dependent threshold levels for N1 activation in development (Guentchev and McKay, 2006) and suggests that transient increases in N1 may instead be responsible for promoting tumour invasion. In fact, activated N1 signalling is known to induce morphological changes consistent with tumour invasion in other tissues (Leong *et al.*, 2007; Timmerman *et al.*, 2004), but has not yet been tested in the CRC context.

Analogous changes in the tissues from IBD patients are highly suggestive of a role for N1 in tumour invasion. As described above, the restitutive epithelium also demonstrates molecular changes consistent with cells found at the invasive front, including reduced expression of the cell adhesion protein E-cadherin (Hanby *et al.*, 1996; Karayiannakis *et al.*, 1998), and was shown here to be strongly positive for N1 (*FIGURE 3-9F-I*). In addition, the granulation tissue was also consistently and strongly positive for N1. Granulation tissue is known to be heavily dependent on the presence of sub-epithelial myofibroblasts (SEMFs), involved in depositing new basement membrane components and growth factors, such as TGF-β, at the ulcerative surface (Andoh *et al.*, 2005). Interestingly, similar mechanisms are now also linked to the promotion of invasive tumour growth through the action of cancer-associated fibroblasts (CAFs) (Micke and Ostman, 2005). Taken together, these results imply a central role for N1 during intestinal repair but also suggest that N1 may be an essential feature of tumour de-differentiation, migration and invasion; defining features of the adenocarcinoma phenotype.

3.3.4 CONCLUDING REMARKS

In summary, these results confirm that N1 is expressed in the human colorectal epithelium and that changes in expression are an important feature of human colorectal disease. Although CRC incidence continues to increase, the majority of these patients still present with late stage disease and novel adjuvant therapies are now essential to help improve the survival. The potential use of Notch inhibitors in the treatment of CRC has been suggested previously (van Es and Clevers, 2005) and this is fully supported by the findings of this study, which demonstrate N1 expression to be significant feature of colorectal tumour malignancy. What currently remains unclear, however, is whether this increased N1 expression arises as a cause or a consequence in these tissues and the functional contribution of N1 in CRC now needs to be defined, with particular focus on both the proliferative and invasive phenotype in these cells. Importantly, this study also highlights other undefined but potentially essential roles for N1 in both normal epithelial maturation and repair, suggesting that N1 may also have tumour suppressor-type roles in this tissue. Therefore, it is clear that, although Notch inhibition is beginning to be tested in other tissue contexts, a much greater understanding of a functional contribution for N1 in the normal and diseased colorectal epithelium is now essential before these treatments should be considered in CRC patients.

CHAPTER 4

NOTCH1 IS INCREASED WITH CELL CYCLE PROGRESSION

4 NOTCH1 IS INCREASED WITH CELL CYCLE PROGRESSION

4.1 INTRODUCTION

4.1.1 NOTCH AND WNT DEMONSTRATE CONSERVED SYNERGY IN A NUMBER OF TISSUES

Deregulation of normal cell cycle control and uncontrolled proliferation are key hallmarks of colorectal carcinogenesis (Hanahan and Weinberg, 2000) and increasing progression along the colorectal adenoma-adenocarcinoma sequence coincides with further increases in proliferative potential (Jackson *et al.*, 1995; Shpitz *et al.*, 1997; Yang *et al.*, 1996). In a similar fashion to Wnt, exogenous N1 activation in the intestinal epithelium is known to induce expansion of the proliferative progenitor pool, while Notch inactivation has been shown to cause loss of proliferation and precocious differentiation (*SECTION 1.5, p37*). Notch activation is also essential for the maintenance of intestinal tumours derived from $Apc^{+/Min}$ mice (van Es *et al.*, 2005). Taken together, these results strongly suggest that Notch is a central mediator of promotion in both the normal and neoplastic human colorectal epithelium; however, functional analysis in human colorectal cancer cells was not previously addressed and a putative oncogenic role for Notch in these tissues remains poorly defined.

The Integration of mitogenic signalling pathways to affect cell fate is a fundamental issue in developmental biology (Hurlbut *et al.*, 2007) and interaction between Notch and Wnt is known to be highly conserved in a number of contexts. In *Drosophila*, coordinated Notch and Wnt signalling is known to control both proliferation and differentiation in the development of various tissues (Baonza and Freeman, 2005; Go and Artavanis-Tsakonas, 1998). Cross talk is believed to occur at receptor, cytoplasmic and nuclear levels, and convergence between Notch and various components of the Wg signalling pathway have been documented, including interaction with the Wg ligand (Wesley, 1999), Dishevelled (Axelrod *et al.*, 1996), Armadillo (β-catenin in mammals) (Hayward *et al.*, 2006; Hayward *et al.*, 2005) and Shaggy (GSK-3β in mammals) (Ruel *et al.*, 1993).

Synergy between Notch and Wnt is thought to be highly conserved and is also a major feature of many mammalian developmental processes (reviewed in Hayward *et al.*, 2008). This is most eloquently demonstrated during control of the segmentation clock, in which transient cycles of activation are essential for correct vertebrae formation (reviewed in Rodriguez-Gonzalez *et al.*, 2007) and Wnt activation is believed to act upstream of Notch (Galceran *et al.*, 2004). It is becoming increasingly clear that continued synergy between these pathways is essential for

homeostasis of a number of adult tissues, including blood (Weerkamp *et al.*, 2006), breast (Collu and Brennan, 2007), skin (Fuchs, 1998) and intestine (reviewed in Nakamura *et al.*, 2007). It should not be surprising therefore that synergy between these two pathways is now increasingly known to be essential to many human neoplasia, including CRC (Nakamura *et al.*, 2007).

As with *Drosophila*, several nodes of potential interaction between mammalian Notch and Wnt pathway components have been proposed, with GSK-3 (Glycogen synthase kinase 3), LEF-1 (lymphoid enhancer binding factor 1) and Maml-1 (Mastermind-like 1) likely to be of greatest importance in the intestine and CRC. As described previously, GSK-3 proteins (GSK-3α/β) form an essential part of the 'β-catenin-destruction' complex in the canonical Wnt pathway (*SECTION 1.2.3, p14*). GSK-3α/β have also been shown to modulate both $N1^{IC}$ (Foltz *et al.*, 2002; Jin *et al.*, 2009b) and $N2^{IC}$ (Espinosa *et al.*, 2003) in other tissues, suggesting that GSK-3 proteins may represent a crucial link between Notch and Wnt in the intestinal epithelium. Furthermore, $N1^{IC}$ has been shown to physically interact with LEF-1, an established Wnt transcriptional co-factor, and is believed to potentiate the transcription of a set of genes distinct from those mediated by β-catenin/LEF-1 (Ross and Kadesch, 2001). This may be of particular relevance in the context of colorectal cancer, since LEF-1 is not normally expressed in the adult intestine (Wong *et al.*, 2002) but *de novo* expression is known to be an essential feature of colorectal tumorigenesis (Hovanes *et al.*, 2001). In a similar fashion, Maml-1 is an essential transcriptional co-factor of active Notch signalling (Wu *et al.*, 2000) but has been shown to promote β-catenin-mediated signalling independently of Notch and is required for the proliferation and survival of colorectal adenocarcinoma cells (Alves-Guerra *et al.*, 2007). In addition, active Wnt signalling may also positively modulate Notch activation at the receptor level by inducing expression of the Notch ligands Delta-like ligand1 (Dll1) (Galceran *et al.*, 2004; Hofmann *et al.*, 2004) and Jagged1 (Jag1) (Estrach *et al.*, 2006), as shown in other tissues. Although it is clear that convergence between Notch and Wnt is highly conserved and both pathways are central features of intestinal tumorigenesis, the mechanistic and functional convergence of the Notch and Wnt pathways in the context of human colorectal cancer remains largely unknown.

4.1.2 NOTCH AND WNT MAY CONVERGE IN CONTROL OF THE CELL CYCLE

In general, proliferation is accompanied by progression through the cell cycle (*FIGURE 4-1*), while differentiation requires cell cycle arrest. Since many of the hallmark features of cancer (*SECTION 1.1.7, p8*) relate directly to increased proliferation and cell survival, cancer cells frequently demonstrate perturbed cell cycle control, which generally occurs through increased mitogenic

signalling and/or alterations to the normal regulatory machinery (reviewed in Evan and Vousden, 2001). In colorectal tumours, the Wnt pathway is well known to promote proliferation and continued cell cycle initiation by over-riding the G1-S transition (van de Wetering *et al.*, 2002). Of the various direct Wnt target genes identified, *c-Myc* (He *et al.*, 1998) and *Cyclin D1* (Shtutman *et al.*, 1999; Tetsu and McCormick, 1999) are the most well defined and have profound effects on G1 progression. Expression of Cyclin D1 directly promotes G1 progression by forming complexes with Cyclin dependent kinases 4 and 6 (CDK4/CDK6) (reviewed in Adams, 2001), while c-Myc directly represses the important CDK inhibitor (CDKI) $p21^{CIP1/WAF1}$ (*CDKN1A*) (van de Wetering *et al.*, 2002). During colorectal differentiation, decreased Wnt activity therefore coincides with increased $p21^{CIP1/WAF1}$ and G0/G1 cell cycle arrest (Evers *et al.*, 1996; Mariadason *et al.*, 2001). Similarly, forced expression of $p21^{CIP1/WAF1}$ alone is sufficient to arrest cells at G0/G1 and up-regulate genes associated with differentiation (Archer *et al.*, 1998; van de Wetering *et al.*, 2002).

The significance of Notch signalling in cell cycle control is yet to be fully appreciated, however preliminary work in *Drosophila* has shown that Notch may be required for the G1/S transition (Baonza and Freeman, 2005; Firth and Baker, 2005). As discussed previously (*SECTION 1.6, p46*), the oncogenic function of N1 in mammals is most well appreciated as a causative agent in T-cell leukaemia (T-ALL) (Pear *et al.*, 1996; Weng *et al.*, 2003). An increasingly apparent feature of T-ALL is deregulation of the G1-S transition through altered expression of various CDKIs and CDKs, as well as c-Myc and Rb (Hebert *et al.*, 1994; Iravani *et al.*, 1997; Rao *et al.*, 2009). N1 activation has been shown to promote proliferation and progression through the G1 phase of the cell cycle in both T-ALL and bone marrow stem cells (Carlesso *et al.*, 1999), while γ-secretase inhibitors (GSIs) arrests T-ALL cell lines in G0/G1 (Lewis *et al.*, 2007; Palomero *et al.*, 2006; Rao *et al.*, 2009; Weng *et al.*, 2004; Weng *et al.*, 2003). In further support of an integrated role for Notch and Wnt in cancer, both c-Myc (Palomero *et al.*, 2006; Sharma *et al.*, 2006; Weng *et al.*, 2006) and Cyclin D (Ronchini and Capobianco, 2001; Sicinska *et al.*, 2003) are identified as direct and necessary targets of N1 in T-ALL cells, and over-expression of c-Myc has been shown to confer further resistance to GSI treatment in these cells (Sharma *et al.*, 2006).

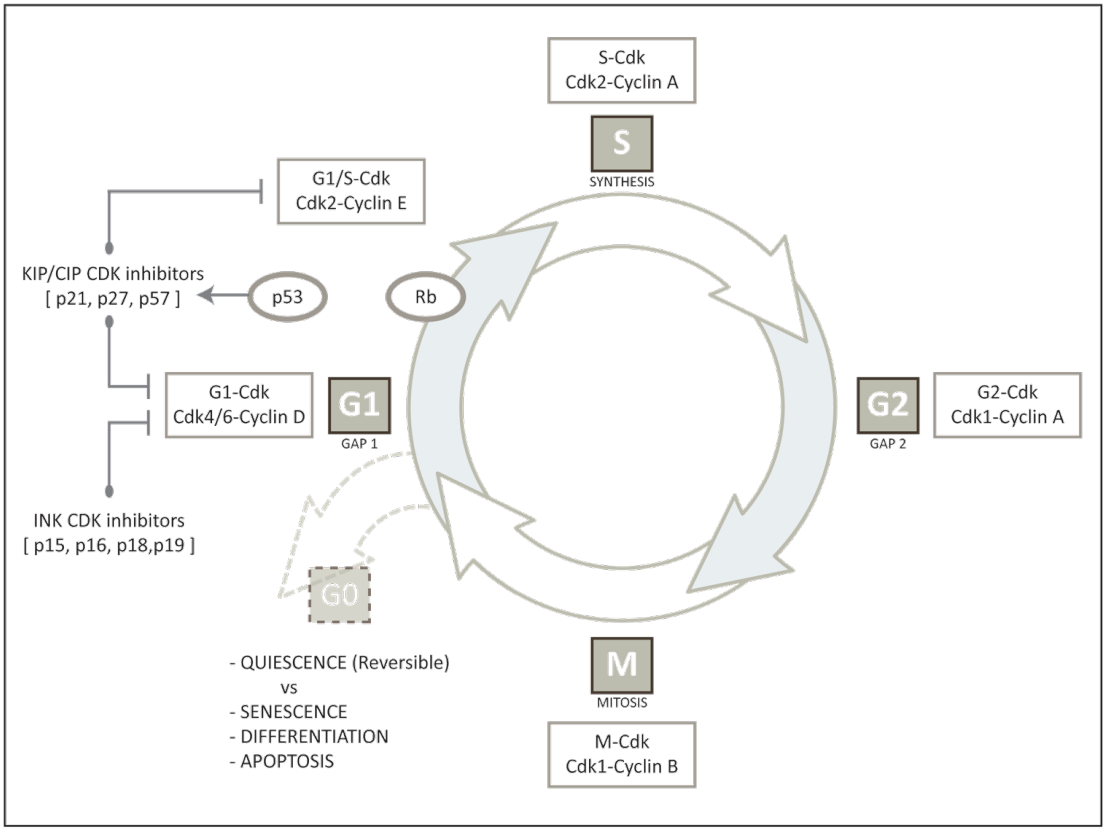


Figure 4-1| *The mammalian cell cycle.* The basic cell cycle is complex but composes of four distinct phases; S (*Synthesis*), M (*Mitosis*), G1 (*Gap1*) and G2 (*Gap2*) (reviewed in Schafer, 1998). The decision to commit to a new cell cycle is largely governed by regulatory mechanisms in G1, in which cells may also reversibly exit the cycle and enter 'quiescence' (G0), or undergo irreversible senescence, terminal differentiation, or apoptosis. Each phase of the cell cycle is driven by specifically timed formation of Cyclin dependent kinase (CDK) complexes, comprised of regulatory 'Cyclin' and catalytic 'CDK' subunits (reviewed in Pines, 1993). The activity of these Cyclin-CDK complexes is further controlled by both the INK4 and CIP/KIP family of CDK inhibitors (CDKIs), which are potent inducers of cell cycle arrest (reviewed in Vidal and Koff, 2000). Numerous cell cycle checkpoints also exist to sense replication defects and DNA damage, with two major checkpoints at the G1/S and G2/M boundaries (reviewed in Hartwell and Weinert, 1989). The G1 checkpoint is crucial in determining whether a cell should divide, differentiate or enter G0, and is famously governed by the action of both the p53 and Rb tumour suppressor proteins (reviewed in: Adams, 2001; Vousden and Lane, 2007).

Deregulated expression of many cell cycle regulatory proteins, namely Cyclin D and the various CDKIs, is associated with colorectal cancer (McKay *et al.*, 2002; Sutter *et al.*, 1997; Yang *et al.*, 2001b). Of these, negative regulation of $p21^{CIP1/WAF1}$ has received particular attention, with inactivation of $p21^{CIP1/WAF1}$ (*CDKN1A*) in the intestines of $Apc^{+/Min}$ mice being shown to promote tumour formation (Yang *et al.*, 2001c). In addition, reduced $p27^{KIP1}$ (*CDKN1B*) is a marker of poor prognosis in CRC (Loda *et al.*, 1997) and its inactivation alone is sufficient to enhance proliferation and tumour formation in the normal intestinal epithelium (Philipp-Staheli *et al.*, 2002; Yang *et al.*, 2005; Yang *et al.*, 2003). While $p21^{CIP1/WAF1}$ expression is classically governed by active Wnt/c-Myc

signalling in these tissues, $p27^{KIP1}$ transcription is instead believed to be under the control of Notch in the normal intestinal epithelium (Riccio *et al.*, 2008). Furthermore, it has been shown that active N1 signalling may also promote the proteasomal degradation of both $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ proteins in other tissues (Dohda *et al.*, 2007; Sarmento *et al.*, 2005).

It is becoming increasingly evident that Notch may govern cell cycle progression in a range of tissues, including the intestinal epithelium. However, to date this has remained largely untested in the context of CRC and it might be hypothesised that in addition to Wnt, Notch concertedly acts to promote cell cycle progression in colorectal cancer cells. This may arise through convergence on a particular cell cycle step/factor or through the complementary promotion of distinct cell cycle stages; however, Notch activation is known to promote the G1-S transition in other tissues and it is possible that active N1 signalling carries out similar function in CRC to complement Wnt in overriding G1.

4.1.3 A MULTIFACETED ROLE FOR NOTCH IN CANCER

In other mammalian tissues, N1 activation is known to induce cell cycle arrest and drive differentiation, suggesting N1 may also function as a tumour suppressor in certain contexts. This has been most clearly defined in human and murine epidermal keratinocytes (reviewed in: Dotto, 2008; Watt *et al.*, 2008), in which the expression of Notch ligands and receptors is markedly increased in the differentiating layers of the epidermis (Rangarajan *et al.*, 2001b) and exogenous N1 activation results in dramatic growth arrest and differentiation (Lowell *et al.*, 2000; Nickoloff *et al.*, 2002; Rangarajan *et al.*, 2001b). Strangely, N1-mediated arrest in these tissues occurs through increased expression of $p21^{CIP1/WAF1}$ (*CDKN1A*), which was further shown to be a positive transcriptional target of active Notch by the presence of CSL binding motifs in its promoter region (Rangarajan *et al.*, 2001b). In these tissues, N1 may be reciprocally regulated by the cell cycle and was shown to be required for $p21^{CIP1/WAF1}$ induced keratinocyte differentiation (Devgan *et al.*, 2005; Rangarajan *et al.*, 2001b). Notch inhibition in the murine epidermis therefore leads to decreased $p21^{CIP1/WAF1}$ expression and increased proliferation, resulting in epidermal hyperplasia (Lee *et al.*, 2007a; Rangarajan *et al.*, 2001b) and squamous cell carcinoma (SCC) formation (Lefort *et al.*, 2007; Nicolas *et al.*, 2003; Proweller *et al.*, 2006). Furthermore, N1 activity has been shown to be significantly reduced in human cases of SCC as well as basal cell carcinomas (BCC), the two major types of non-melanoma skin cancer (Thelu et al., 2002; Lefort et al., 2007).

Notch signalling elicits a pro-differentiation and tumour suppressor role in an increasing number of tissues (reviewed in: Radtke and Raj, 2003; Roy *et al.*, 2007) and N1 has been shown to

promote growth arrest in various cell types, including adipocytes (Garces *et al.*, 1997), erythroid cells (Shelly *et al.*, 1999), myeloid cells (Weijzen *et al.*, 2002b), mesenchymal cells (Sakamoto *et al.*, 2002) endothelial cells (Noseda *et al.*, 2004a), liver cells (Croquelois *et al.*, 2005) and osteoblasts (Tezuka *et al.*, 2002). N1 also elicits growth inhibitory effects in a number of cancers, including multiple myeloma (Nefedova *et al.*, 2004), prostate cancer (Shou *et al.*, 2001), small cell lung cancer (Sriuranpong *et al.*, 2001; Sriuranpong *et al.*, 2002) and B-cell malignancies (Morimura *et al.*, 2000; Zweidler-McKay *et al.*, 2005). Interestingly, although N1 inactivation in epidermal keratinocytes results in dramatic hyperplasia, inhibition in follicular keratinocytes (derived from the same stem cell origin) results in a completely opposing hypoplastic phenotype (Lee *et al.*, 2007a). Similarly, N1 activation strongly promotes the formation of T-cell leukaemia (Pui *et al.*, 1999), but is also known to inhibit the growth of B-cell malignancies (Morimura *et al.*, 2000; Zweidler-McKay *et al.*, 2005), even though T-cells and B-cells originate from a common hematopoietic stem cell. Therefore, the distinct tumour promoting and suppressor roles of Notch may even operate within the same tissue and in cells derived from a common progenitor, depending on cell lineage, plus positional and/or pathophysiological context.

N1 has largely been proposed to promote tumorigenesis in the intestinal epithelium (Nakamura *et al.*, 2007) but, as described in the previous chapter, N1 activation also promotes differentiation in the normal murine intestinal epithelium (Zecchini *et al.*, 2005) and it was shown that N1 is expressed by differentiating cells at the top of the normal human colonic crypt (*SECTION 3.2.2, p92*). Taken together these results potentially highlight additional but largely undefined tumour suppressive functions in the intestinal epithelium. The functional significance of the Notch pathway in CRC cells remains to be established, but it is possible that both tumour promoting and tumour suppressing functions operate in these tissues under different contexts and is likely to be a major consideration for Notch-mediated therapies in these patients.

4.1.4 HYPOTHESIS AND SPECIFIC AIMS

The hypothesis guiding this chapter was that N1 signalling may cooperate with aberrant Wnt in colorectal cancer cells to promote proliferation and cell cycle progression, but under different cellular contexts N1 may associate with cells either demonstrating evidence of proliferation or demonstrating evidence of functional differentiation.

The aims of this chapter were therefore;

1. To determine the downstream effects of Notch1 pathway activation in colorectal cancer cells.
2. To determine the effects of modulating Notch1 signalling on colorectal cancer cell proliferation and cell cycle dynamics.
3. To determine changes in Notch1 expression in response to changes in cell cycle distribution.
4. To determine potential interactions between Notch and Wnt in the context of colorectal cancer.
5. To determine changes in Notch1 expression in response to the induced and spontaneous differentiation of colorectal cancer cells.

4.2 RESULTS

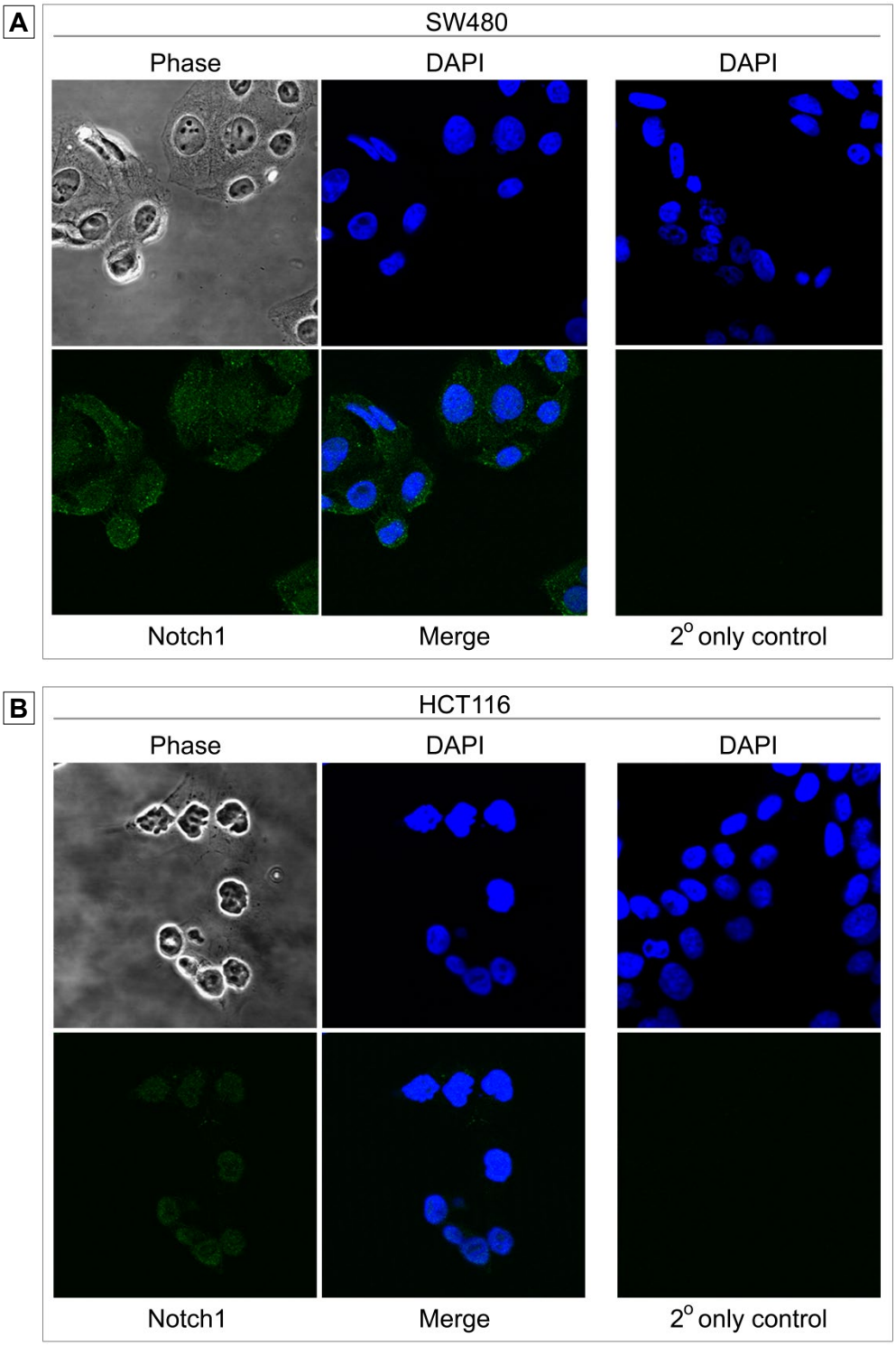
4.2.1 SUB-CELLULAR LOCALISATION DEFINES THE ACTIVATION STATUS OF NOTCH1

Nuclear translocation is an important regulatory mechanism for many transcription factors, including Notch (Struhl and Adachi, 1998; Vandromme *et al.*, 1996), with nuclear localisation and transcriptional activation being essential features for Notch-induced oncogenic transformation (Jeffries *et al.*, 2002; Ronchini and Capobianco, 2000). In line with studies performed in other tissues (Baldi *et al.*, 2004; Veeraraghavalu *et al.*, 2004), immunohistochemical analysis of N1 expression in the previous chapter demonstrated N1 immunoreactivity to be predominantly cytoplasmic in colorectal tissues, except for small regions of some tissues where nuclear N1 was also observed (*SECTION 3.2.6, p108*). Analysis of N1 expression using colorectal tumour cell lines strongly suggested that these *in vitro* cells faithfully model the *in vivo* scenario (*SECTION 3.2.7, p111*) and therefore enable the functional manipulation of Notch signalling in human colorectal tumour tissues. In order to further define the sub-cellular localisation and activation status of endogenous N1 in CRC cells, the SW480 and HCT116 colorectal adenocarcinoma cell lines were selected as positive controls for cleaved ($N1^{TM/IC}$) and full length ($N1^{FULL}$) protein expression respectively. Both cell lines were grown to sub-confluency and fluorescently immunolabelled for endogenous N1 protein expression (*SECTION 2.6, p68*). Cell nuclei were highlighted using blue DAPI counter-stain and N1 sub-cellular localisation was visualised by combined confocal and phase contrast microscopy.

In SW480 cells (*FIGURE 4-2A*), N1 immunoreactivity was observed in both the cytoplasm and nucleus at roughly equal concentration, although numerous small and strongly positive cytoplasmic puncta were also observed, suggesting potential protein accumulation in these structures. In agreement with previous western blotting analysis (*SECTION 3.2.7, p111*), the HCT116 cell line (*FIGURE 4-2B*) demonstrated a relatively lower level of cytoplasmic (cleaved) N1 immunoreactivity and all N1 expression was localised primarily to the nucleus. Strangely, the HCT116 cell line did not appear to express readily detectable amounts of the $N1^{FULL}$ isofom, as might be expected in the trans-Golgi network. Similarly, no membranous N1 localisation was detected in either cell line, suggesting that this isoform may be difficult to detect without the denaturing conditions associated with western blotting analysis. These findings do however support analysis using *in vivo* tissues, in which virtually all N1 immunoreactivity using this antibody (C20/sc6014; *Santa Cruz, USA*) was observed in the cytoplasm (*SECTION 3.2.6, p108*) and never at the cell surface or in Golgi-like structures.

In order to further clarify this, both $N1^{FULL}$ and $N1^{IC}$ protein isoforms were exogenously expressed in the SW480 and HCT116 cell lines by transient transfection (*SECTION 2.3.1, p61*) (Aster *et al.*, 1997). Increased expression of both isoforms (~300kDa $N1^{FULL}$ and ~110kDa $N1^{IC}$) was confirmed by western blotting analysis (*FIGURE 4-3A*) and transfected cells were immunofluorescently labelled for N1. Sub-cellular localisation was determined by confocal microscopy and the $N1^{FULL}$ protein was found to be clearly evident as punctuate dots in the presumptive Golgi-apparatus and secretory vesicles of both cell lines (*FIGURE 4-4*). Increased immunoreactivity was also frequently evident at the cell membrane and in the cytoplasm of some cells, reflecting potential downstream processing to $N1^{TM}$ and $N1^{IC}$ isoforms by S1-S3 cleavage events. Cytoplasmic immunoreactivity was strongly increased following exogenous expression of the $N1^{IC}$ protein, which was also found to be heavily accumulated in the nucleus (*FIGURE 4-5*). This is in agreement with analysis of basal N1 expression in these cells (*FIGURE 4-2*) and confirms that in CRC cells $N1^{IC}$ rapidly translocates to the nucleus, but may also accumulate in the cytoplasm, although mechanisms governing this are not yet entirely clear.

Figure 4-2| Endogenous *Notch1 expression is predominantly cytoplasmic and nuclear.* Immunocytochemical analysis of the SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma-derived cell lines, fluorescently labelled for endogenous Notch1 using green Alexafluor488 secondary antibody (*Molecular Probes, USA*). Cells were analysed by confocal microscopy and detail of cell structure was provided by blue DAPI nuclear counter-stain and phase contrast imaging. N1-specific immunoreactivity is confirmed in both cell lines by secondary only controls. *(Magnification x40)* ▶


A
SW480
Phase
DAPI
DAPI
Notch1
Merge
2° only control
B
HCT116
Phase
DAPI
DAPI
Notch1
Merge
2° only control

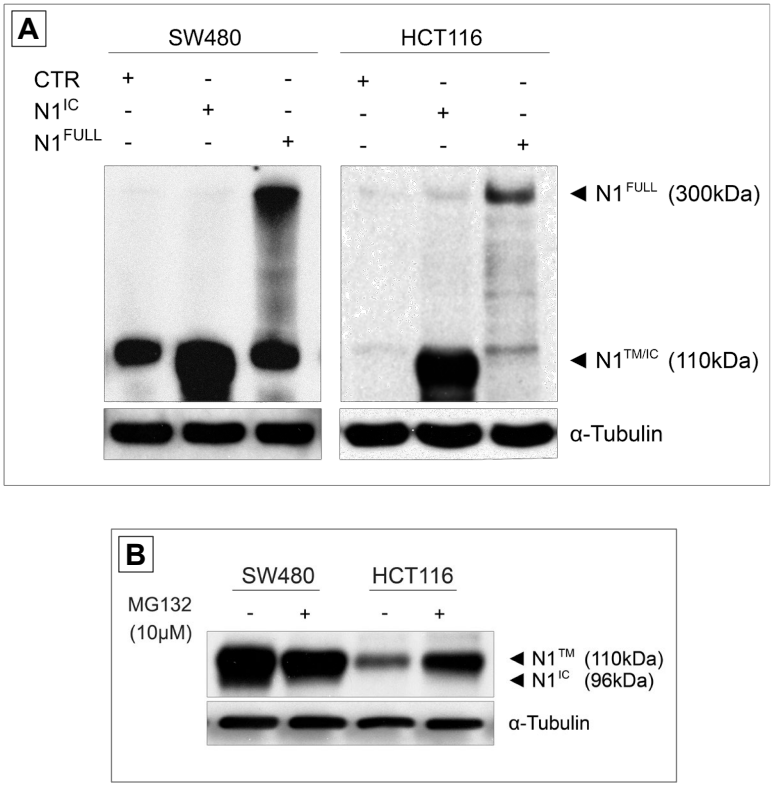


Figure 4-3| *Full length and active Notch1 protein forms can be exogenously expressed.* [**A**] Western blotting analysis of SW480 and HCT116 cells following 24hr transient transfection with empty vector control (CTR) or plasmids encoding either activated intracellular Notch1 ($N1^{IC}$) or unprocessed full length Notch1 ($N1^{FULL}$) proteins. Equal protein loading was confirmed by analysis of α-Tubulin. [**B**] Western blotting analysis of cleaved N1 protein ($N1^{TM}$ and $N1^{IC}$) in the SW480 and HCT116 colorectal adenocarcinoma-derived cell lines following γ-secretase inhibition by 24h treatment with MG132 (10μM), vs. vehicle control.

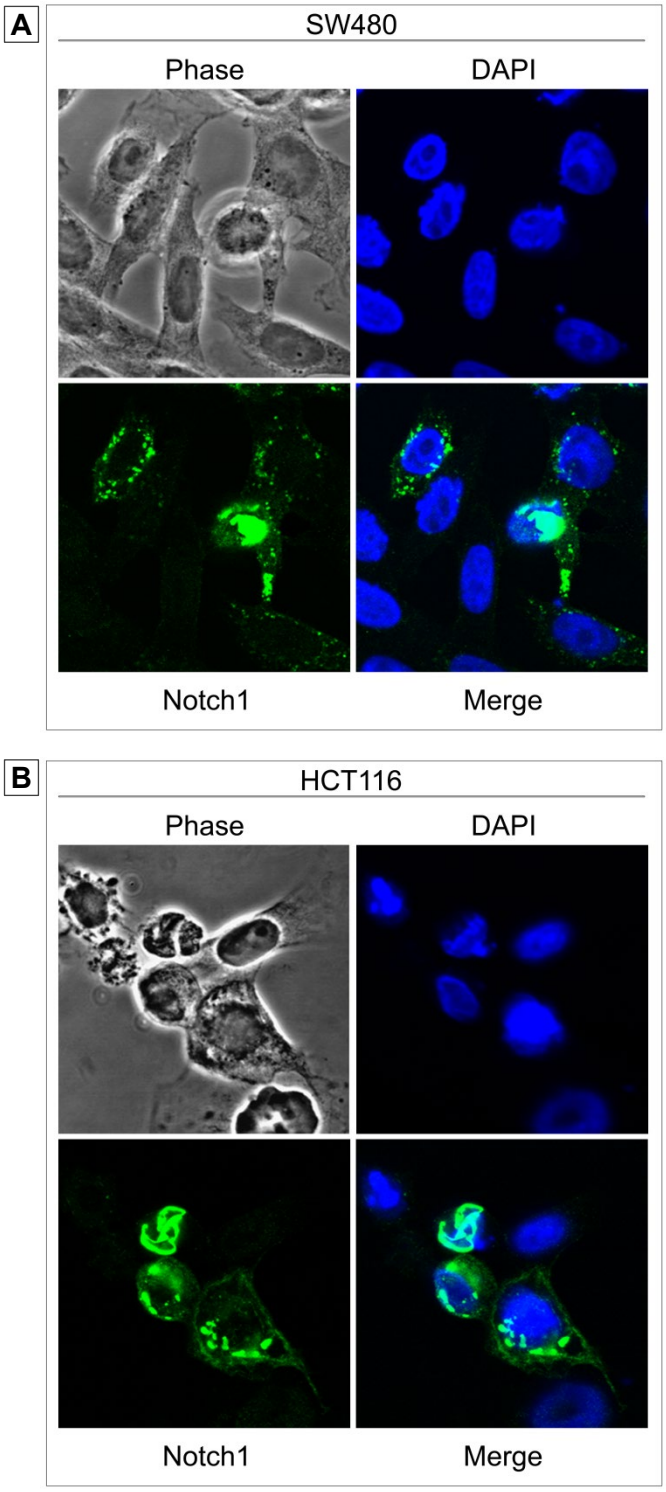


Figure 4-4| *Exogenous full length Notch1 leads to increased vesicular, membranous and cytoplasmic protein localisation.* Immunocytochemical analysis of the SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma-derived cell lines following 24h transient transfection with plasmid encoding $N1^{FULL}$. Cells were fluorescently labelled for N1 using green Alexafluor488 secondary antibody (*Molecular Probes, USA*) and nuclei were highlighted using blue DAPI nuclear counter-stain, prior to analysis by confocal and phase contrast microscopy. *(Magnification x 80)*

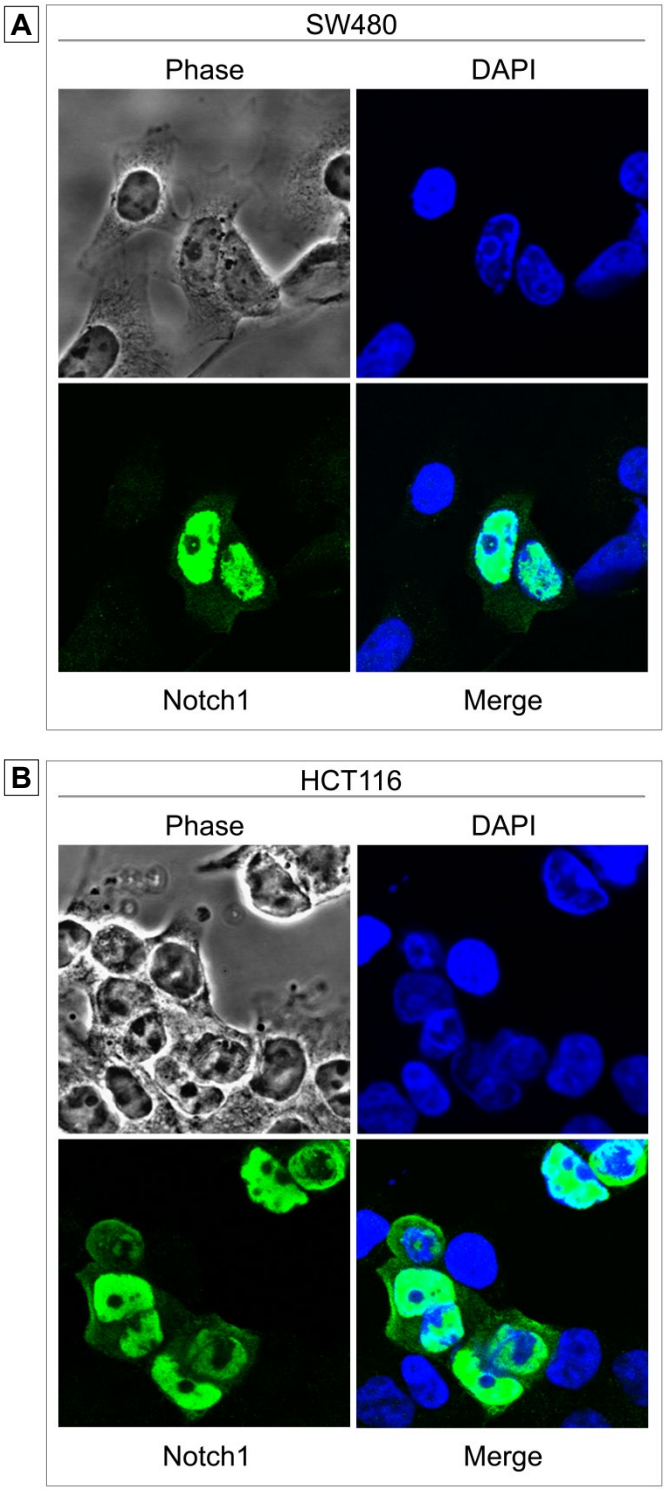


Figure 4-5| *Exogenous intracellular Notch1 expression accumulates in the cell cytoplasm and nucleus.* Immunocytochemical analysis of the SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma-derived cell lines following 24h transient transfection with plasmid encoding active $N1^{IC}$. Cells were fluorescently labelled for N1 using green Alexafluor488 secondary antibody (*Molecular Probes, USA*) and nuclei were highlighted using blue DAPI nuclear counter-stain, prior to analysis by confocal and phase contrast microscopy. *(Magnification x 80)*

4.2.2 THE NOTCH1 PROTEIN BANDING PATTERN IS CONFIRMED BY MG132 TREATMENT

$N1^{TM}$ and $N1^{IC}$ protein isoforms are known to be difficult to distinguish by western blotting but previous studies suggest that these distinct proteins may be identifiable as bands at 110kDa and 96kDa respectively (Blaumueller *et al.*, 1997; Weijzen *et al.*, 2002a). Consistent with this, transient transfection of the SW480 and HCT116 CRC cell lines with $N1^{IC}$ results in the expression of a slightly smaller protein than the endogenous N1 protein expressed in these cells (*FIGURE 4-3A*). MG-132 is a widely used broad-spectrum (peptide aldehyde) protease inhibitor, which has previously been shown to act as a potent inhibitor of the γ-secretase complex (De Strooper *et al.*, 1999; Higaki *et al.*, 1995; Klafki *et al.*, 1995; Skovronsky *et al.*, 2000; Steinhilb *et al.*, 2001; Zhang *et al.*, 1999). MG132 treatment therefore strongly inhibits the processing of $N1^{TM/\Delta E}$ to $N1^{IC}$ (De Strooper *et al.*, 1999) and significantly decreases *Hes1* activation (Oberg *et al.*, 2001). Therefore, in order to investigate the endogenous levels of N1 activation in colorectal cancer cells, sub-confluent cultures of SW480 and HCT116 were treated for 24 hours with 10μM of MG132 or vehicle control and the relative levels of cleaved N1 were assessed by western blotting analysis (*FIGURE 4-3B*). In support of the hypothesis that cleaved N1 (~110kDa) constitutes two distinct bands ($N1^{TM}$ at 110kDa and $N1^{IC}$ at 96kDa), MG132 treatment of the HCT116 cell line resulted in a dramatic up-regulation of the 110kDa band while the SW480 cell line demonstrated a substantial decrease in the lower 96kDa form. Together, these results confirm both the 110kDa $N1^{TM}$ and 96kDa $N1^{IC}$ isoforms to be present in these cells. While the SW480 cell line appears to strongly express both forms, HCT116 cells do not express detectable levels of $N1^{IC}$. This suggests that $N1^{TM}$ is the principal isoform expressed in most colorectal tumour cell lines (*SECTION 3.2.7, p111*) and is consistent with the short half life for activated $N1^{IC}$ (De Strooper *et al.*, 1999; Oberg *et al.*, 2001; Qiu *et al.*, 2000; Wu *et al.*, 2001) and cell fractionation analysis in other tissues using this antibody (Blaumueller *et al.*, 1997).

4.2.3 HES1 AND HES5 ARE TRANSCRIPTIONAL TARGETS OF ACTIVATED N1

It is generally accepted that *Hes1* is the predominant target of active Notch signalling in many tissues, including the intestine (Fre *et al.*, 2005; Jensen *et al.*, 2000), although only a small number of other Notch target genes have so far been identified (reviewed in Iso *et al.*, 2003). From analysis in animal models, Hes1 is also believed to be a significant target of active Notch signalling in intestinal tumours (van Es *et al.*, 2005), although the downstream effects of Notch activation in human CRC cells is very poorly defined. In order to investigate possible downstream targets of active N1 signalling in CRC cells, the HCT116 cell line was selected for further studies

due to the relatively low levels of cleaved N1 and Hes1 protein expressed by this cell line (*SECTION 3.2.7, P111*). Cells were transiently co-transfected with expression plasmids for either $N1^{IC}$ or empty vector control, plus reporter constructs containing full length promoter regions from mammalian *Hes1*, *Hes2*, *Hes3* or *Hes5* (Nishimura *et al.*, 1998). Mutated Hes1 promoter constructs, comprising only the $N1^{IC}$/CSL (CBF1/RBP-J in mammals, Su(H) in *Drosophila*, Lag-1 in C. Elegans) responsive region (*CSL responsive*) or lacking this region (*CSL unresponsive*), were also included as controls. In these cells, both *Hes1* and *Hes5,* but not *Hes2* or *Hes3* were significantly trans-activated by $N1^{IC}$ (*FIGURE 4-6A*), confirming both *Hes1* and *Hes5* to be targets of active Notch signalling in human colorectal cancer. This is consistent with functional analysis in the normal murine intestinal epithelium, which has identified both Hes1 and Hes5 as targets of $N1^{IC}$ (Fre *et al.*, 2005; Jensen *et al.*, 2000; Zecchini *et al.*, 2005) and with the previous identification of CSL binding sites in both the *Hes1* and *Hes5* promoters from analysis in neurogenesis (Nishimura *et al.*, 1998). Furthermore, it was shown that *Hes1* promoter activation occurs through the canonical N1/CSL pathway, since the CSL-responsive but not CSL-unresponsive promoter-reporter construct was also up-regulated in the presence of $N1^{IC}$.

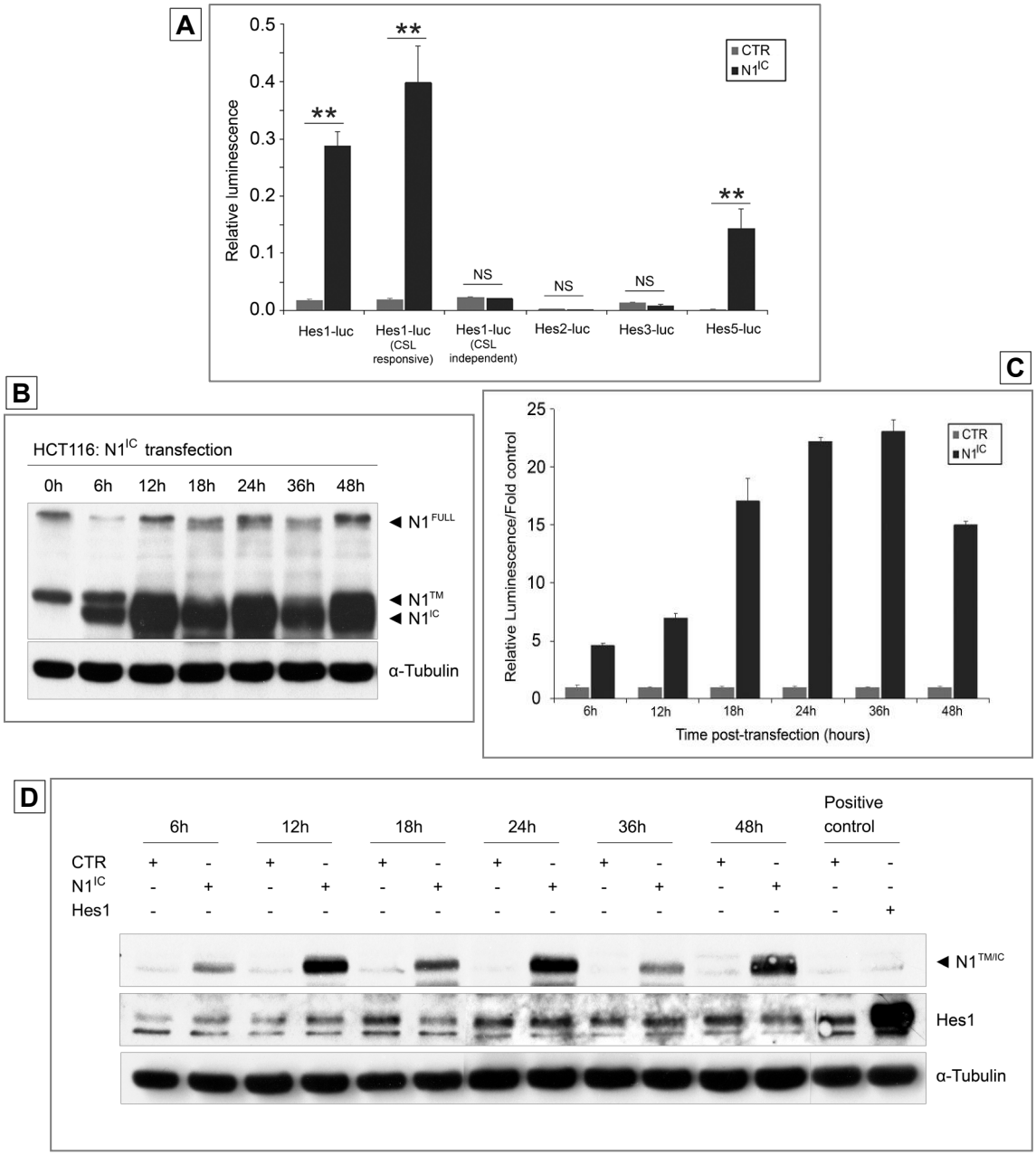


Figure 4-6| *Hes1 and Hes5 are immediate targets of Notch1 activity in colorectal cells.* [**A**] Trans-activation of *Hes1* and *Hes5* promoter-reporter constructs by intracellular-Notch1 ($N1^{IC}$) in the HCT116 colorectal adenocarcinoma cell line. The CSL-responsive and CSL-unresponsive *Hes1* promoter reporters were included as controls. Values were normalised to *Renilla* readout and data represents two experiments performed in triplicate. [**B**] Time-course of N1 protein expression following transient transfection of the HCT116 cell line with $N1^{IC}$ expression vector. [**C**] Time-course of *Hes1* promoter-reporter activation by $N1^{IC}$, normalised to *Renilla* and expressed as fold control. [**D**] Western blotting analysis of $N1^{IC}$ and Hes1 protein expression over time-course of $N1^{IC}$ expression vs. empty vector control. (*Statistical analysis was performed using the Student's t-test, where degrees of significance are stated as *: p<0.05, **: p<0.01, ***: p<0.001 and error bars represent SD*).

To further investigate the dynamics of Notch target gene activation in these tissues, the relative levels of *Hes1* promoter transactivation and subsequent protein expression were assessed in the HCT116 cell line over a time-course ranging from 6 to 48 hours post-transfection. Western blotting confirmed the appearance of the lower 96kDa $N1^{IC}$ protein band (*FIGURE 4-6B*), which was evident as a 'doublet' band with endogenous $N1^{TM}$ within 6 hours and was sustained throughout the course of the experiment. Interestingly, forced activation of N1 in this cell line resulted in a coincidental decrease in endogenous $N1^{FULL}$/$N1^{TM}$ protein expression at the 6 hour time-point, possibly reflecting the action of negative feedback mechanism in these cells. Endogenous protein levels were restored again by 12 hours, but decreased and increased again in a cyclic fashion, as described previously in developmental systems such as vertebrate somitogenesis (Palmeirim *et al.*, 1997), which begins to allude to the existence of possible cyclic mechanisms in these cells. A significant increase in *Hes1* transcriptional activity was also observed within 6 hours and maintained throughout (*FIGURE 4-6C*). Expression of the Hes1 protein was also assessed by western blotting analysis at each time point for comparison with untransfected cells and positive controls expressing exogenous *Hes1* (*FIGURE 4-6D*). Surprisingly, despite the significant increases in promoter activity, no significant increase in Hes1 protein was observed at any time-point. Taken together, these results confirm that both *Hes1* and *Hes5* are transcriptional targets of active N1 in colorectal cancer but suggest that the expression and translation of these genes may be complex and tightly regulated by other additional mechanisms.

4.2.4 BASAL HES1 TRANSCRIPTION IS MAINTAINED BY NOTCH1 AND TCF4 ACTIVITY

Deregulated Wnt signalling is a central player in colorectal carcinogenesis (*SECTION 1.2, p12*) and conserved synergy between Notch and Wnt is established in many contexts, including the intestinal epithelium (*SECTION 4.1.1*). *Hes1* mRNA was also previously shown to be strongly and uniformly expressed in adenomas derived from $Apc^{+/Min}$ mice (van Es *et al.*, 2005), suggesting *Hes1* is up-regulated early in intestinal tumorigenesis; however, it was shown in the previous chapter that N1 expression remains low in the majority of colorectal adenomas and increases significantly with malignant progression (*SECTION 3.2.5, p103*). Taken together, these findings suggest that active Wnt signalling may drive or potentiate the expression of Hes1 in these tissues.

In order to determine if basal levels of *Hes1* expression in colorectal cancer cells are Notch and/or Wnt dependent, the HCT116 cell line was treated with siRNA targeting either *N1*, *CSL*, or *CTNNB1* (β-catenin) vs. scrambled negative control (*Dharmacon/Autogen*) (*SECTION 2.3.3, p62*). Transient co-transfection of these cells with the *Hes1* promoter-reporter demonstrated a

significant but incomplete inhibition of *Hes1* transcriptional activity following N1-specific (*siRNA N1*) or pan-Notch (*siRNA CSL*) inhibition (*FIGURE 4-7A*). A lack of complete ablation in these cells may reflect the established low level activation of *Hes1* by Notch/CSL proteins (Schroeter *et al.*, 1998) but also implies that *Hes1* expression might be concertedly maintained by other Notch-independent signalling factors in colorectal cancer. In support, siRNA mediated knockdown of *CTNNB1* in HCT116 cells also resulted in a highly significant decrease in *Hes1* promoter-reporter activity, even exceeding that observed following siRNA-mediated knockdown of *N1* and *CSL* in this cell line (*FIGURE 4-7B*), suggesting that β-catenin/Wnt may have a substantive role in promoting *Hes1* expression in colorectal cancer cells.

The transcriptional effects of β-catenin are classically mediated by co-factors of the LEF/TCF family of transcription factors, of which TCF-4 is the prominent TCF family member in the intestinal epithelium (Korinek *et al.*, 1998a; Korinek *et al.*, 1997). To investigate a possible mechanistic role for β-catenin regulation of *Hes1*, the SW480 and HCT116 cell lines were co-transfected with expression plasmid encoding a dominant negative form of TCF-4 (*dnTCF4*) or empty vector control and the *Hes1* promoter-reporter or LEF/TCF responsive reporter (TOPFLASH; Korinek *et al.*, 1998a). In both cell lines, inhibition of canonical TCF-4 activity was confirmed by a significant decrease in TOPFLASH reporter readout (*Upstate Biotechnology, USA*) (*FIGURE 4-8*), and in agreement with results attained from siRNA-mediated knockdown of *CTNNB1*, a highly significant decrease of *Hes1* promoter-reporter activity was also observed in the presence dnTCF-4. Furthermore, a significant decrease in the CSL responsive *Hes1* promoter was observed following treatment with dnTCF4 in both cell lines, indicating that β-catenin/TCF-4 may promote *Hes1* expression either i) indirectly by altering factors associated with the canonical Notch pathway, or ii) directly by acting in the *Hes1* promoter region containing the CSL consensus sequences.

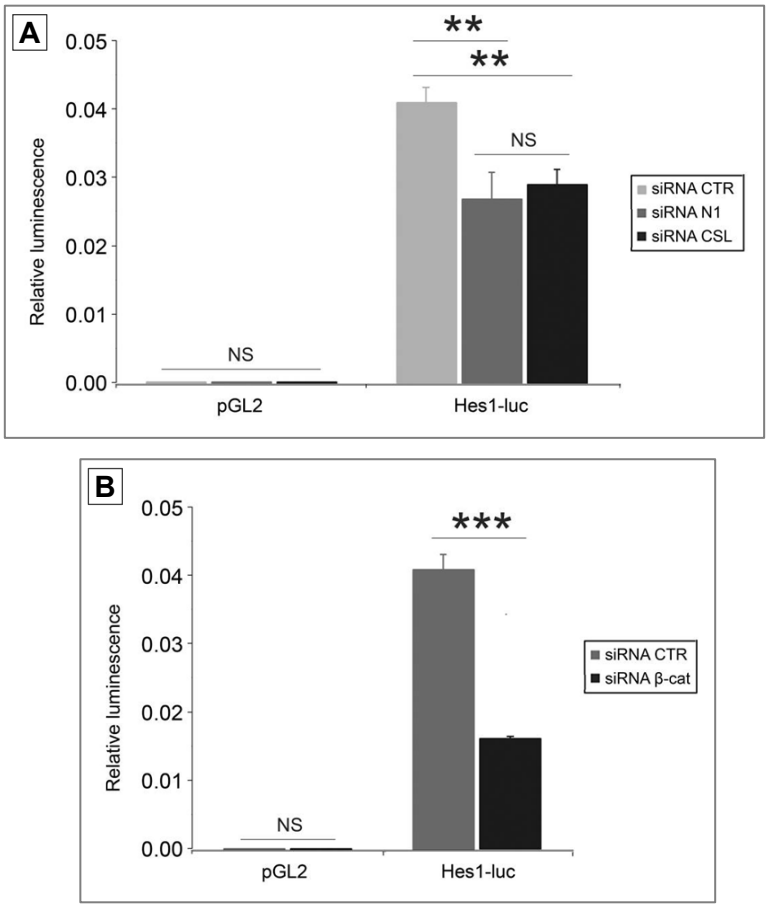


Figure 4-7| *Hes1 transcription is reduced following siRNA targeting of Notch1, CSL or CTNNB1.* [**A**] Inhibition of the *Hes1* promoter-reporter following inhibition of N1 or pan-Notch signalling in colorectal cancer cells. The HCT116 colorectal adenocarcinoma cell line was treated with either scrambled siRNA control or siRNA targeting either *N1* or *CSL* (*Dharmacon*). [**B**] Inhibition of the *Hes1* promoter-reporter construct following specific down-regulation of canonical Wnt signalling in HCT116 cells by siRNA targeting of β-catenin (*CTNNB1*) (*Autogen*). The promoter-less pGL2 vector is included as a control. All readings are normalised to *Renilla* control and data represents three independent experiments performed in triplicate. (*Statistical analysis was performed using the Student's t-test, where degrees of significance are stated as *: p<0.05, **: p<0.01, ***: p<0.001 and error bars represent SEM*).

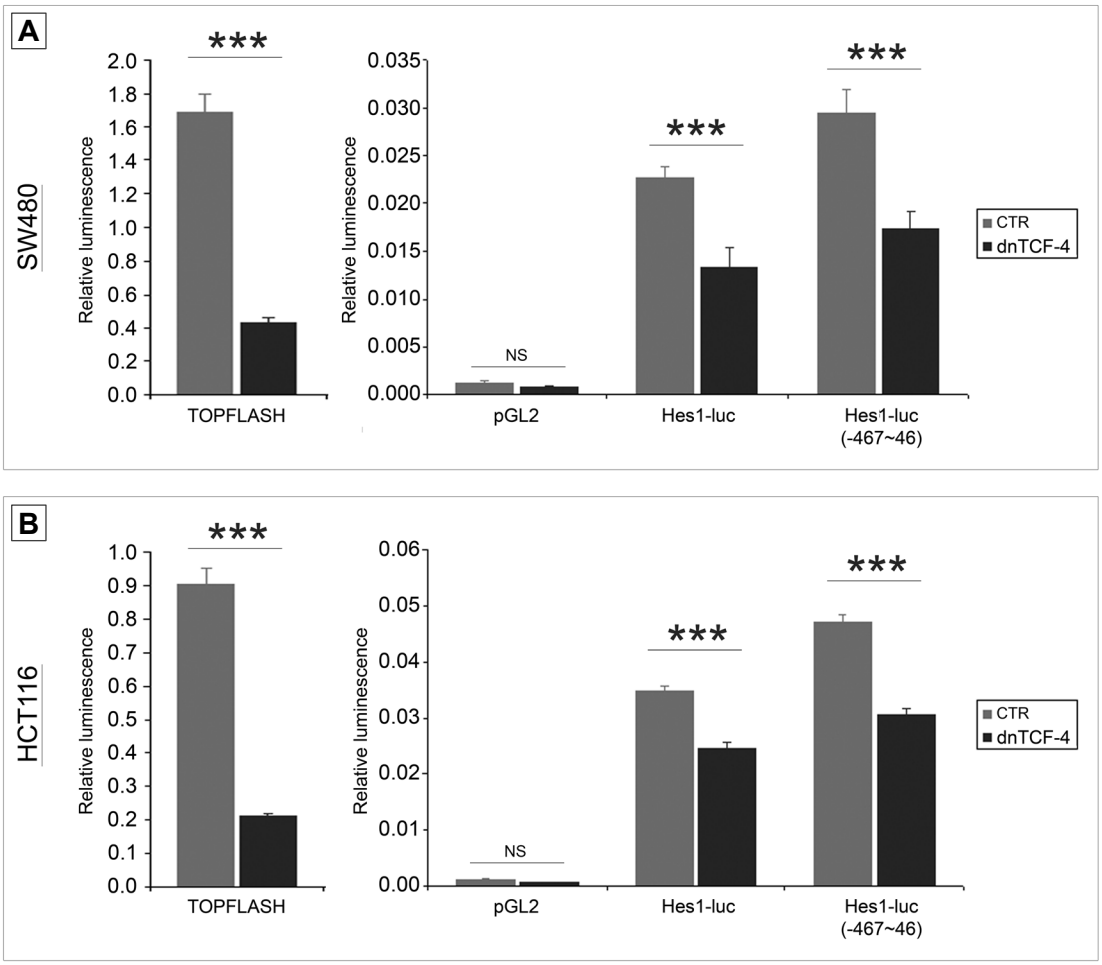


Figure 4-8| *Basal Hes1 activation is reduced by dominant-negative TCF4.* Inhibition of *Hes1* transcriptional activation following down-regulation of TCF-4 activity in colorectal cancer cells. SW480 [**A**] and HCT116 [**B**] cells were transiently transfected with plasmid encoding *dnTCF-4* or empty vector control. Cells were also co-transfected with full length *Hes1*, or CSL-responsive *Hes1* promoter reporters (vs. promoter-less pGL2 vector), or TOPFLASH (LEF/TCF responsive) reporter. Inhibition of TCF-4 activity was confirmed by a significant decrease in TopFlash reporter activity. Readings are normalised to *Renilla* control and data represents average values from three experiments performed in triplicate. (*Statistical analysis was performed using the Student's t-test, where degrees of significance are stated as *: p<0.05, **: p<0.01, ***: p<0.001 and error bars represent SEM*).

4.2.5 THE HES1 PROMOTER CONTAINS MULTIPLE LEF/TCF BINDING SITES

The *Hes1* gene is located at chromosome position *3q28-29* in humans (*Entrez gene ID 3280; 193,853,934-193,856,396*) (*FIGURE 4-9A*) and *16b2* in the mouse (*Entrez gene ID 15205; 300,654,43-300,678,82*). The genomic sequence 1000 base pairs upstream of the *Hes1* transcriptional start site (ATG) was identified (*SECTION 2.4, p64*) and this 5' regulatory promoter/enhancer region in both species was analysed for putative transcription factor binding sites (TFBSs) using PROMO3.0 software (Farre *et al.*, 2003; Messeguer *et al.*, 2002). To determine the degree of conservation, both human and murine sequences were then also aligned by EMBOSS pairwise alignment algorithms (Rice *et al.*, 2000; Sarachu and Colet, 2005) (*FIGURE 4-10*).

Although the pattern of *Hes* gene expression is complex, its promoter region is known to be remarkably simple. The Hes1 5' regulatory region contains a transcriptional start site 31 nucleotides downstream of a TATA box (TATATA), and transcription initiation occurs ~250 nucleotides upstream of the translation initiation codon (ATG) (Takebayashi *et al.*, 1994) (*FIGURE 4-9B*). CSL recognises the general DNA sequence CGTGGGAA (Tun *et al.*, 1994) and, consistent with numerous previous reports, the *Hes1* promoter was shown here to contain two CSL binding elements in reverse orientation (Rodilla *et al.*, 2009; Takebayashi *et al.*, 1994; Wettstein *et al.*, 1997). The Hes1 protein is unusual in that it exhibits a preference for binding N-box DNA motifs [CACNAG], unlike most other bHLH transcription factors, which bind E-box motifs [CANNTG] (Sasai *et al.*, 1992). The *Hes1* promoter contains three N-box sequences, through which Hes1 autoregulation (negative feedback) is thought to occur (Takebayashi *et al.*, 1994). Analysis of the *Hes1* promoter/enhancer region using PROMO3.0 software also highlighted several novel LEF/TCF binding sites (2 conserved, 3 partially conserved), which correspond specifically to LEF-1 and TCF-4E (*FIGURE 4-10*), supporting the hypothesis that β-catenin/TCF-4 activity might functionally promote *Hes1* transcription.

Importantly, these LEF-1/TCF-4E binding elements in the *Hes1* promoter are contained within a small region, 400-500bp upstream of the transcriptional start site, which has previously been shown to be responsible for almost all aspects of *Hes1* control during *Drosophila* development (Cooper *et al.*, 2000). Moreover, at least one of the conserved LEF-1/TCF-4E sites is contained within the promoter region of the CSL-responsive *Hes1* reporter construct *(-467 - +46*). Taken together, these results confirm that *Hes1* expression is closely regulated by active Wnt signalling in CRC cells and this may occur through the direct action of β-catenin/TCF-4 and the presence of at least two novel LEF-1/TCF-4E consensus sites identified in the *Hes1* promoter.

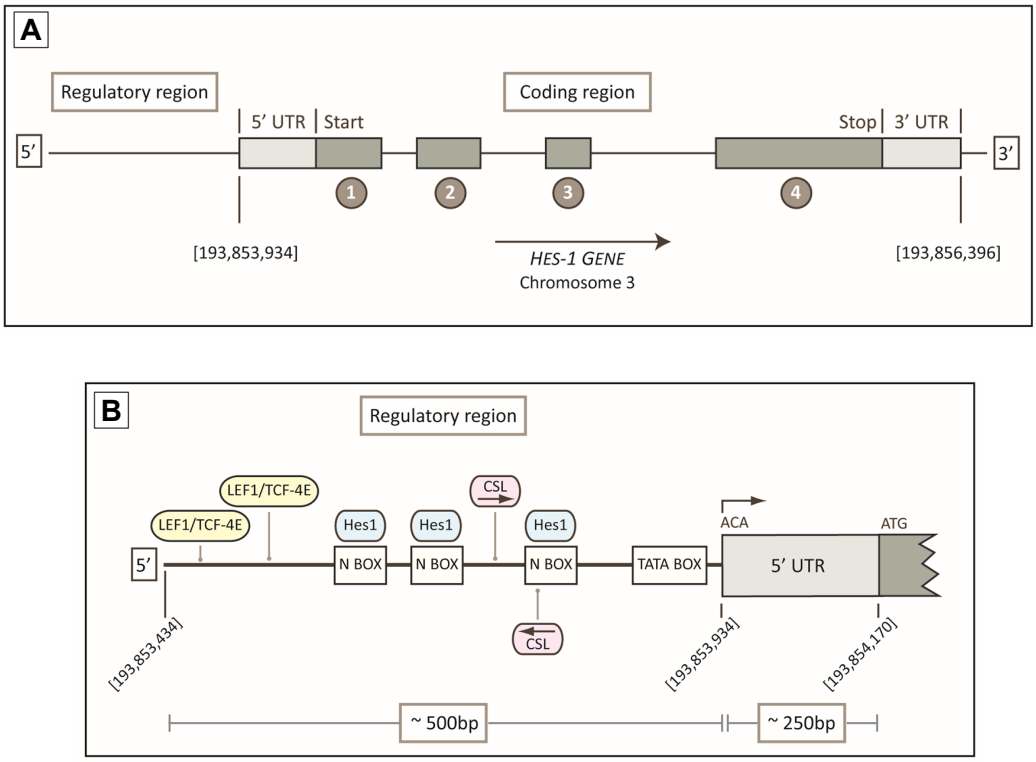


Figure 4-9| *Schematic representation of the Hes1 gene and 5' regulatory region.* [**A**] The human *Hes1* gene is positioned in chromosome 3 (*3q29*; *193,853,934-193,856,396*), consisting of 4 exons. Murine *Hes1* has a highly corresponding gene structure and is located at *16b2* (*300,654,43-300,678,82*). Transcriptional initiation of both human and murine *Hes1* begins ~250bp upstream of the translation initiation site (ATG), constituting the 5' untranslated region (UTR). Transcription is governed by a relatively small 5' regulatory promoter region of only 400-500bp. [**B**] The *Hes1* 5' regulatory region consists of a TATA box (TATATA) 31bp upstream of the transcription initiation site. *Hes1* autoregulation is coordinated by the presence of three N-box DNA motifs (CACNAG). Two $N1^{IC}$/CSL binding elements are present in reverse orientation, while at least 2 novel but highly conserved LEF-1/TCF-4 motifs are also identified upstream of the transcription initiation site.

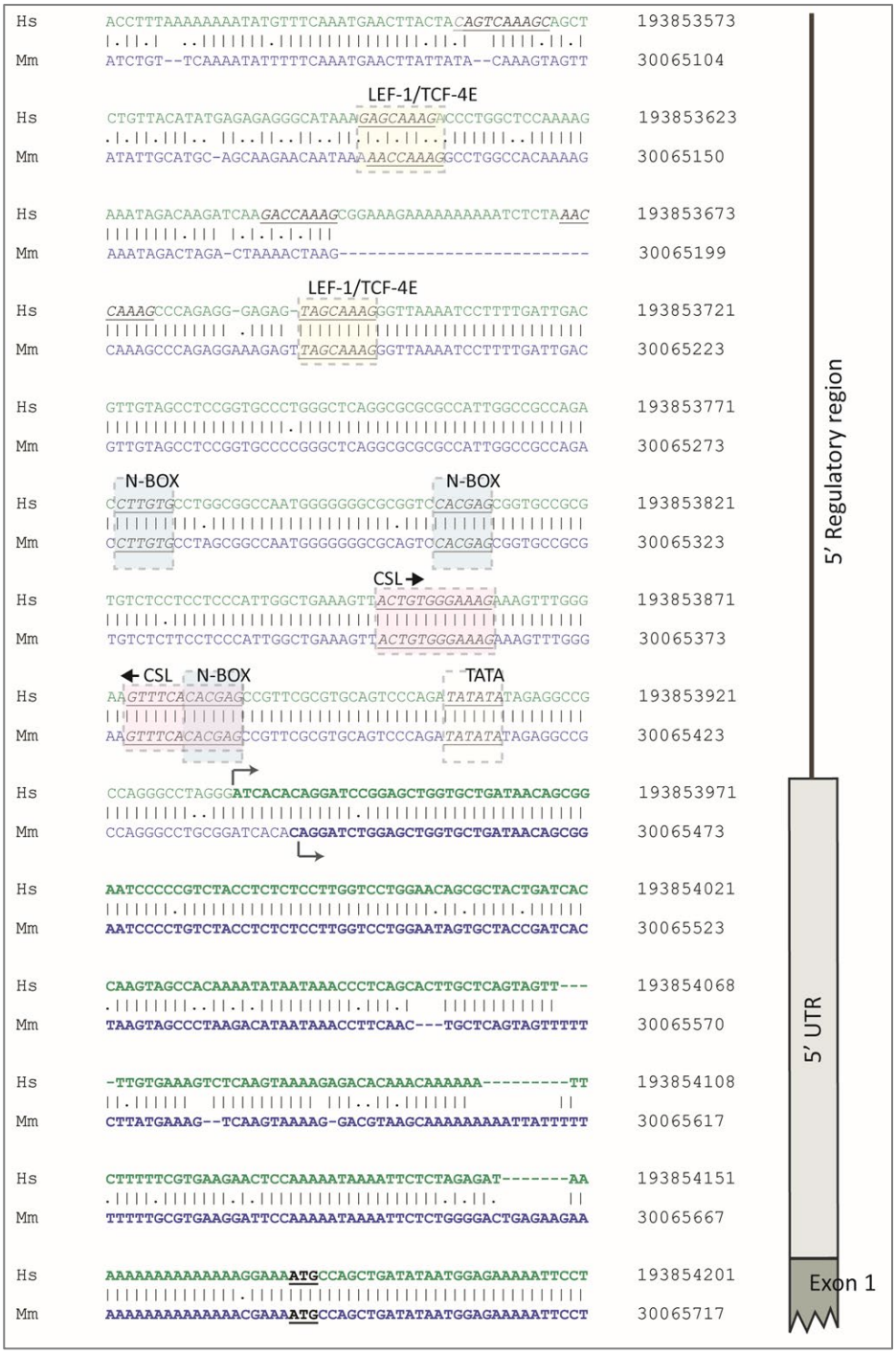


Figure 4-10| ***The 5' region of the Hes1 promoter contains multiple CSL and LEF/TCF binding sites.*** Computational analysis of the 5' region upstream of the human and murine *Hes1* transcriptional start site (ATG) by PROMO3.0 putative transcription factor binding site software. This demonstrated both CSL and LEF/TCF binding sites in the *Hes1* promoter, consistent with convergent regulation by both Notch and Wnt signalling pathways. Comparison of human and murine sequence homology was performed using the on-line EMBOSS Pairwise Alignment Algorithms and demonstrates that both the 5' upstream region of *Hes1* and at least 2 of the novel LEF-1/TCF-4E binding sites are highly conserved.

4.2.6 ACTIVATED NOTCH1 SIGNALLING DECREASES LEF/TCF ACTIVITY

Having identified a potential mechanism by which the Wnt pathway may directly impact on the transcriptional output of active Notch signalling, the potential effect of active N1 on Wnt target gene activation was also tested to determine if this regulation is reciprocal. The SW480 and HCT116 colorectal adenocarcinoma cell lines were transiently co-transfected with the $N1^{IC}$ expression construct and the TOPFLASH (LEF/TCF binding site) reporter construct (Korinek *et al.*, 1997). In addition, parallel cells were also co-transfected with the established FOPFLASH vector control or with the *Hes1* promoter reporter (Nishimura *et al.*, 1998). HCT116 cells express wild-type *APC* but harbour mutant β-catenin; alternatively SW480 cells express mutant *APC* and have a particularly high basal activity due to defective β-catenin nuclear export (Ilyas *et al.*, 1997; Rosin-Arbesfeld *et al.*, 2003; Rowan *et al.*, 2000). In agreement with differences in the mutational status of these cells, the SW480 cell line was shown here to exhibit considerably higher basal LEF/TCF activation compared to HCT116 when normalised to *Renilla* controls (*FIGURE 4-11A,B*). Exogenous $N1^{IC}$ protein expression was confirmed by western blotting analysis and pathway activation was confirmed by a significant increase in *Hes1* promoter activity (*FIGURE 4-11A-C*). Interestingly, increased N1 activation resulted in a moderate but significant decrease in LEF/TCF activity in both cell lines (an average of 40% in SW480, 43% in HCT116). However, this decrease was not sufficient to result in a discernable change in the expression of either c-Myc or $p21^{CIP/WAF1}$ proteins by immunoblot after 24h (*FIGURE 4-11C*). Taken together, these results imply potential competition between the Notch and Wnt pathway for downstream transcriptional effectors and further suggest that transcriptional effectors of the Wnt pathway may converge on the *Hes1* promoter in the presence of $N1^{IC}$ on the *Hes1* promoter but suggest that β-catenin and LEF-1/TCF-4 may be recruited to the Hes1 promoter in the presence of $N1^{IC}$.

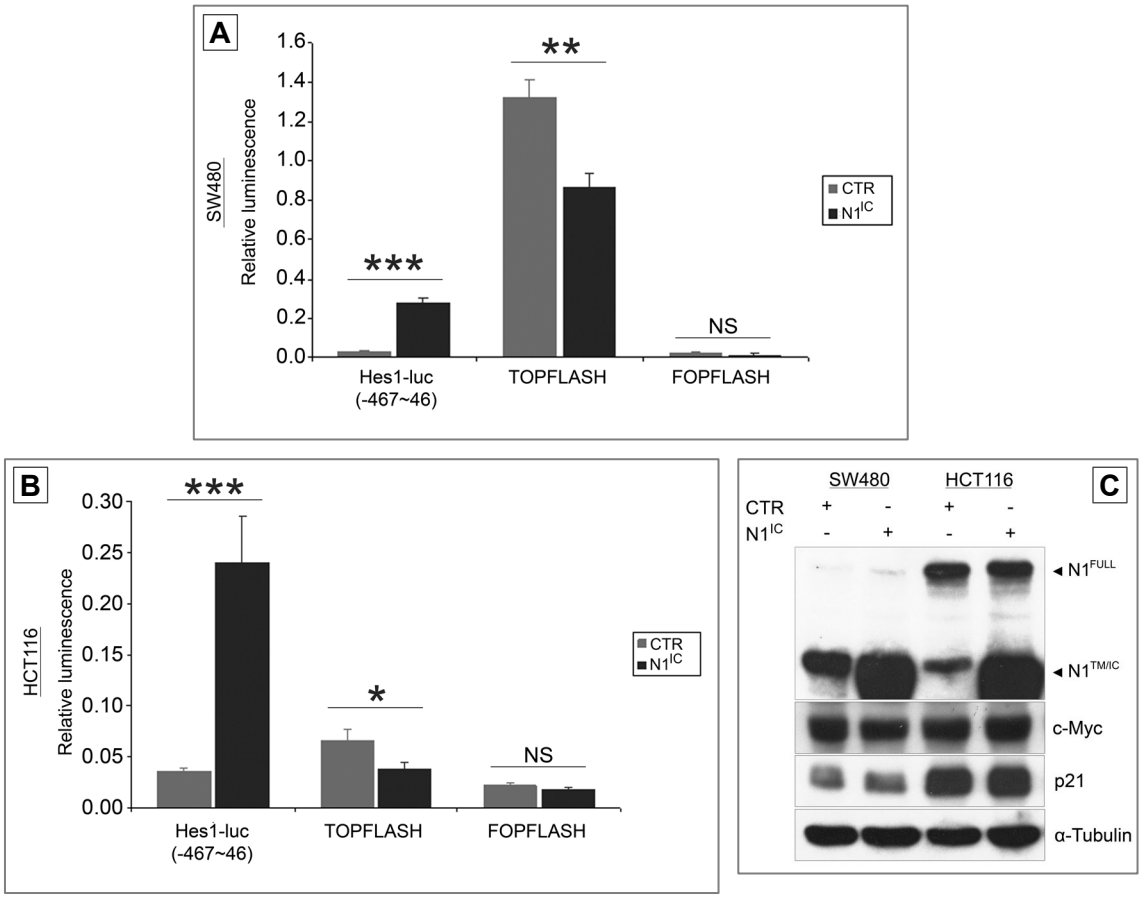


Figure 4-11| *Increased Notch1 signalling decreases LEF/TCF activity in colorectal cancer cells.* N1 activity was increased in the SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma cell lines by 24 hour transient transfection with expression plasmid encoding $N1^{IC}$, vs. empty vector control. Cells were also co-transfected with *Hes1*-promoter reporter, TOPFLASH (LEF/TCF) reporter construct or FOPFLASH mutant counterpart. Values are normalised to *Renilla* control and these data are representative of three experiments performed in triplicate. (*Statistical analysis was performed using the Student's t-test, where degrees of significance are stated as *: p<0.05, **: p<0.01, ***: p<0.001 and error bars represent SEM*). [**C**] Western blotting analysis of N1 and the established Wnt targets c-Myc and $p21^{Cip1/Waf1}$ in response to transient $N1^{IC}$ transfection. α-Tubulin is included as a loading control.

4.2.7 NOTCH1 OVER-EXPRESSION DOES NOT PROMOTE CRC CELL GROWTH

Evidence from functional analysis in the intestinal epithelium of animal models has led to a hypothesised role for active Notch signalling as a promoter of proliferation in the human colon and CRC. As described above, increasing evidence from other tissues also suggests a key role for Notch signalling in control of the cell cycle (*SECTION 4.1.2, p126*) but the functional contribution of active Notch signalling on either proliferation and/or cell cycle progression in CRC has not previously been tested. Therefore, in order to determine the effects of active Notch signalling on colorectal cancer cell proliferation and survival, exogenous $N1^{IC}$ or constitutively active *CSL* (*caCSL*) was expressed in the SW480 and HCT116 colorectal adenocarcinoma cell lines by transient transfection and compared with empty vector controls. Although these cell lines represent relatively high and low endogenous levels of cleaved N1 expression respectively, both were previously shown to be responsive to exogenous N1 activation (*FIGURE 4-5, Figure 4-6*).

Transfected cells were grown under standard conditions and counts of both adherent and floating cell number were determined after 48 hours (*FIGURE 4-12*) and transfection efficacy was confirmed by western blotting analysis (*data not shown*). It has previously been shown that attached and floating cell number are indicative of changes in the proliferation and/or apoptosis of colorectal tumour cell lines because apoptotic cells detach and float into the growth medium (Bracey *et al.*, 1995; Hague *et al.*, 1993). The degree of apoptosis is determined as the percentage of floating cells in the total cell population (attached and floating) (Elder *et al.*, 1996). The seeding density and timing used here ensured that cells remained sub-confluent and therefore proliferative at the end of the experiment and averaged results are presented from three separate experiments performed in triplicate. In contrast to a pro-proliferative role for N1 in these cells, no significant change in adherent or floating cell number was observed in either cell line following transient N1 or pan-Notch/CSL activation. Serum is known to stimulate basal N1 activation in cell culture conditions and this experiment was also repeated in both cell lines at a reduced (2%) serum concentration, although cell number continued to remain unaltered (*data not shown*).

Propidium Iodide (PI) DNA counter-stain is routinely used for cell cycle analysis of DNA and enables the percentage of cells in each phase of the cell cycle to be determined on the basis of DNA content (Crissman and Steinkamp, 1973). Transfected SW480 and HCT116 cells were stained using PI and analysed by flow cytometry (*SECTION 2.7, p69*). The percentage of cells in each phase was determined by ModFit analysis software (*Verity software, USA*) and, in agreement with

analysis of cell number, no significant change in cell cycle position was observed between vector control and $N1^{IC}$ transfected cells. Representative images (*FIGURE 4-13A,B*) and averaged data are presented here from three independent experiments (*FIGURE 4-13C*).

N1 pathway activation is known to be active in the presence of extremely low level N1 protein expression (Conboy *et al.*, 2005; Schroeter *et al.*, 1998) and, although HCT116 cells express relatively low levels of the cleaved N1 protein, it is possible that these cells may be growth-insensitive to further increases in N1 activity. For this reason, positive Notch modulation was also attempted in the HT29 adenocarcinoma cell line as well as various adenoma-derived cell lines, which express relatively lower endogenous levels of N1 (*SECTION 3.2.7, p111*). Unfortunately, these cell lines are comparatively recalcitrant to transfection and sufficient pathway activation could not be achieved by transient methods. Therefore, clones of these cell lines stably expressing either $N1^{IC}$, $N1^{FULL}$ or empty vector control were established by G418 selection. Surprisingly, N1 activation in these cells appeared to have negative effects on clone survival and cells demonstrating stable expression could not be maintained for more than one passage (*data not shown*); suggesting that sustained N1 activation may actually be antagonistic to growth and survival in these cells.

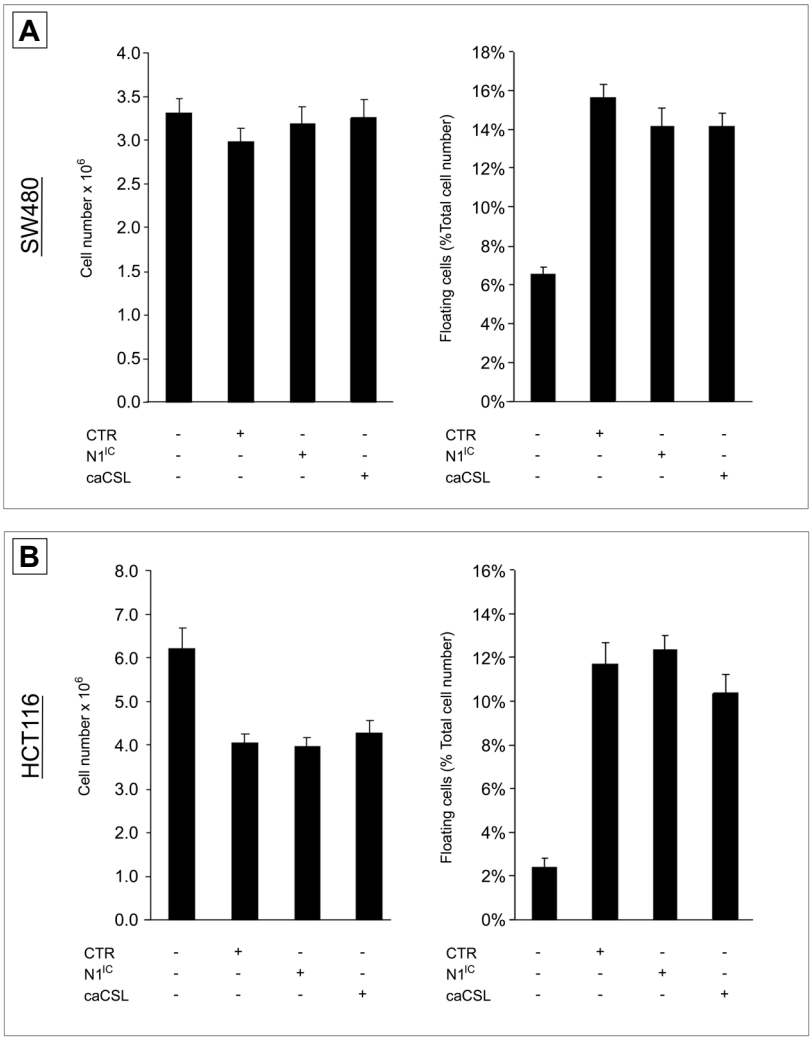


Figure 4-12| ***Exogenous Notch activation in colorectal cancer cells does not alter cell growth.*** Analysis of cellular proliferation and apoptosis in the SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma cell lines following 48 hour transient transfection with *N1^{IC}* or constitutively active *CSL* (*caCSL*). Cellular proliferation is indicated by the number of adherent cells and the percentage apoptosis determined as floating cell number relative to the total population (attached and floating). Mock and empty vector transfections were performed as controls. Data represents averaged results from three separate experiments performed in triplicate (*Error bars = SEM*).

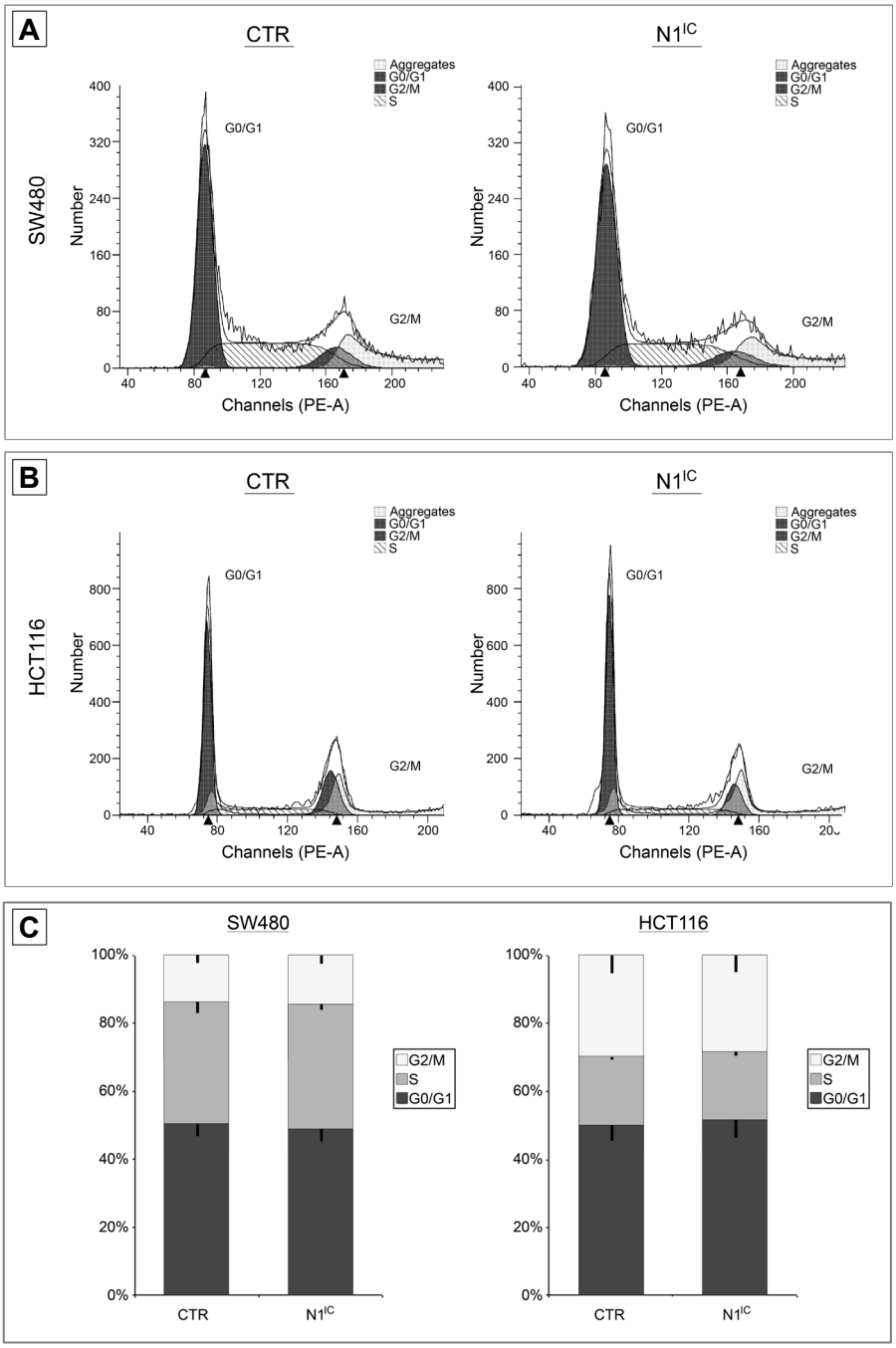


Figure 4-13| ***Notch1 activation does not increase cell cycle progression.*** Cell cycle analysis of the SW480 [**A**] and HCT116 [**B**] cell lines following 48 hour transfection with *N1^{IC}* expression construct vs. empty vector control. Propidium Iodide staining of DNA content was used to produce cell cycle histograms from which the percentage of cells in each phase of the cell cycle was determined using ModFit analysis software (*Verity software, USA*). The average data are presented from three independent experiments in each cell line [**C**].

4.2.8 DOWN-MODULATION OF NOTCH SIGNALLING DOES NOT ALTER CRC CELL GROWTH

Notch inhibition in the normal intestine has previously been performed by intestine-specific targeting of *CSL* or through the application of γ-secretase inhibitors (GSIs), while neither method has previously been employed in human colorectal cancer cell lines. GSIs are known to elicit highly pleiotropic effects and have been shown to affect a large number physiologically important transmembrane proteins (Grosveld, 2009). The γ-secretase complex has also been shown to profoundly influence β-catenin signalling (Zhang *et al.*, 1998), a major determinant of proliferation in these cells. For this reason, and to enable N1-specific targeting, pathway inhibition was instead achieved by siRNA-mediated knockdown of *N1* or *CSL* (*Dharmacon*) in HCT116 cells (*SECTION 2.3.3, p62*). Scrambled negative siRNA controls (*Dharmacon*) and mock transfections were also performed to ensure that cellular proliferation and apoptosis were not affected by the presence of siRNA oligonucleotides in these cells. Effective siRNA transfection was confirmed by a significant reduction in the *Hes1* promoter-reporter in each case (*FIGURE 4-7A*). Adherent and floating cell number was determined 72 hours post-transfection, and results are representative of average values from three separate experiments performed in triplicate. These results show that siRNA-mediated knockdown of N1 and pan-Notch activity in HCT116 cells does not significantly alter cellular proliferation or apoptosis (*FIGURE 4-14*). In accordance with this, *N1* or *CSL* specific knockdown were shown not to affect cell cycle distribution in these cells (*FIGURE 4-15*). These results support findings from exogenous N1 activation and continue to suggest that modulating Notch activity does not have a significant functional effect on either the proliferation and/or apoptosis of colorectal cancer cells.

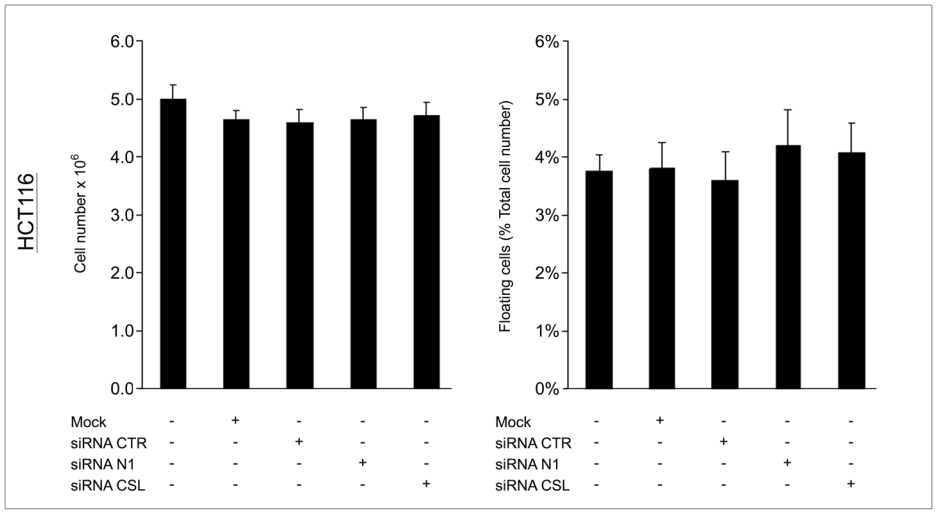


Figure 4-14| ***Down-modulation of Notch signalling does not significantly alter cell growth.*** HCT116 cells were transiently transfected with scrambled negative siRNA control or sequences targeting either *N1* or *CSL* (*Dharmacon*), leading to N1 and pan-Notch inhibition. Counts of both adherent and floating cell number were performed 72 hours post-seeding and the percentage of cellular apoptosis is shown as the number of floating cells relative to the total cell number. These data are representative of average readings from three separate experiments performed in triplicate. (*Error bars = SEM*)

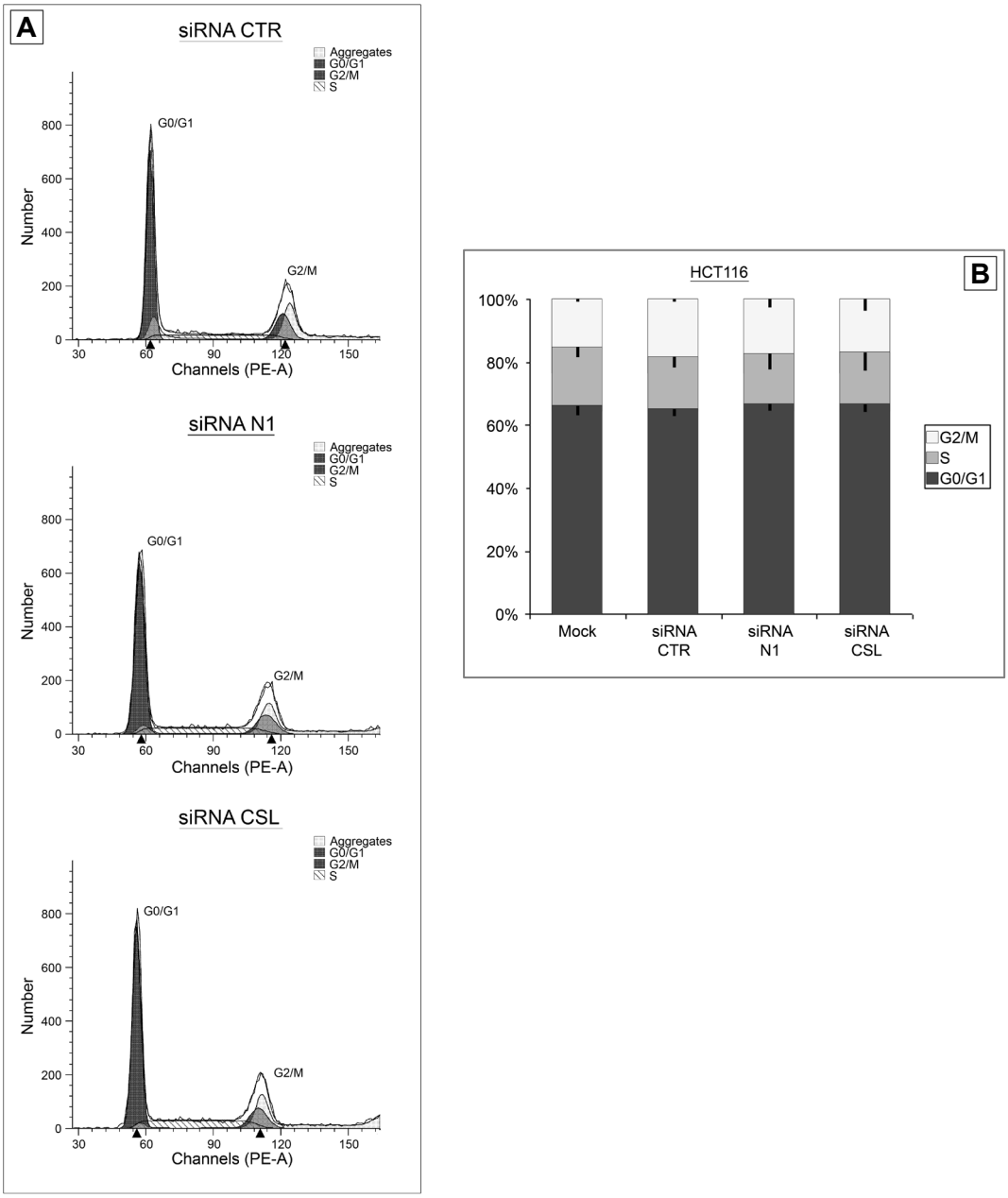


Figure 4-15| ***Notch inhibition does not alter cell cycle distribution.*** Cell cycle analysis of HCT116 cells following 72 hour transient transfection with scrambled negative siRNA or sequences targeting either *N1* or *CSL* (*Dharmacon*). Propidium Iodide staining of DNA content was used to produce cell cycle histograms and the percentage of cells in each phase of the cell cycle was determined by ModFit analysis software (*Verity software, USA*). These data are representative of three separate experiments. (*Error bars = SEM*)

4.2.9 NOTCH1 EXPRESSION CORRELATES WITH CELLS IN G2/M

In order to investigate a potential reciprocal relationship between N1 expression and cell cycle progression, untreated SW480 and HCT116 cells were fluorescently immunolabelled for endogenous N1 protein expression and counter-stained using PI. Cells were then analysed by flow cytometry (*SECTION 2.7, p69*), enabling direct comparisons to be made between N1 immunoreactivity and cell cycle position. Single, non-apoptotic cells were selected on the basis of forward scatter (FSC-A) vs. side scatter (SSC-A), plus DNA dot-plot distributions (*FIGURE 4-16A,B, FIGURE 4-17A,B*). Fluorescently labelled cells were gated on the basis of 'low' or 'high' endogenous N1 immunoreactivity (*FIGURE 4-16C,D, FIGURE 4-17C,D*) for comparison with cell cycle distribution. Interestingly, a direct correlation between N1 protein expression and cell cycle position was observed in both cell lines, with 'low' N1 expressing cells predominating in the G0/G1 phase of the cell cycle and 'high' expressing cells in G2/M (*FIGURE 4-16E,F, FIGURE 4-17E,F*). These data therefore suggest that endogenous N1 expression is increased in actively dividing CRC cells and may in fact be highly dependent on transient changes in cell cycle position.

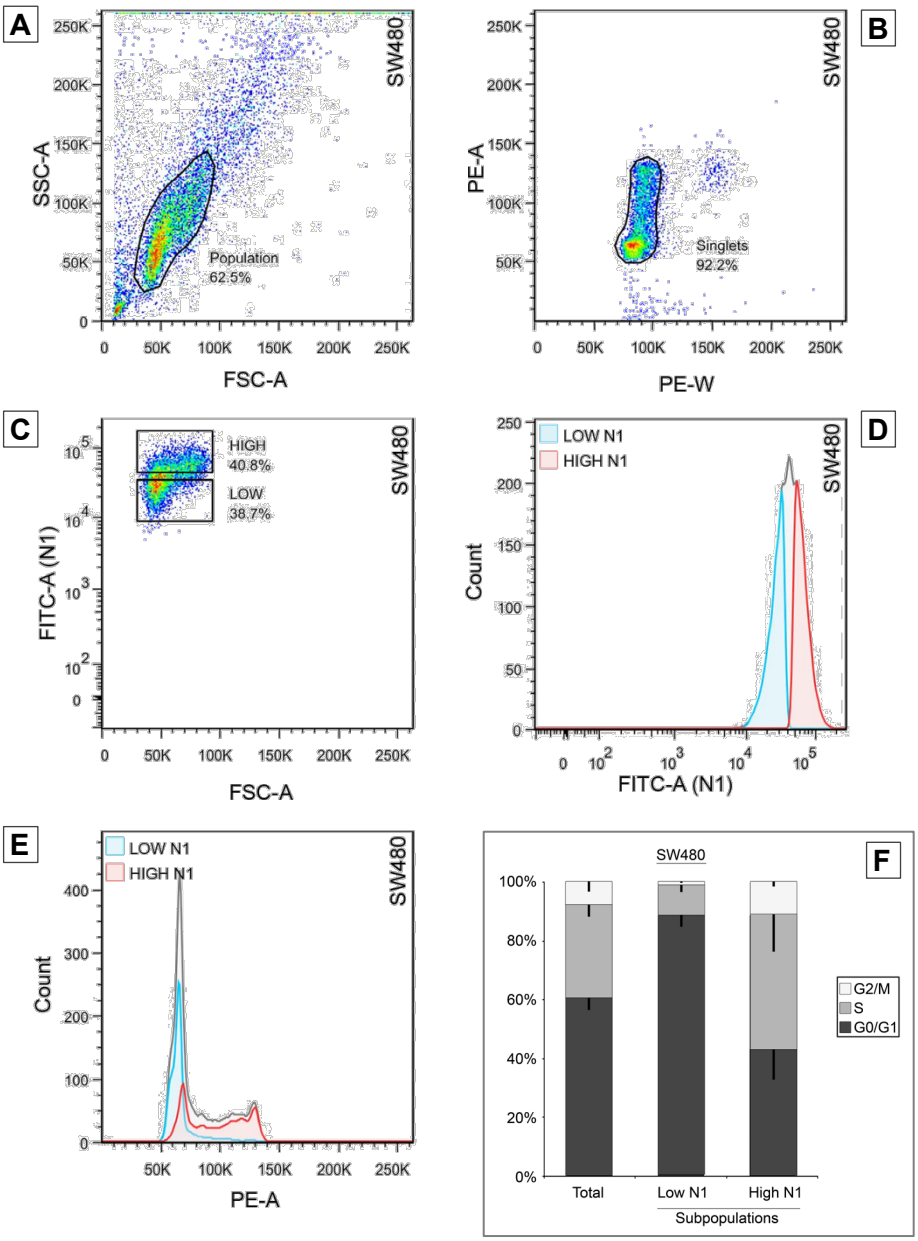


Figure 4-16| *Notch1 protein expression correlates with SW480 cells in G2/M.* Flow analysis of the SW480 colorectal adenocarcinoma cell line following immunofluorescent dual stain for basal N1 protein expression (by green Alexafluor488 secondary*; Molecular Probes, USA*) and DNA content (by Propidium Iodide). Non-apoptotic cells were gated on the basis of FSC-A vs. SSC-A [**A**], from which single cells were determined by DNA dot-plot distribution [**B**]. Stained cells were gated on the basis of 'high' (red) or 'low' (blue) basal N1 expression [**C-D**]. High N1 expression correlates directly with cells in the G2/M phase of the cell cycle [**E-F**]. These data are representative of three independent experiments.

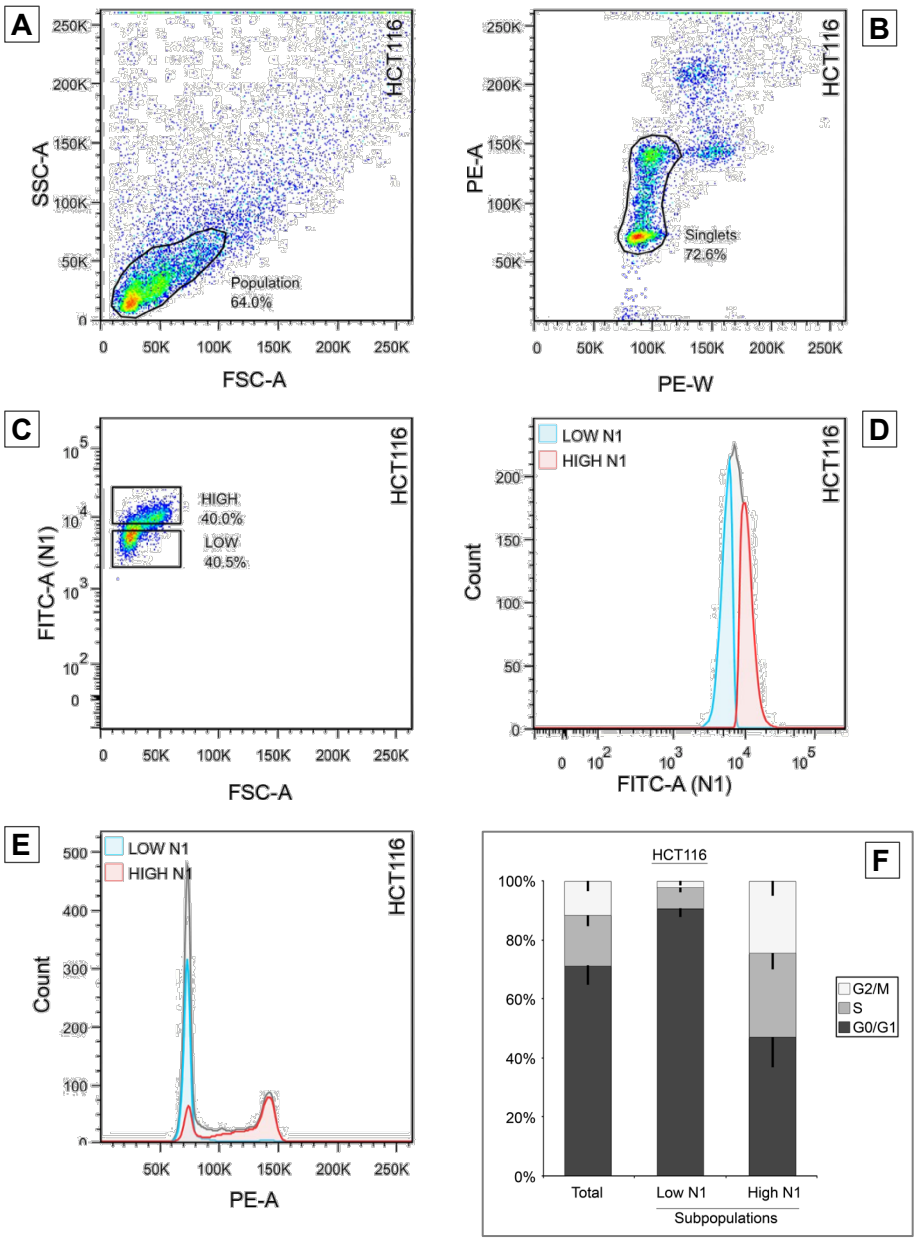


Figure 4-17| *Notch1 protein expression correlates with HCT116 cells in G2/M.* Flow analysis of the HCT116 colorectal adenocarcinoma cell line following immunofluorescent dual stain for basal N1 protein expression (by green Alexafluor488 secondary*; Molecular Probes, USA*) and DNA content (by Propidium Iodide). Non-apoptotic cells were gated on the basis of FSC-A vs. SSC-A [**A**], from which single cells were determined by DNA dot-plot distribution [**B**]. Stained cells were gated on the basis of 'high' (red) or 'low' (blue) basal N1 expression [**C-D**]. High N1 expression correlates directly with cells in the G_2/M phase of the cell cycle [**E-F**]. These data are representative of three independent experiments.

4.2.10 SELECTIVE CYTOSTASIS DOES NOT ALTER NOTCH1 EXPRESSION

In order to further define changes in endogenous N1 expression in response to cell cycle position, different cytotoxic drugs were employed to enrich colorectal cancer cells in either the G2/M or G0/G1 phases of the cell cycle. Colcemid is a well defined metaphase inhibitors (reviewed in Rieder and Palazzo, 1992) that prevents spindle fibre formation (Taylor, 1965) and is frequently used in cell cycle analysis and cytogenetic techniques (Gray and Darzynkiewicz, 1987). Previous studies have shown that cells treated with Colcemid accumulate in the G2/M phase of the cell cycle (Dosik *et al.*, 1981) and, in line with previous studies (Barlogie and Drewinko, 1980; Bezrookove *et al.*, 2003; Jha *et al.*, 1994), sub-confluent SW480 and HCT116 cells were treated with 0.03µg.ml^{-1} of Colcemid over a time-course of 6, 12 and 24 hours (*SECTION 2.1.5, p56*). As expected, both cell lines were found to accumulate in G2/M, compared to untreated controls (harvested at 24 hours) (*FIGURE 4-18*). Increased treatment duration in other CRC cells has previously been shown to induce a distinctive rounded cell morphology, compared to a flattened, post-mitotic interphase configuration (Jha *et al.*, 1994). Consistent with this, SW480 and HCT116 cells were found to be increasingly rounded following treatment, as shown by phase contrast microscopy (*FIGURE 4-19A*). In order to assess relative N1 protein expression in these cells, lysates from parallel flasks of cells were analysed by western blotting and, in contrast to a hypothesised increase in N1 expression, forced enrichment of both SW480 and HCT116 cells in G2/M instead resulted in a notable decrease in N1 protein expression relative to duration (*FIGURE 4-19B*).

For comparison, SW480 and HCT116 cells were also halted in the G1/S phase of the cell cycle using another chemostatic reagent, Hydroxyurea. The use of Hydroxyurea in cell cycle modulation is well established and known to cause cellular arrest at G1/S by potent inhibition of DNA synthesis (reviewed in Yarbro, 1992). SW480 and HCT116 cells were treated with 2mM Hydroxyurea for 6, 12 and 24 hours and compared with untreated controls (*SECTION 2.1.5, p56*) (Maurer-Schultze *et al.*, 1988). Cell cycle analysis of both cell lines confirmed a dramatic increase in the number of cells in G1/S within 6 hours and by 12-24 hours almost all cells were found to be arrested in this phase of the cell cycle (*FIGURE 4-20A-C*). Parallel flasks of cells were analysed for N1 expression by western blotting analysis, however, no discernable change in either full length ($N1^{FULL}$) or cleaved ($N1^{TM/IC}$) N1 protein expression was evident following treatment with Hydroxyurea and G1/S enrichment in these cells (*FIGURE 4-20D*).

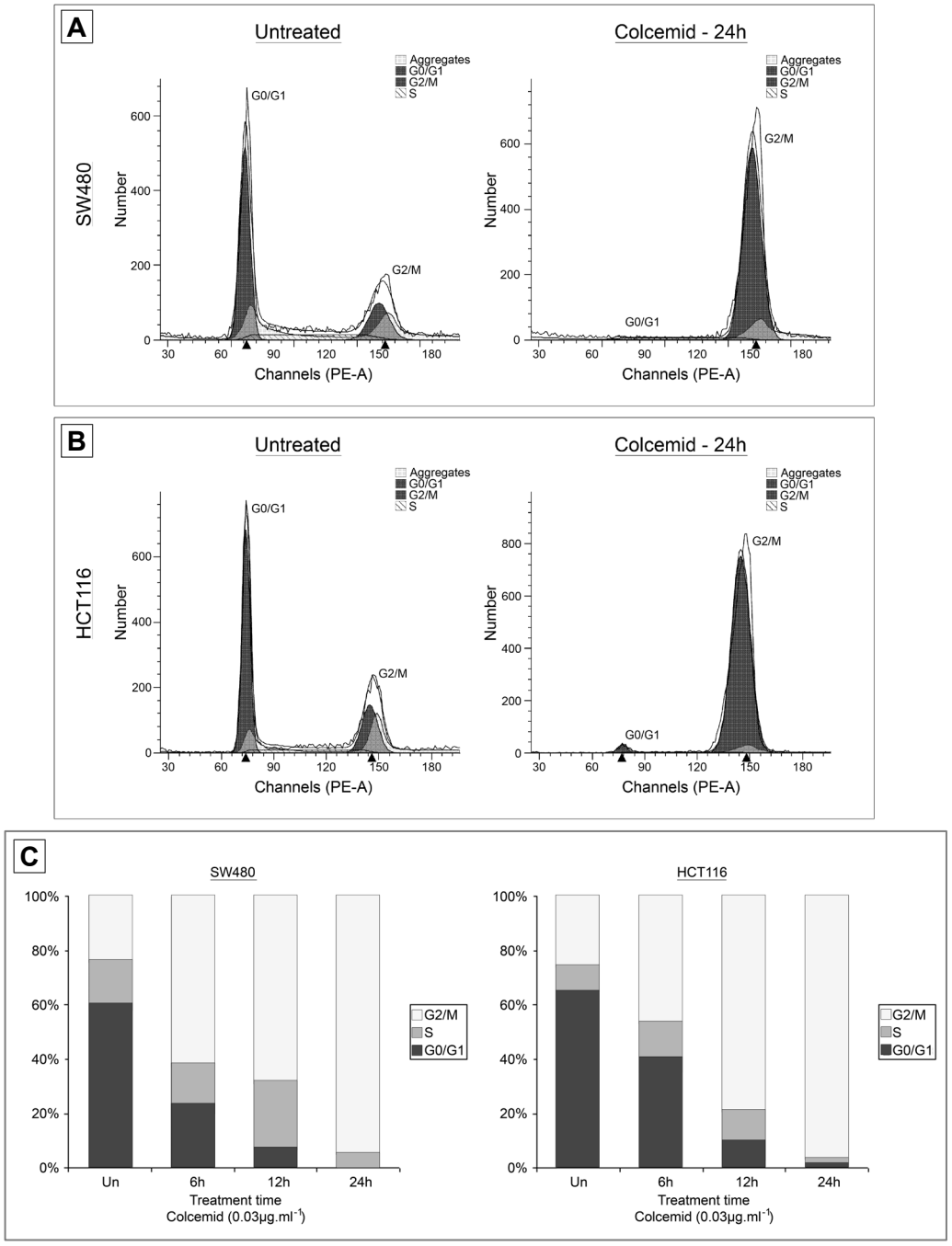


Figure 4-18| ***Colcemid treatment is mitostatic in colorectal cancer cells.*** Cell cycle analysis of the SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma cell lines following a time-course of treatment with Colcemid, a G2/M cytostatic drug. Cells were treated at a dose of 0.03µg.ml^{-1} for either 6, 12 or 24 hours and compared to untreated control. The percentage of cells in each phase of the cell cycle was determined by ModFit analysis software (*Verity software, USA*) and both cell lines were found to increasingly accumulate in the G2/M phase of the cell cycle, relative to treatment duration [**C**].

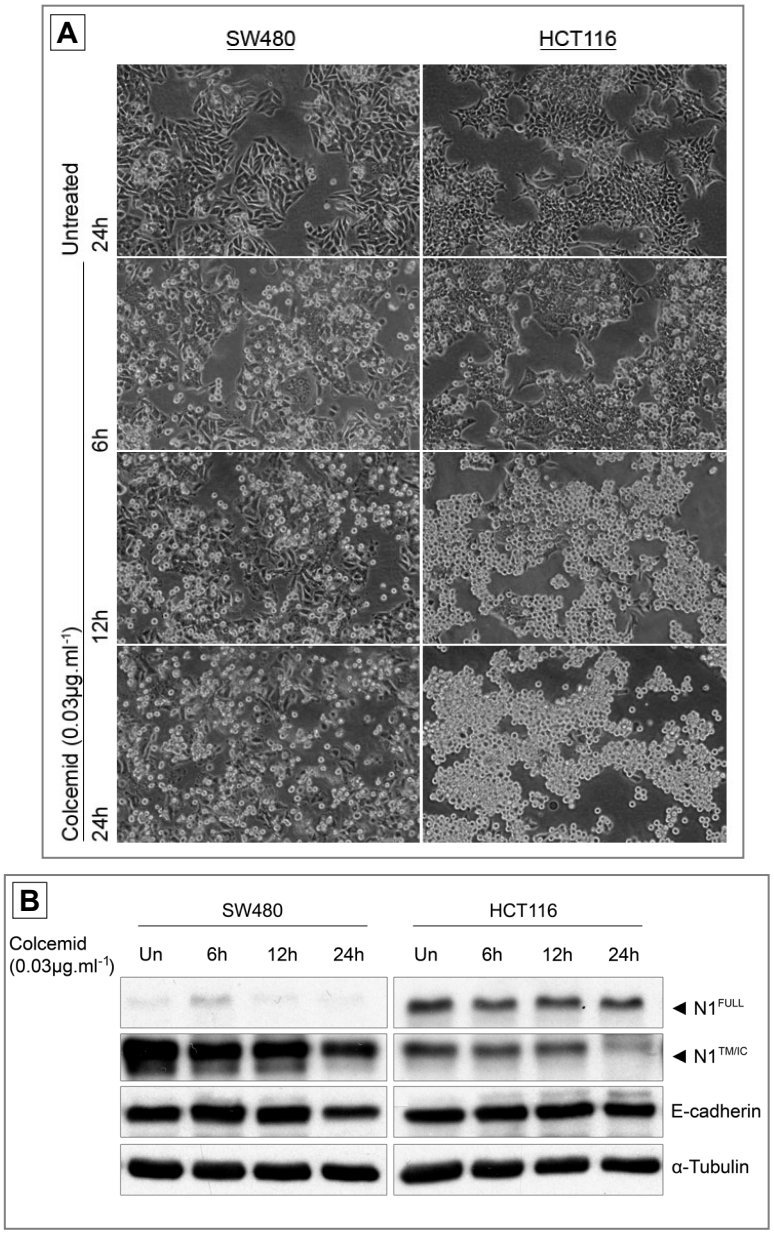
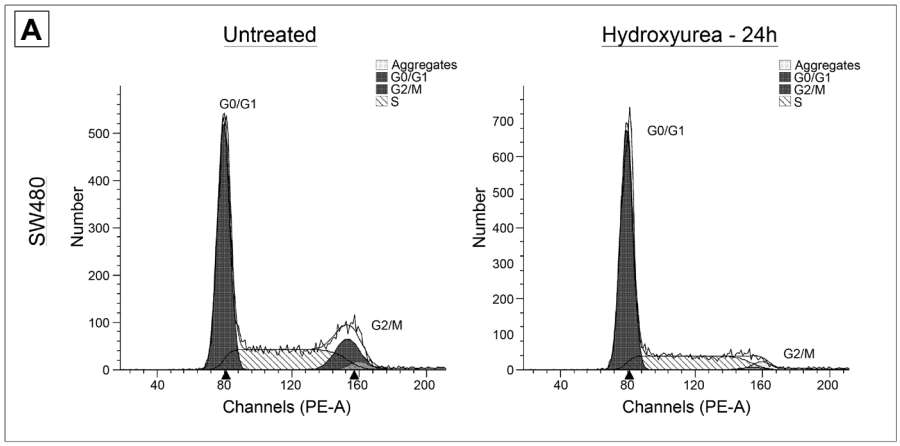
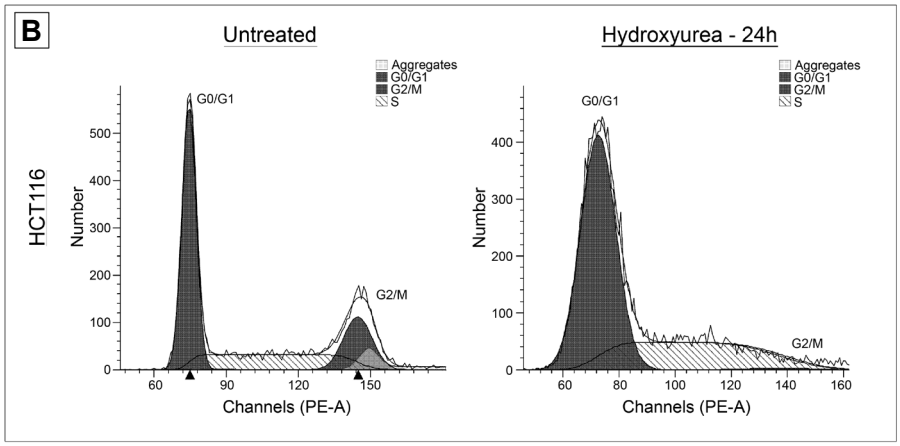
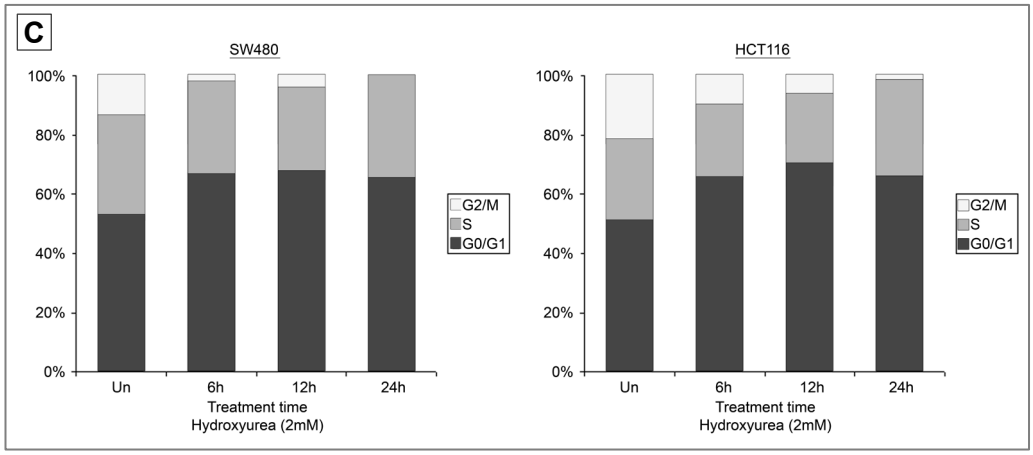


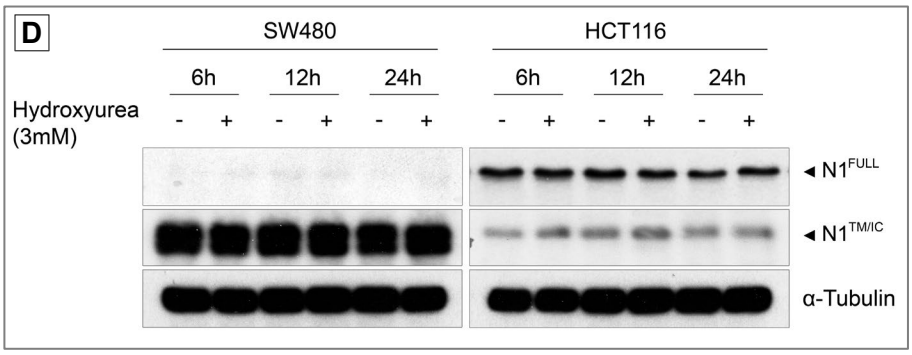
Figure 4-19| ***Mitostasis of colorectal cancer cells results in decreased Notch1 expression.*** [**A**] Phase contrast microscopy of SW480 and HCT116 colorectal adenocarcinoma cell lines halted in the G2/M phase of the cell cycle by treatment with 0.03µg.ml^{-1} Colcemid. Increased treatment duration was accompanied by an increase in rounded mitotic cell morphology compared to a flattened post-mitotic interphase configuration. [**B**] Corresponding western blotting analysis of full length (N1^{FULL}) and cleaved N1 (N1$^{TM/IC}$) protein expression in SW480 and HCT116 cells following a time-course of Colcemid treatment. α-Tubulin was assessed to ensure equal protein loading.

Figure 4-20| ***G1/S cell cycle arrest does not decrease N1 expression in colorectal cancer cells.*** Cell cycle analysis of the SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma cell lines following a time-course of treatment with Hydroxyurea, a G1/S cytotoxic drug. Cells were treated at 2mM for 6, 12 or 24 hours, compared to untreated controls. An increasing accumulation of cells in G1/S was confirmed relative to treatment duration [**C**]. Western blotting analysis of full length and cleaved N1 protein expression in SW480 and HCT116 following Hydroxyurea treatment [**D**]. α-Tubulin is included as a loading control. ▶


A
Untreated
Hydroxyurea - 24h
SW480
Number
Channels (PE-A)
Aggregates
G0/G1
G2/M
S
G0/G1
G2/M


B
Untreated
Hydroxyurea - 24h
HCT116
Number
Channels (PE-A)
Aggregates
G0/G1
G2/M
S
G0/G1
G2/M


C
SW480
HCT116
100%
80%
60%
40%
20%
0%
Un
6h
12h
24h
Treatment time
Hydroxyurea (2mM)
G2/M
S
G0/G1


D
SW480
HCT116
6h
12h
24h
Hydroxyurea
(3mM)
- + - + - + - + - + - +
◄ N1FULL
◄ N1TM/IC
α-Tubulin

4.2.11 NOTCH1 EXPRESSION IS INCREASED IN ACTIVELY CYCLING CELLS

Although an effective enrichment of cells in G2/M was achieved by Colcemid treatment, it is important to note that this mitotic accumulation results from cell cycle arrest at a very specific cell cycle phase, suggesting that cells need to be actively cycling to elicit an increase in N1. Alternatively, and in possible explanation of results obtained with Hydroxyurea, N1 may be stimulated during S-phase rather than G2/M. In order to investigate this further, it was necessary to obtain enrichment of cells in the different phases of the cell cycle without inducing cell cycle arrest. Hydroxyurea is known to induce cell death in S-phase when used as a chemotherapeutic agent, but is also known induce cell cycle synchronization in the fraction of cells that survive (Yarbro, 1992). Temporary induction of cell cycle arrest by Hydroxyurea therefore enables *in vitro* cell cycle synchronisation and enrichment of cycling cells in the progressive phases of the cell cycle. It has previously been used to obtain synchronous enrichment of various *in vitro* cell types, including colorectal cells (Gupta and Singh, 1994; Sato *et al.*, 1995; Wang *et al.*, 2002).

In line with previous studies, sub-confluent SW480 and HCT116 cells were treated for 10 hours with 1mM and 2mM Hydroxyurea respectively and cell cycle analysis was used to confirm the accumulation of both cell lines at G0/G1. Treated cells were trypsinised and parallel flasks were re-seeded in either normal growth medium or growth medium containing 2mM Hydroxyurea (*SECTION 2.1.6, p57*). Cells were harvested at 2 hour intervals, for up to 14 hours, and cell cycle analysis was used to confirm progressive and synchronous transition through the cell cycle or maintained arrest at G0/G1 (*FIGURE 4-21*). No obvious change in cleaved N1 ($N1^{TM/IC}$) protein expression was observed between arrested and actively cycling cells at any time point in either cell line by western blotting analysis (*FIGURE 4-22A,B*). In contrast, full length N1 ($N1^{FULL}$) was virtually undetectable in the SW480 cell line under standard culture conditions but was shown here to increase in cells synchronously passing through the cell cycle at 2, 4, 6 and 8 hours post-seeding. An increase in $N1^{FULL}$ protein expression in the SW480 cell line was confirmed by densitometry (*FIGURE 4-22C*) and the maximum increase was shown to occur at 6-8 hours post-treatment release (230-300% increase relative to arrested counterpart). Interestingly, this time point correlates with the greatest increase in S-phase cells (*S=48-56%*) in the SW480 cell line. In contrast, the maximum enrichment of cells at G2/M was instead shown to occur at 10 hours following treatment removal (*G2/M=32%*), at which point N1 levels have again become equalised with control. Taken together these results further demonstrate that N1 expression is transiently modulated by cell cycle progression and preliminary data presented here suggests that induction of N1 occurs in the later stages of S-phase, rather than during mitosis.

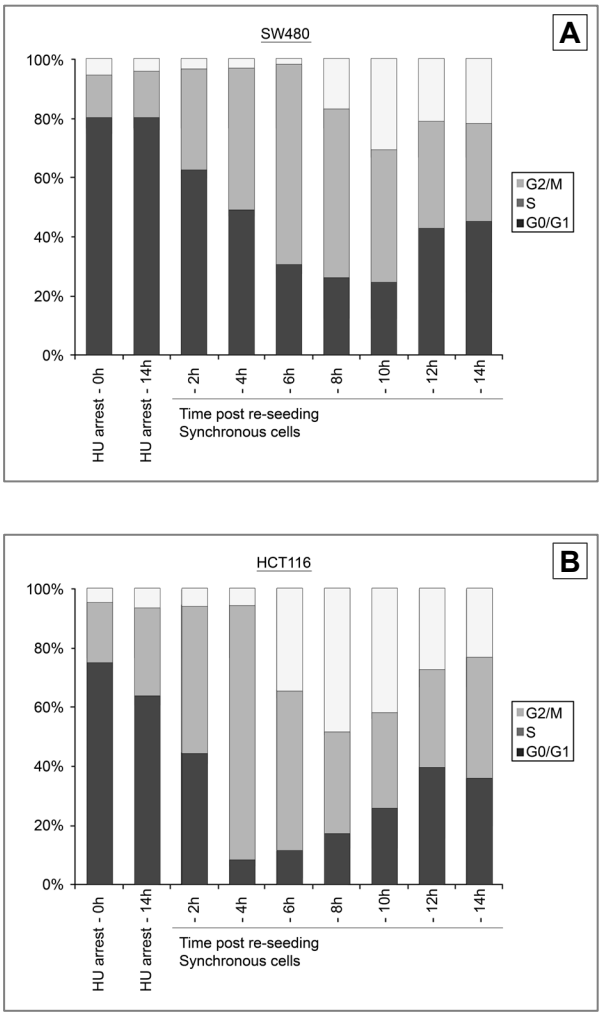


Figure 4-21| ***Transient Hydroxyurea treatment results in synchronous cell cycle enrichment.*** A synchronous enrichment of SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma cells in the different phases of the cell cycle was attained by transient Hydroxyurea treatment. Cells were initially arrested in G1/S by 10h treatment with 1mM and 2mM Hydroxyurea respectively. Following reseeding and treatment removal, cells synchronously resumed cell cycle progression. Cell cycle analysis was performed at 2 hour intervals and the percentage of cells in each phase of the cell cycle was determined by ModFit analysis software (*Verity software, USA*).

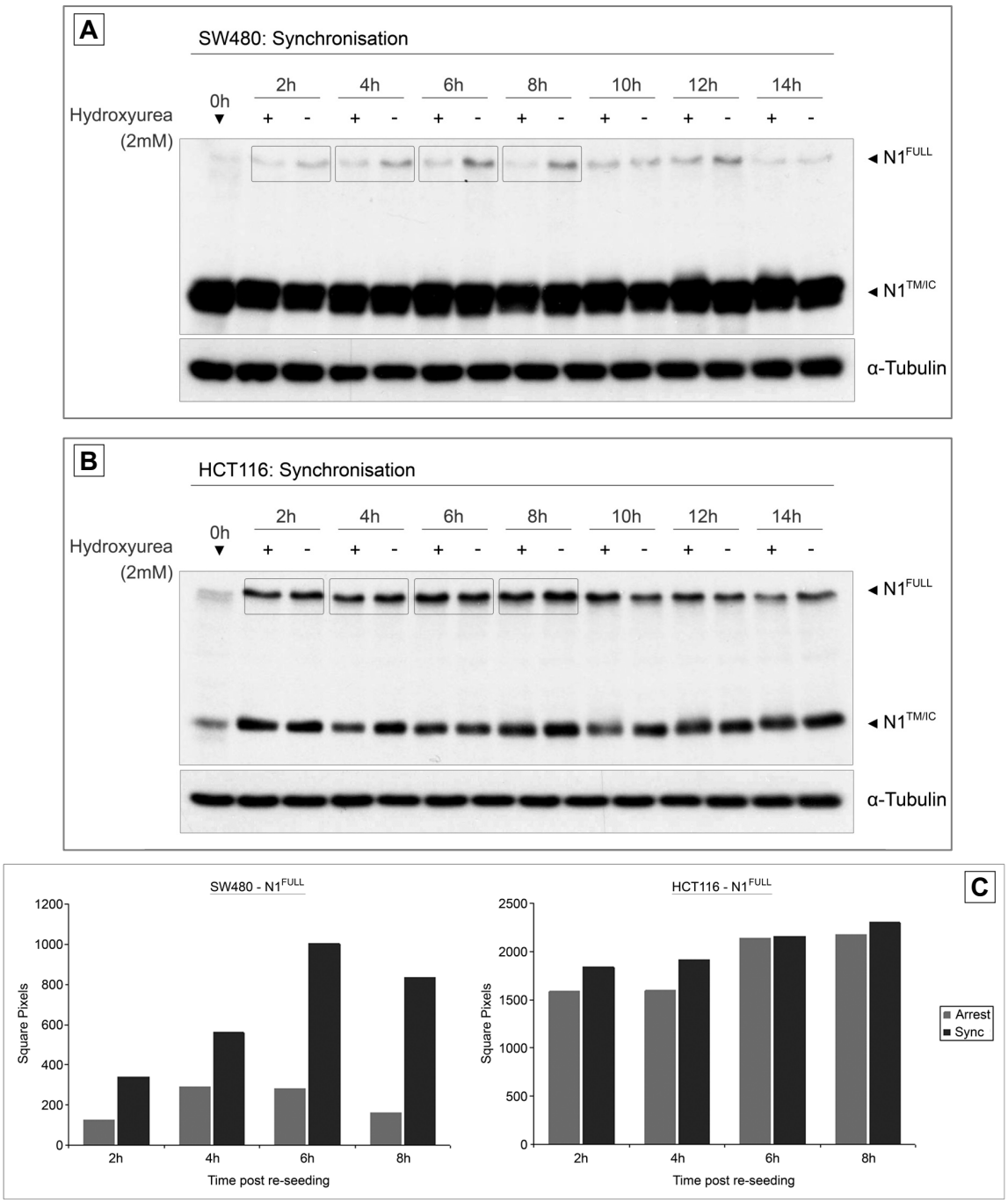


Figure 4-22| ***Notch1 protein expression is increased in mitotic SW480 cells.*** Western blotting analysis of N1 expression in synchronous cultures of SW480 [**A**] and HCT116 cells [**B**]. Following an initial arrest in G1/S using Hydroxyurea, cells were either maintained in G1/S arrest (+Hydroxyurea) or allowed to synchronously progress through the cell cycle following removal of treatment (-Hydroxyurea). The relative expression of N1 protein was assessed by western blotting analysis. α-Tubulin was also assessed to ensure equal protein loading. [**C**] Densitometric analysis of $N1^{FULL}$ protein expression in synchronous cultures of SW480 and HCT116 cells, compared with G1/S arrested controls.

4.2.12 NOTCH1 EXPRESSION IS REDUCED FOLLOWING SODIUM BUTYRATE TREATMENT

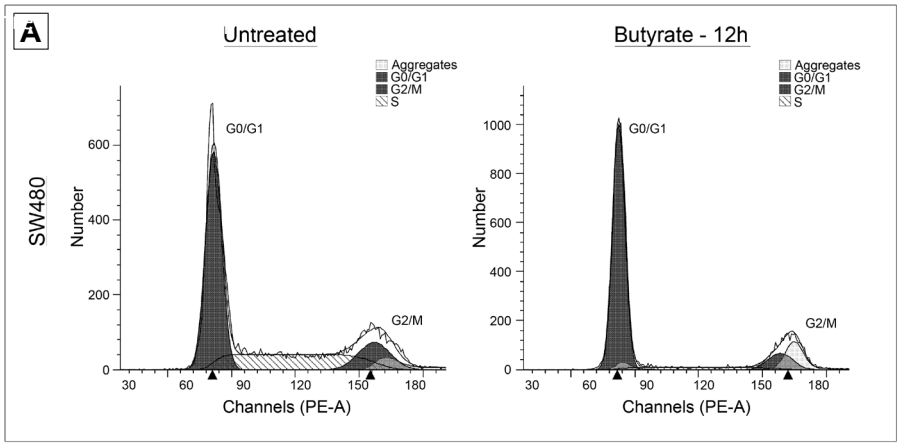
In addition to cytogenetic drugs, other naturally occurring and physiological agents have routinely been used to modulate proliferation and cell cycle progression in colorectal cancer cells. For example, microbial fermentation of dietary fibre in the intestine leads to the production of various short-chain fatty acids (SCFAs: acetate, propionate and sodium butyrate), all of which are known to be present at millimolar concentrations in the lumen of the large intestine (Cummings *et al.*, 1987). Of these, sodium butyrate has been shown to demonstrate the most profound effect on cell growth (Whitehead *et al.*, 1986) through its effects as a potent histone deacetlase (HDAC) inhibitor, leading to histone hyper-acetylation and altered gene expression (Hebbes *et al.*, 1988; Kruh, 1982; Moore *et al.*, 1979; Sealy and Chalkley, 1978). In the intestine, butyrate is known to act in a paradoxical manner, being an essential colonocyte respiratory fuel (Roediger, 1980) but also acting to induce colonic cell maturation (reviewed in Augenlicht *et al.*, 1995). As such, butyrate is an established and highly effective inhibitor of proliferation and has been shown to be a potent inducer of cell cycle arrest, differentiation and apoptosis in a variety colorectal cell lines (Barnard and Warwick, 1993; Butt *et al.*, 1997; Hague *et al.*, 1995; Hague *et al.*, 1993; Heerdt *et al.*, 1994; Mariadason *et al.*, 2000; Qualtrough *et al.*, 2002; Whitehead *et al.*, 1986). Butyrate treatment has also been shown to modulate β-catenin/Tcf activity (Bordonaro *et al.*, 1999) and alter the expression of c-Myc (Barnard and Warwick, 1993; Taylor *et al.*, 1992). As such, butyrate increases $p21^{CIP1/WAF1}$ and induces cell cycle arrest at G0/G1 (Archer *et al.*, 1998; Litvak *et al.*, 1998; Nakano *et al.*, 1997; Richon *et al.*, 2000). Butyrate is also known to initiate apoptosis through induction of differentiation in these cells, increasing the expression and activity of the two key intestinal differentiation markers, alkaline phosphatase and E-cadherin (Barshishat *et al.*, 2000; Butt *et al.*, 1997; Gum *et al.*, 1987; Heerdt *et al.*, 1994).

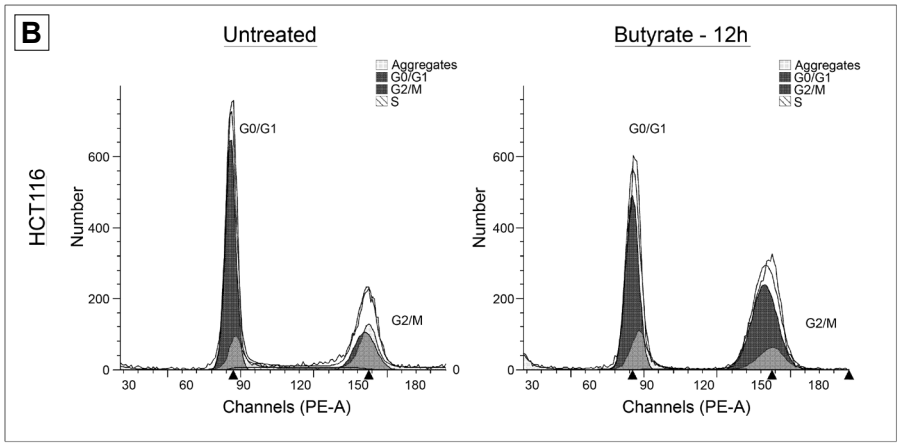
Treatment with butyrate has previously been shown to induce p21-mediated growth arrest in both the SW480 and HCT116 cell lines (Archer *et al.*, 1998; Daly *et al.*, 2005; Lee *et al.*, 2005a; Mahyar-Roemer and Roemer, 2001; Wilson *et al.*, 2006). In line with these studies, SW480 and HCT116 cells were treated with a physiologically relevant (and non-toxic) dose of butyrate (3mM) for 48 hours. In agreement with analyses in other colorectal cell lines (Qualtrough *et al.*, 2002), both SW480 and HCT116 cell lines showed visual evidence of approximately 30-50% growth inhibition after 48 hours of treatment. SW480 cells were shown to undergo a typical G0/G1 arrest following butyrate treatment, while HCT116 cells were shown to accumulate predominantly at G2 (*FIGURE 4-23A-C*), as described previously (Wilson *et al.*, 2006). Crucially, the number of cells in S-phase was dramatically reduced in both cell lines following butyrate treatment. In support of

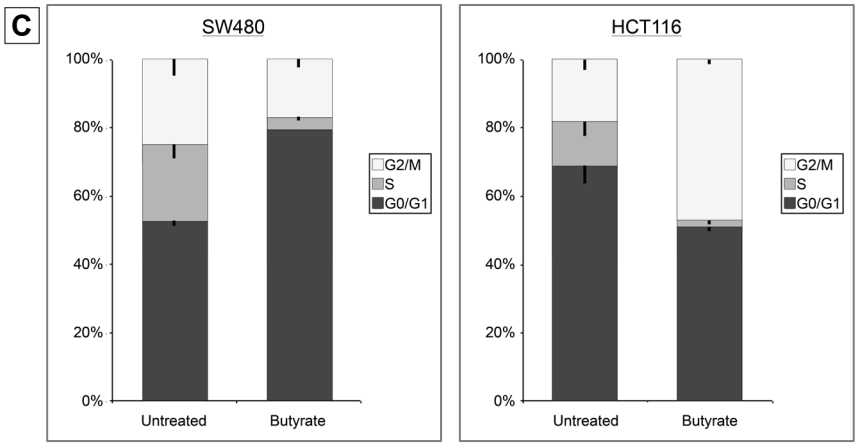
the previous finding that N1 is increased in actively cycling cells, N1 protein expression was shown to decrease in both the SW480 and HCT116 cell lines following butyrate induced cell cycle arrest (*FIGURE 4-23D*). Furthermore, a loss of cells in S-phase further supports the suggestion that N1 expression may be increased during S-phase rather than during G2/M.

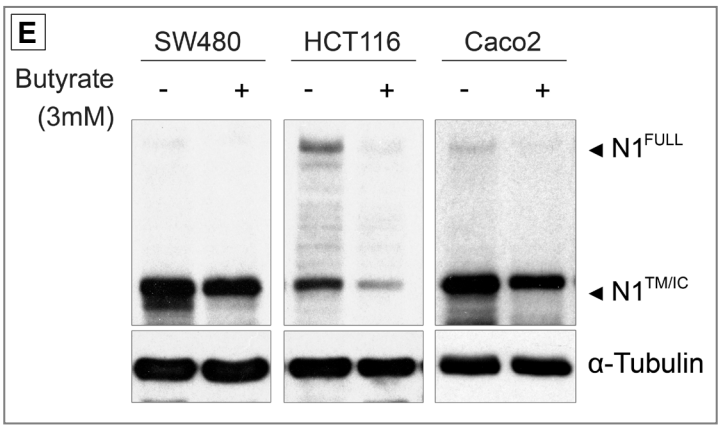
Furthermore, the effects of butyrate treatment on CRC induction of differentiation have been particularly well characterised in the Caco2 colorectal adenocarcinoma cell line (Litvak *et al.*, 1998; Mariadason *et al.*, 2000) and, consistent with findings in both SW480 and HCT116 cells, sodium butyrate treatment of the Caco2 cell line was shown to result in a substantial reduction in N1 expression by western blotting analysis (*FIGURE 4-23D*). Importantly, a reduction in full length N1 protein ($N1^{FULL}$) was evident in all three cell lines, suggesting potential transcriptional down-regulation of *N1* following butyrate treatment, albeit only marginally detectable in the SW480 cell line (*FIGURE 4-23D*). Given that butyrate treatment of *in vitro* colorectal tumour cells is believed to mimic normal differentiation in the intestinal epithelium *in vivo* (Butt *et al.*, 1997; Mariadason *et al.*, 2002; Mariadason *et al.*, 2000), these results, therefore, also imply that N1 expression is decreased with the onset of differentiation. This potentially contrast with previous findings from analysis of N1 in the normal human colorectal epithelium (*SECTION 3.2.2, p92*), although it is important to note that butyrate treatment in these cells reflects chemically induced tumour cell differentiation and is not entirely analogous to the physiological differentiation observed in the normal epithelium.

Figure 4-23| ***Notch1 protein is down-regulated in response to treatment with sodium butyrate.*** Cell cycle analysis of SW480 [**A**] and HCT116 [**B**] colorectal adenocarcinoma cells following 48 hour treatment with 3mM butyrate, vs. water-only control. Cells were stained for DNA content using Propidium Iodide prior to analysis by flow cytometry. [**C**] The percentage of cells in each phase of the cell cycle was determined by ModFit analysis software (*Verity software, USA*). [**D**] Western blotting analysis of full length and cleaved Notch1 ($N1^{FULL}$ & $N1^{TM/IC}$) protein expression. α-Tubulin was used to ensure equal protein loading. Results are representative of three independent experiments. ▶


A
Untreated
Butyrate - 12h
SW480
Aggregates
G0/G1
G2/M
S
Number
Channels (PE-A)


B
Untreated
Butyrate - 12h
HCT116
Aggregates
G0/G1
G2/M
S
Number
Channels (PE-A)


C
SW480
HCT116
100%
80%
60%
40%
20%
0%
G2/M
S
G0/G1
Untreated
Butyrate


E
SW480
HCT116
Caco2
Butyrate
(3mM)
-
+
N1FULL
N1TM/IC
α-Tubulin

4.2.13 NOTCH1 IS INCREASED IN POST-CONFLUENT CELL DIFFERENTIATION

A number of the colorectal adenoma- and adenocarcinoma-derived cell lines used in this study undergo spontaneous changes in morphology and protein expression consistent with intestinal differentiation when maintained in post-confluent culture. Of these, the Caco2 cell line has received particular notoriety, being well defined and widely used as an *in vitro* model of functional intestinal differentiation for both functional and pharmalogical studies (reviewed in Sambuy *et al.*, 2005). This colorectal adenocarcinoma cell line undergoes spontaneous differentiation in normal culture, but particularly in long-term post-confluent conditions, where cells form a polarised monolayer and express several morphological and biochemical characteristics of enterocytic differentiation (Grasset *et al.*, 1985; Grasset *et al.*, 1984; Rousset *et al.*, 1985). Caco2 differentiation has been shown to be accompanied by a parallel decrease in cell proliferation and a gene expression profile that defines cell maturation in the intestinal epithelium (Mariadason *et al.*, 2002). Post-confluent Caco2 cells also demonstrate increased p21 expression (Evers *et al.*, 1996) and G0/G1 cell cycle arrest (Hara *et al.*, 1993; Mariadason *et al.*, 2002; Mariadason *et al.*, 2000). Proteome analysis in these cells has confirmed that expression changes represent a shift from the colorectal cancer phenotype towards a more normal intestinal profile (Stierum *et al.*, 2003), while maximal phenotypic and functional differentiation has been shown to occur following 21 days of post-confluent growth (Hara *et al.*, 1993; Hidalgo *et al.*, 1989; Mariadason *et al.*, 2000).

In previous studies, a wide range of initial seeding densities have been reported (reviewed in Sambuy *et al.*, 2005), however, an optimal seeding density of 6 x 10^4 cells/cm^2 has been shown to result in reproducible and uniform differentiation after 21 days in confluent culture (Behrens and Kissel, 2003). Caco2 cells were seeded at this density and maintained under normal growth conditions (10% DMEM) for harvest at 48 hours, 96 hours, 1 week, 2 weeks, 3 weeks and 4 weeks post-seeding. The medium was changed 48 hours prior to each time point and 100% confluency was uniformly attained at about 120 hours post-seeding, ensuring that cells in the final fraction had remained in confluent growth for at least 21 days. Functional differentiation and cellular polarization of the post-confluent Caco2 monolayer was confirmed by the increasing formation of cellular 'domes', first appearing within the first 14 days of post-confluent growth (*FIGURE 4-24A; WHITE ARROW*). Cellular doming is a characteristic observed in a number of colonic cell lines and results from active and uni-directional ion and water transport to the basal layer with functional absorptive differentiation in these cells (Fantini *et al.*, 1986; Kirkland, 1985; Lever, 1982).

The relative expression of N1 protein at each time point was determined by western blotting analysis, where moderate but readily detectable levels of N1 protein were observed at 48-96 hours post-seeding (*FIGURE 4-24B*) and, consistent with a decline in cellular proliferation in these cells, decreasing N1 expression was observed with increasing cellular confluency, becoming virtually undetectable at 2 and 3 weeks post-seeding. However, in direct support of a potential secondary role for N1 in post-mitotic intestinal differentiation, N1 protein expression was dramatically restored again at 4 weeks post-seeding, therefore corresponding with more than 21 days of post-confluent growth.

In addition, PC/AA/C1 (AA/C1) is a clonogenic adenomatous cell line, which was originally derived from the descending colon of a familial adenomatous polyposis (FAP) patient (Paraskeva *et al.*, 1984). Comparable to the Caco2 adenocarcinoma cell line, AA/C1 cells have previously been shown to undergo spontaneous differentiation and doming when maintained in post-confluent growth conditions (Guy *et al.*, 2001; Qualtrough *et al.*, 2002). Parallel flasks of AA/C1 were seeded and maintained under standard growth conditions (20% DMEM; *SECTION 2.1.2, p55*) for harvest at 48 hours, 96 hours, 1 week, 2 weeks, 3 weeks and 4 weeks post-seeding, with the medium being changed 48 hours prior to each time point. Given the slower growth rate of this adenoma-derived cell line, cells were seeded at an increased density of 16 x 10^4 cells/cm^2 to ensure that 100% confluency was also attained at approximately 120 hours post-seeding. Functional differentiation was again confirmed by the appearance of domes in these cells within 14 days of post-confluent growth (*FIGURE 4-24C; WHITE ARROW*). Similar to Caco2 cells, N1 protein expression was readily detectable in sub-confluent AA/C1 cells but was shown to decrease with increasing cell confluency, being undetectable at 96 hours and 1 week post-seeding (*FIGURE 4-24D*). In further agreement with results obtained using Caco2 cells, N1 protein re-expression was observed at two weeks post-seeding and was equally maintained at all later time points.

A number of other colorectal cell lines have also been shown to undergo spontaneous differentiation when maintained in post-confluent growth (Kirkland, 1985; Pizao *et al.*, 1992), suggesting this phenomenon may be a common feature of *in vitro* colorectal cells in general. The effect of post-confluent growth on SW480 and HCT116 had not previously been assessed, but post confluent growth had been used in the HCT116 cell line to induce cellular arrest (Archer *et al.*, 1998). Experiments in SW480 and HCT116 cells were carried out in line with analysis in the Caco2 cells and 100% confluency was uniformly attained at about 120 hours post-seeding. In agreement with Caco2 and AA/C1 cells, cleaved N1 protein expression decreased in both the

SW480 and HCT116 cell lines with increasing confluency (*FIGURE 4-25*). N1 was not re-expressed in the SW480 cell line at 4 weeks; however, both full length and cleaved N1 expression were shown to increase in the HCT116 cell line with increasing time in post-confluent growth.

Taken together these results further demonstrate that the N1 protein is highly expressed in actively dividing colorectal tumour cells, but decreases in cells undergoing loss of proliferation and cell cycle arrest. Given that these colorectal tumour cells are also known to undergo changes reminiscent of differentiation in the normal epithelium, findings presented here also strongly support *in vivo* analysis of N1 expression in the normal colorectal epithelium, adding further weight to the hypothesis that N1 may have additional secondary roles in the post-mitotic differentiation of these normal and neoplastic tissues.

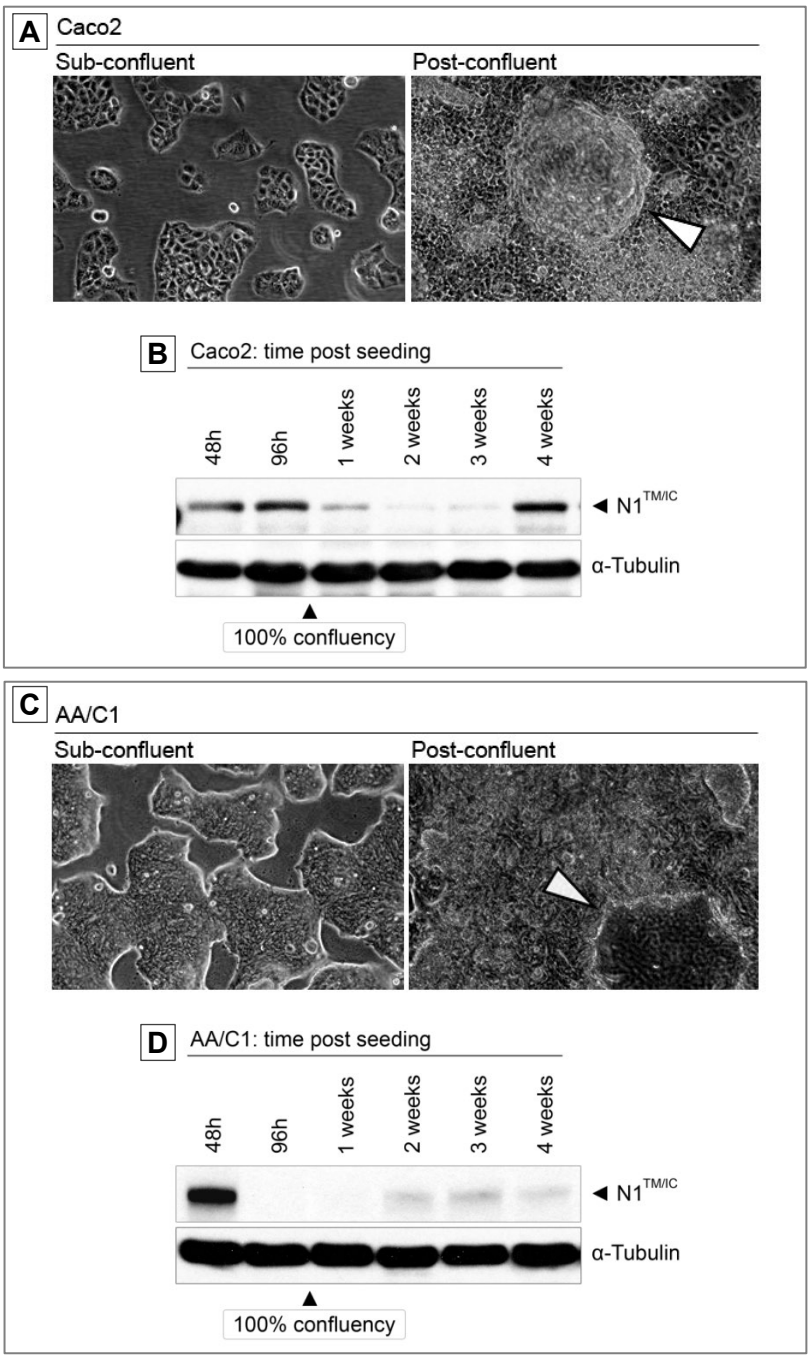


Figure 4-24| ***Notch1 is up-regulated in established in vitro models of intestinal differentiation.*** Analysis of N1 protein expression in the Caco2 adenocarcinoma-derived [**A-B**] and AA/C1 adenoma-derived [**C-D**] colorectal cell lines following induction of spontaneous differentiation by post-confluent growth. Functional differentiation was confirmed by phase-contrast microscopy and the post-confluent formation of 'domes' (*White arrows*; *Magnification x10*). N1 protein expression was determined by western blotting analysis in both cell lines. α-Tubulin expression was assessed to ensure equal protein loading and results are representative of three separate experiments.

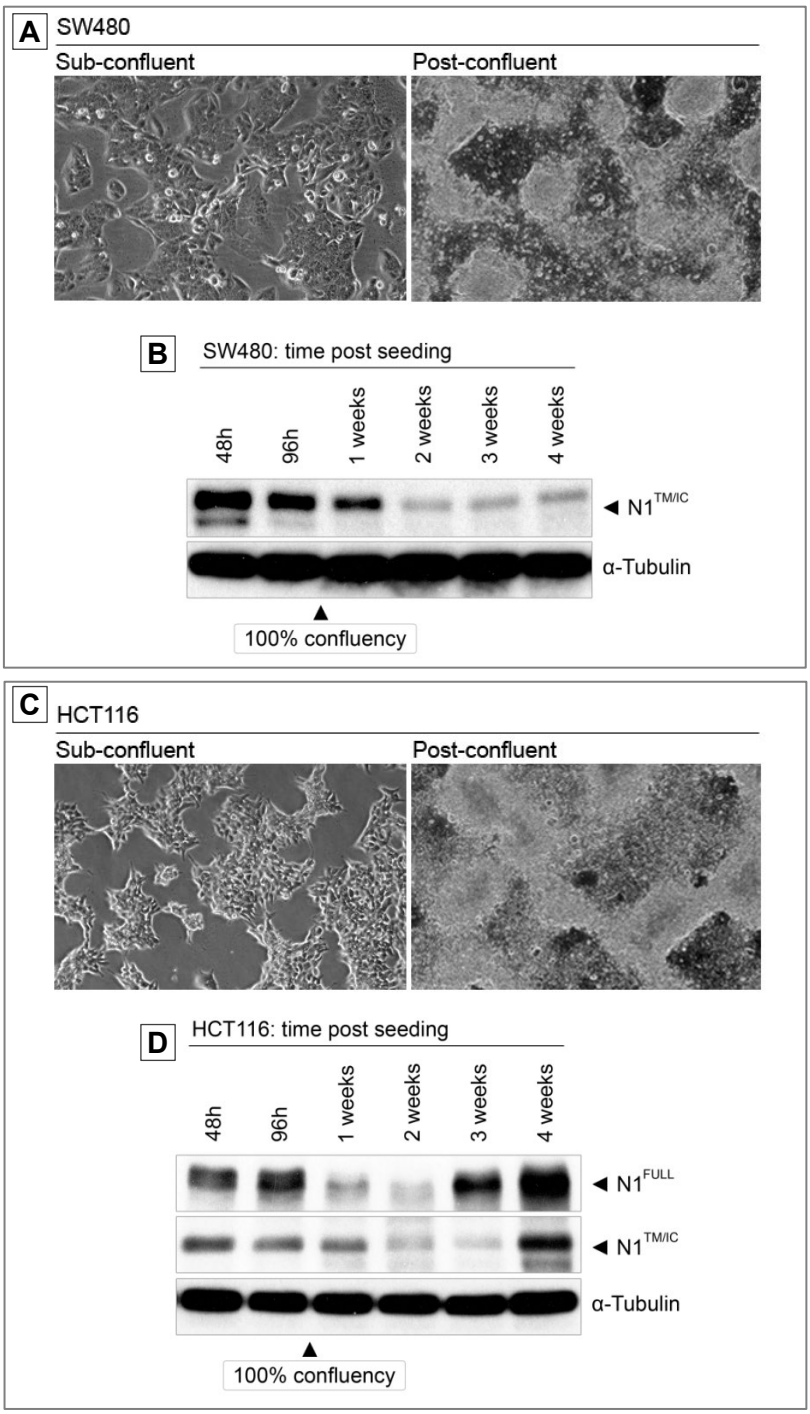


Figure 4-25 | ***Notch1 is up-regulated with post-confluency in HCT116 but not SW480 colorectal cancer cells.*** Analysis of N1 protein expression in the SW480 [**A-B**] and HCT116 [**C-D**] adenocarcinoma colorectal cell lines, following induction of spontaneous differentiation by post-confluent growth. Post-confluency lead to multilayered growth in both cell lines, as shown by phase-contrast microscopy (*Magnification x5*) and N1 protein expression was determined by western blotting analysis. α-Tubulin was included to ensure equal protein loading and results are representative of two experiments.

4.3 DISCUSSION

Correlative associations between N1 expression and carcinogenesis have now been demonstrated in an increasing number of tissues (reviewed in Leong and Karsan, 2006) and, from results in the previous chapter, it is now clear that up-regulated N1 is a significant feature of CRC tissues. For the most part, however, the downstream effects or functional contribution of Notch signalling in these tissues has continued to remain elusive and poorly defined. Although results from the small intestine of animal models have led to a hypothesised role for N1 in aberrant proliferation, the putative contribution of N1 to human CRC cells was largely untested and was the major focus of this chapter. In addition to testing the effects of Notch pathway modulation on proliferative potential and cell cycle progression in human CRC cells, the results of this chapter also go on to potentially identify a reciprocal relationship between N1 and both proliferative and differentiating phenotypes in these cells. Furthermore, this study also begins to highlight complex and previously unknown mechanisms of Notch target regulation and interplay with the Wnt pathway, suggesting a more complex and dynamic basis to Notch signalling in these tissues than currently appreciated.

4.3.1 HES1 IS A HIGHLY DYNAMIC TARGET OF ACTIVE NOTCH SIGNALLING

Only a limited number of Notch target genes have so far been identified and many of these appear to be context-dependent in both their activation and expression (Iso *et al.*, 2003). It is widely accepted that *Hes1* is the predominant target of active Notch signalling in many tissues, including both the normal and neoplastic intestinal epithelium (Jensen *et al.*, 2000; van Es *et al.*, 2005); however, it was shown here that, in addition to *Hes1, Hes5* is also a functional target of active N1 signalling in colorectal cancer cells (*FIGURE 4-6A*). Although previously unknown, the expression of *Hes5* in these tissues is consistent with previous N1 activation studies in the murine intestinal epithelium (Zecchini *et al.*, 2005) and is noteworthy because *Hes1* and *Hes5* are known to be evolutionarily the most closely related of all *Hes* homologues (Takebayashi *et al.*, 1995), although distinct functions between these two factors in the intestinal epithelium or CRC are not yet defined.

Hes1 has previously been used as a marker of sustained N1 activation in the normal murine intestinal epithelium (Fre *et al.*, 2005), but results presented here begin to suggest that a simple and direct relationship may not be applicable in the context of human colorectal tumour tissues. While N1 activation resulted in a significant up-regulation of *Hes1* transcription, no corresponding increase in Hes1 protein expression was observed in these CRC cells (*FIGURE 4-6*),

supporting generally complex and tightly controlled mechanisms of *Hes1* expression in other contexts. A lack of increased Hes1 protein expression may be explained by the identification of *Hes1* as an 'immediate early gene' (IEG) during embryonic development (Feder *et al.*, 1993). IEGs represent a set of genes, including *fos, jun* and *myc,* that are rapidly and transiently induced upon growth factor stimulation and undergo continued transcriptional activation in the absence of new protein synthesis (Sheng and Greenberg, 1990). This is an essential feature for coordination of normal mammalian developmental processes, such as vertebrate segmentation, which are highly dependent on transient and synchronous oscillatory cycles of Notch activation (Jiang *et al.*, 2000; Jiang *et al.*, 1998; Pourquie, 1999). In this context, rapid degradation and negative feedback events limit the half-lives of Hes1 mRNA and protein to about 24 and 22 minutes respectively (Hirata *et al.*, 2004; Hirata *et al.*, 2002). Nevertheless, these small and transient increases in Hes1 are able to elicit dramatic transcriptional changes due to the positive autoregulation demonstrated by many downstream target genes (Kuroda *et al.*, 1999; Thayer *et al.*, 1989). Hes1 also negatively regulates its own transcription and that of other *Hes* genes (Cooper *et al.*, 2000; Fior and Henrique, 2005; Hirata *et al.*, 2004) through the presence of multiple N-box motifs in the promoter (Takebayashi *et al.*, 1994), as confirmed by analysis in the present study (*FIGURE 4-9*). These auto-regulatory mechanisms therefore limit Hes1 expression within tightly controlled and oscillatory thresholds.

Furthermore, it is now increasingly understood that a significant degree of expression control occurs outside the convention of transcriptional activation (reviewed in Mattick, 2004). *Hes1* and other related mRNAs are also known to be regulated post-transcriptionally through their 3' UTR regions (Davis *et al.*, 2001; Lai *et al.*, 1998) and may result from non-coding RNA transcripts (ncRNA), such as micro RNAs (miRNAs) or small-interfering RNAs (siRNAs) (reviewed in Gebauer and Hentze, 2004). This is believed to be in response to changing Hes1 protein concentrations and, in support of this and auto-regulatory mechanisms, it has previously been shown that the Hes1 protein minimises *Hes1* mRNA levels even in response to continued activating stimulus (Kuroda *et al.*, 1999; Takebayashi *et al.*, 1994). Taken together this highlights further unappreciated levels of complexity in control of the Notch signalling output, which are likely to have considerable implications for both future Notch-related research and also any potential Notch-associated therapies.

4.3.2 NOTCH AND WNT SYNNERGISE IN THE CONTROL OF HES1 EXPRESSION

These results may also be partially explained by the possible dependence of Hes1 expression on factors independent of $N1^{IC}$ or Notch/CSL activity. For example, the expression of *Hes1* was found to be positively modulated by β-catenin/TCF-4 activity in these CRC cells (*FIGURE 4-7* - *FIGURE 4-10*). These findings add weight to an implied synergy between the Notch and Wnt pathways in colorectal tumours and have been underscored by several recent complementary studies. As described previously, Fre and colleagues (2009) demonstrate that N1 activation enhances the tumorigenic effect of de-regulated Wnt in the intestine of animal models ($N1^{IC}/Apc^{+/Min}$ mice). These compound transgenic mice also present a large number of dysplastic lesions in the colon, which are not normally observed in the $Apc^{+/Min}$ background (Fre *et al.*, 2009). Importantly, this is one area of contention between these animal models and the human scenario, since the majority of human intestinal neoplasia rarely occur in the SI (Yamada and Mori, 2007) and suggests that combined N1 and Wnt activation may be essential for neoplasia in the colonic epithelium. Furthermore, despite uniform Hes1 expression, this enhanced effect suggests that high level N1 activation may not be an endogenous feature of $Apc^{+/Min}$ mouse tumours. This is therefore consistent with observations presented here demonstrating low level N1 protein expression in human colorectal adenomas (*SECTION 3.2.5, p103*) and implies that the action of aberrant Wnt activity on *Hes1* expression may be responsible for its increase early in tumorigenesis.

Recent microarray analysis has also shown that a significant proportion of genes in CRC are under the combinatorial control of both Notch and Wnt, including *Hes1* (Rodilla *et al.*, 2009). In support of results presented here (*FIGURE 4-8*) it was shown that *Hes1* is significantly reduced in CRC cells by the forced expression of dnTCF4. The present study extends these findings by demonstrating that reduced *Hes1* transactivation occurs via the CSL-responsive region of the *Hes1* promoter and therefore Wnt-mediated influence on *Hes1* expression may occur via alteration to the canonical Notch pathway or by direct transcriptional control close to the CSL transcription factor binding site. Expression of the Jag1 ligand is known to be positively regulated by active Wnt in other tissues (Estrach *et al.*, 2006) and is now believed to provide a significant pathological link between Wnt and Notch signalling in CRC (Guilmeau *et al.*, 2009; Rodilla *et al.*, 2009). However, findings presented here also found that the predominant cleaved N1 isoform expressed in CRC is $N1^{TM}$, rather than $N1^{IC}$ (*FIGURE 4-2*), suggesting that more direct mechanisms may also be involved.

This was confirmed by computer-aided analysis performed here, which demonstrated multiple novel LEF-1/TCF-4E binding elements in the *Hes1* promoter/enhancer region (*FIGURE 4-10*). It is therefore likely that β-catenin and/or LEF-1/TCF-4E proteins directly promote or potentiate *Hes1* transcription in colorectal tumour cells. Importantly, it was also shown that the most highly conserved of these binding sites lies within the CSL responsive region of the *Hes1* promoter, suggesting close association between these factors. In further support of these findings, it has recently been shown in neural precursor cells that intracellular N1 physically binds β-catenin (Jin *et al.*, 2009a; Shimizu *et al.*, 2008) and siRNA targeting of *CTNNB1* results in a significant decrease in *Hes1* activation (Shimizu *et al.*, 2008). It was shown that β-catenin is incorporated as part of a molecular complex with $N1^{IC}$/CSL at the *Hes1* promoter region but that transcriptional activation is ultimately dependent on the presence of active $N1^{IC}$ (Shimizu *et al.*, 2008). This highlights a role for β-catenin in potentiating rather than driving Hes1 transcription, possibly through enhancing the recruitment of transcriptional co-activators or the release of negative regulators. In fact, the interaction between β-catenin and $N1^{IC}$ is further enhanced in the presence of CBP/p300 proteins (Hayward *et al.*, 2005; Shimizu *et al.*, 2008) and both β-catenin and the E-tail domain of TCF-4 have been shown to interact with CBP/p300 (Hecht and Stemmler, 2003; Takemaru and Moon, 2000). These co-factors are normally recruited to the $N1^{IC}$/CSL complex by another transcriptional co-factor, Maml-1 (Fryer *et al.*, 2002) and Maml-1 has now been shown to be essential for canonical β-catenin/TCF activity in CRC cells (Alves-Guerra *et al.*, 2007), suggesting yet further complex and largely undefined levels of interdependence between the Notch and Wnt pathways in these tissues. What is clear is that, further to preliminary results presented here, integration between Notch and Wnt in many tissues including CRC looks set to remain the focus of extensive and interesting research over future years.

The identification of LEF-1 and TCF-4E consensus sites (*FIGURE 4-10*) may be significant given the importance of these two factors in CRC. The TCF-4 protein (encoded by the *TCF7L2* gene) demonstrates three distinct isoforms, B, C and E. The E-tail form contains a β-catenin-dependent transcriptional activation domain (Atcha *et al.*, 2003; Hecht and Stemmler, 2003) and is necessary for the activation of a select subset of Wnt target genes involved in cell growth, including *LEF-1* (Atcha *et al.*, 2007). Importantly, the predominant TCF forms in human CRC contain E-tails (Atcha *et al.*, 2007) and TCF-4E has previously been selected for studies involving CRC cell lines (Batlle *et al.*, 2002; van de Wetering *et al.*, 2002). TCF-4E is also essential for progenitor proliferation and regulation of c-Myc/$p21^{CIP1/WAF1}$ in the normal intestine (van de Wetering *et al.*, 2002) and the dnTCF-4 plasmid used here (*FIGURE 4-8*) corresponds to the E-tail isoform (Korinek *et al.*, 1998a).

LEF-1 is less well characterised, but *de novo* protein expression is an established feature of colorectal cancer and is believed to reflect potential reversion of these neoplastic tissues to a more embryonic state (Hovanes *et al.*, 2001; Wong *et al.*, 2002).

Although LEF1 and TCF-4E are known to recognise the same DNA sequence, their affinities for different sites and their target gene profile varies considerably (Hecht and Stemmler, 2003). Forced expression of LEF-1 has previously been shown to diminish the positive effects of β-catenin on the *Hes1* promoter in other tissues (Jin *et al.*, 2009a), indicative of potential competitive antagonism between *Hes1* and the LEF-1 target genes. Similar results are demonstrated here, since a reciprocal decrease in LEF-1/β-catenin activity was observed in the presence of exogenous $N1^{IC}$ (*FIGURE 4-11*), possibly due to competition for β-catenin. Although β-catenin is a critical transactivator of LEF/TCF target genes in the canonical Wnt pathway (Behrens *et al.*, 1996), it has previously been proposed that β-catenin carries out 'dual-function' to synergistically promote proliferation and suppress differentiation in neural precursor cells through regulation of Wnt and Notch target genes respectively (Shimizu *et al.*, 2008). From results presented here, it is feasible that a similar model may apply to the intestinal epithelium and active Notch and Wnt may carry out complementary but distinct functions in these cells. However, this model does not account for possible Notch-mediated effects in the absence of active Wnt signalling and results presented here also begin to suggest that Notch exhibits a contextual switch under these conditions and may then carry out alternative and potentially paradoxical functions in the intestinal epithelium.

4.3.3 NOTCH1 DOES NOT MODULATE COLORECTAL CANCER PROLIFERATION

The outcome of active N1 may therefore be context-dependent and heavily influenced by the degree of Wnt activation. In the normal murine intestine it was recently shown that physiologically active Wnt signalling is necessary for the pro-proliferative effects of exogenous $N1^{IC}$, while the inhibitory action of $N1^{IC}$ on goblet cell differentiation occurs independently of the Wnt cascade (Fre *et al.*, 2009). Similarly, N1 activation in the absence of active Wnt signalling has been shown not to induce proliferation in CRC cells lacking active Wnt but continued to strongly suppress the secretory differentiation of these cells (Rodilla *et al.*, 2009). In the present study, activation or inhibition of N1 or pan-Notch signalling was shown not to mediate proliferation or cell cycle progression in human colorectal cancer cells (*FIGURE 4-12* - *FIGURE 4-15*). This suggests that Notch signalling is no longer necessary for proliferation and survival in late stage colorectal carcinogenesis and N1 may be responsible for alternate functions in these adenocarcinoma

tissues. Support for this was provided by another recent study, in which GSI treatment or siRNA-mediated targeting of either *N1* or *CSL* had no significant effect on the growth or cell cycle progression in human CRC cells, namely SW480 (Akiyoshi *et al.*, 2008). In contrast, another recent study demonstrated that transient expression of $N1^{IC}$ in the HT29 colorectal adenocarcinoma cell line increased cell number, while GSI treatment or siRNA-mediated knockdown results in significant decrease in cell growth (Ghaleb *et al.*, 2008). Importantly, N1 protein expression was shown here to be largely undetectable in HT29 cells, being the only adenocarcinoma cell line with basal N1 levels lower than those of adenoma origin (*SECTION 3.2.7, p111*).

As described previously, Notch pathway activation is known to be active at extremely low N1 concentrations (Conboy *et al.*, 2005; Schroeter *et al.*, 1998) and it is known from a number of development contexts that different functional outcomes arise depending on dose-dependent threshold levels of N1 activation (Guentchev and McKay, 2006). It should also be noted that adenomas represent a more 'normal' phenotype and HT29 is an extremely well differentiated adenocarcinoma cell line. This may be an important consideration, since one of the major findings of the present study is that N1 is up-regulated with tumour progression and therefore Notch may carry out dose-dependent functional roles at different stages of colorectal carcinogenesis. It is plausible therefore that the SW480 and HCT116 adenocarcinoma cell lines used here have become insensitive to the N1-mediated growth effects observed in the naïve intestinal epithelium or early intestinal adenomas. The functional contribution of increased N1 expression in adenocarcinoma tissues therefore remains to be determined, although analysis in other tissues now implies that increased pathway activation with malignancy may influence other factors, such as tumour invasion (Leong *et al.*, 2007).

4.3.4 NOTCH1 IS TRANSIENTLY INCREASED IN ACTIVELY CYCLING CELLS

Although Notch signalling was shown not to modulate proliferation or cell cycle distribution in colorectal cancer cells under the conditions used, it was consistently shown that mitotic cells (in G2/M) express relatively higher levels of basal N1 compared to those in G0/G1 (*FIGURE 4-16, FIGURE 4-17*), suggesting that increased N1 expression may occur as a consequence rather than cause of cell cycle progression. This may not be surprising since it has been predicted for many years that developmental programs are subordinately governed by changes in the cell cycle (Foe, 1989) and in *Drosophila,* the exons encoding *Notch* and *Hairy* have been shown to demonstrate close sequence homology to cell cycle genes, suggesting common cyclic mechanisms underlying their expression (Breeden and Nasmyth, 1987; Hartley *et al.*, 1988).

It was originally hypothesised from analysis in T-ALL that Notch may be involved in the G1/S transition but, consistent with findings presented here for CRC cells, much evidence also supports a conserved function for N1 in G2/M. For example, analysis in the *Drosophila* wing has shown that active Notch promotes the G2/M transition (Johnston and Edgar, 1998) and Notch-mediated neural cell fate in *Drosophila* has also been shown to be highly dependent on G2 cell cycle position (Negre *et al.*, 2003). Similarly, in the vertebrate neuroepithelium, the levels of neurogenic and proneural genes oscillate as neural precursor cells pass through the cell cycle (Latasa *et al.*, 2009). In particular, the capacity to differentially express N1 and Dll1 in a cell cycle-dependent fashion has recently been suggested as an intrinsic characteristic of these cells, with maximal expression of both factors and *Hes1/5* during the G2/M phase of the cell cycle (Cisneros *et al.*, 2008; Murciano *et al.*, 2002). This is thought to be an essential feature for the selection of neural precursors and for their commitment to differentiate along specific neural fates and, given that intestinal progenitor cell fate determination is dependent on the same pro-neural factors (*SECTION 1.5.8, p45*), it might also be predicted that similar mechanisms govern Notch activation in early intestinal progenitors and associated tumours.

The cyclic expression of N1 demonstrated in this study (*FIGURE 4-16, FIGURE 4-17*) is interesting because surprisingly little is known about the transcriptional control of the genes encoding the Notch receptors in any tissue, despite its paramount importance to normal and aberrant Notch activity. In possible explanation of these results, DNA-remodelling during replication is known to render certain genomic regions more available for transcription than in other phases of the cell cycle (Holtzer *et al.*, 1975). For example, it is known that in undifferentiated embryonic stem cells, master regulatory genes associated with maintaining pluripotency and cell fate determination are differentially expressed during S-phase (Azuara *et al.*, 2006). The increased expression of N1 observed in G2/M may therefore result from transcriptional initiation in the later stages of S-phase rather than during G2/M. Accordingly, it was shown here that N1 expression was reduced following a loss of S-phase cells by treatment with Colcemid (G2/M arrest) (*FIGURE 4-18, FIGURE 4-19*) or sodium butyrate (G0/G1 & G2/M arrest) (*FIGURE 4-23*), but N1 expression was sustained in cells arrested in G1-S using Hydroxyurea (*FIGURE 4-20*). Furthermore, the greatest increase in full length N1 expression in synchronous culture of the SW480 cell line was observed just prior to entry into G2/M (*FIGURE 4-22*). Although a substantial enrichment of cells in the progressive phases of the cell cycle was achieved by this technique, this represents only a partial increase in the proportion of each cycle phase and a much greater increase in N1

expression may be achieved by more effective and specific enrichment and synchronisation methods, such as such centrifugal elutriation (Gillespie and Henriques, 2006).

The functional significance of N1 in the G2/M phase of the cell cycle is yet to be determined, although it has previously been suggested to prevent execution of the differentiation program during cell cycle phases prior to entering G0/G1 (Latasa *et al.*, 2009). It has recently been shown that GSI treatment induces a G2/M arrest in breast cancer cells (Rasul *et al.*, 2009) and, although Notch inhibition was shown here and elsewhere not to elicit a cell cycle arrest in CRC cells (*Figure 4-13*), GSI treatment has been shown to consistently result in arrest at G2/M when combined with other chemotherapeutic agents (Akiyoshi *et al.*, 2008). The contrasting G0/G1 arrest observed in T-ALL cells might be explained since N1 activation is associated with both c-Myc (Palomero *et al.*, 2006; Sharma *et al.*, 2006; Weng *et al.*, 2006) and Cyclin D (Ronchini and Capobianco, 2001) in these tissues. Both of these factors are potent activators of the G1/S transition and are known to be essential for Notch-mediated leukaemogenesis (Palomero *et al.*, 2006; Sicinska *et al.*, 2003).

This is in agreement with the general consensus that oncoproteins need to partner other oncoproteins in order to cause cancer and, in addition to c-Myc and Cyclin D, N1 also partners an array of other oncoproteins including E1A (Capobianco *et al.*, 1997), HPV E6 and E7 (Rangarajan *et al.*, 2001a), RAS (Fitzgerald *et al.*, 2000), other MYC proteins (Girard *et al.*, 1996) and SV40T (Bocchetta *et al.*, 2003). The common feature attributed to all of these factors is their ability to over-run the G1-S checkpoint and promote continued proliferation, therefore, it seems unlikely that the contribution of Notch to the tumorigenic process will be cell cycle initiation or induction of proliferation. Instead, active Notch signalling may provide complementary oncogenic features to maintain the progenitor phenotype and promote colorectal carcinogenesis. For example, expression profiling experiments in neural and hematopoietic stem cells suggest a common role for N1 signalling in maintaining 'stemness' rather than promoting proliferation (Ivanova *et al.*, 2002; Ramalho-Santos *et al.*, 2002) and the potential contribution of N1 to colorectal cancer stem cells is likely to be the focus of many future studies in this field. Whatever the contribution of N1 to colorectal carcinogenesis, like many CRC-associated pathway changes, it is likely that this may reflect aberrant recapitulation of N1 function in the normal intestinal epithelium.

4.3.5 PARADOXICAL SIGNIFICANCE OF NOTCH1 IN POST-MITOTIC GROWTH

Results presented in this chapter support the notion that N1 functions as a general arbiter of cell fate in a manner dependent on cellular context and integration with other signalling pathways

(Miele and Osborne, 1999). It is known that many CRC cells undergo morphological and functional differentiation processes similar to normal tissue (Zweibaum, 1993) and retain a stem-progenitor-differentiation hierarchy reminiscent of the normal tissue from which they derive (Visvader and Lindeman, 2008). Therefore, *in vitro* tumour cells have provided an invaluable model of cellular maturation in the normal intestinal epithelium.

It is shown here that N1 is re-expressed during post-confluent growth of the Caco2 cell line, a well established *in vitro* model of tumour and intestinal differentiation (*FIGURE 4-24*) (reviewed in Sambuy *et al.*, 2005). Crucially, N1 expression is diminished with increasing cellular confluency but is dramatically re-expressed again following 21 days post-confluent growth, consistent with spontaneous differentiation in this cell line. In the Caco2 cell line, it is known that increasing cellular confluency is accompanied by a loss of proliferation, cell cycle arrest at G0/G1 and increased expression of $p21^{CIP1/WAF1}$ (Evers *et al.*, 1996; Hara *et al.*, 1993; Mariadason *et al.*, 2000), although it is likely that this is a phenotypic change common to most cells in culture. In analogy to the normal intestinal crypt (van de Wetering *et al.*, 2002), β-catenin-TCF activity is maximal in rapidly dividing, undifferentiated Caco2 cells, but is also progressively down-regulated with increasing time in post-confluent growth and induction of differentiation (Mariadason *et al.*, 2001). This was further confirmed from analysis in the AA/C1 adenoma cell line, which has also previously been used as an *in vitro* model of post-confluent differentiation (Guy *et al.*, 2001; Qualtrough *et al.*, 2002), along with the HCT116 adenocarcinoma cell line, which undergoes similar proliferative changes with increased confluency (Archer *et al.*, 1998). These results therefore add further weight to the finding that the N1 protein is most highly expressed in mitotically active colorectal cancer cells passing through the G2/M phase of the cell cycle (*FIGURE 4-16, FIGURE 4-17*) and N1 may therefore initially be expressed relative to cellular proliferation.

Alternatively, the consistent re-expression of N1 in post-confluent colorectal tumour cells implicates a potential secondary role for N1 in post-mitotic cells in the normal epithelium and intestinal tumours. This supports *in vivo* findings from resected human colorectal tissues, in which N1 was shown to be highly expressed in cells towards the top of the crypt, thought to be un-proliferative and undergoing differentiation (*SECTION 3.2.2, p92*). Taken together with other recent findings (Fre *et al.*, 2009; Rodilla *et al.*, 2009), these results imply that N1 may mediate highly distinct and reiterative roles within the intestinal epithelium, being responsible for both Wnt interdependent and independent functions along the length of the crypt axis. As described in the previous chapter, Notch may therefore be a critical mediator of different cellular

phenotypes and different cellular compartments within the crypt, with this characteristic likely to extend to heterogeneous populations of cells found within intestinal tumours.

Notch is increasingly appreciated to carry out diverse functions in a range of tissues (Roy *et al.*, 2007), although how one pathway might carry out such diverse functions is not fully understood. It is possible that, in the intestinal epithelium at least, this may relate to context-dependent differences in transcriptional targets, since N1 activation in the embryonic murine epithelium results in proliferative expansion through increased expression of *Hes1* but not *Hes5* (Fre *et al.*, 2005), while N1 activation in the adult murine epithelium drives post-mitotic differentiation through the increased expression of *Hes5* but not *Hes1* (Zecchini *et al.*, 2005). In fact, it has previously been suggested that a considerable amount of Notch-mediated events occur independently of *Hes1* and it has been suggested that *Hes5* may be a conserved target responsible for these alternative functions (de la Pompa *et al.*, 1997; Shawber *et al.*, 1996b). Since it is also shown here that β-catenin/TCF positively modulate *Hes1* transcription, possibly through direct transcriptional modulation (*FIGURE 4-7 - FIGURE 4-10*), it is possible that distinction between *Hes1* and *Hes5* expression may be mediated by relative levels of Wnt activation in these cells. In support of findings in the present study, it has previously been suggested that *Hes1* is less dependent on Notch activity than *Hes5* (de la Pompa *et al.*, 1997) and it has also been shown that while *Hes1* is a Notch and Wnt responsive gene, *Hes5* is responsive to Notch activation only (Espinosa *et al.*, 2003; Rodilla *et al.*, 2009) (*L. Espinosa, personal correspondence*). While an increase in *Hes5* expression in the *Hes1*$^{-/-}$ intestinal epithelium is thought to signify functional redundancy between these factors (Jensen *et al.*, 2000), this may also demonstrate that these two factors are mutually exclusive and may occur through the presence of N-box motifs in the *Hes5* promoter (Takebayashi *et al.*, 1994).

In the previous chapter it was suggested that a post-mitotic role for N1 might be influenced by a switch in exposure from Wnt to TGF-β/BMP activation along the crypt-lumen axis. In agreement with differential roles for *Hes1* and *Hes5,* BMP stimulation in neuroepithelial cells has been shown to enhance N1^{IC} activation of *Hes5* (Takizawa *et al.*, 2003), while the *Hes5* promoter has also been shown to contain several Smad consensus binding sequences, implying that similar regulation may also occur through TGF-β (Nakashima *et al.*, 2001). In surprising similarity to the convergence on *Hes1* suggested here for N1/β-catenin, Smad1 appears to potentiate the *Hes5* promoter by forming a complex with N1/CSL in the presence of p300 (Takizawa *et al.*, 2003). The true contribution of these two prominent Notch targets in the normal intestinal epithelium and in

colorectal tumour cells remains to be determined; however, in the previous chapter it was shown that Hes1 protein is also expressed in cells at the top of the normal colonic crypt (*SECTION 3.2.2, p92*) and both *Hes1* and *Hes5* are concomitant targets of active N1 in colorectal cancer cells (*FIGURE 4-6*). Taken together, these findings suggest largely unexplored Notch functions in these tissues and highlight increasing levels of complexity for this once considered simple pathway.

4.3.6 CONCLUDING REMARKS

Notch plays opposing but conserved and coordinated roles in various tissues during development, where depending on dose and context, Notch may inhibit or induce differentiation (Artavanis-Tsakonas *et al.*, 1999). Although the general aspects of Notch-ligand interaction are known to be extraordinarily conserved, it appears that the biological consequences of active signalling remain extremely cell context- and cell signalling-dependent. From results presented here, it would appear that a complex and multifaceted role for Notch is applicable in the normal colorectal epithelium and associated tumour tissues, where it was shown that increased N1 expression is induced by both the proliferative and differentiating phenotype. Furthermore, this study also demonstrates novel mechanisms by which the transcriptional outcome of active Notch signalling might be influenced by the signalling context imposed by other key pathways, namely Wnt. Therefore, Notch signalling may mediate distinct and contrasting functions in the normal colonic crypt, and in a similar fashion may govern the heterogeneous distributions of cells found in colorectal tumours; however, the definitive contribution of active Notch signalling in these tissues continues to remain elusive. It is clear that the major challenge in the study of Notch in this and any tissue will be to understand how this relatively simple pathway might yield such a variety of different cellular outcomes (Maillard and Pear, 2003).

CHAPTER 5

NOTCH1 IS INCREASED WITH CANCER DE-DIFFERENTIATION

5 NOTCH1 IS INCREASED WITH CANCER DE-DIFFERENTIATION

5.1 INTRODUCTION

5.1.1 MOLECULAR MECHANISMS OF TUMOUR CELL INVASION

As described previously, tissue invasion and metastasis is one of the key hallmarks of cancer (Hanahan and Weinberg, 2000) and is the principal cause of death in most cancer patients (Sporn, 1996; Young and Rea, 2000). Despite its unquestionable clinical significance (*SECTION 3.1.2, p80*), the molecular basis behind the malignant colorectal phenotype remains poorly understood. Unrestrained growth does not, by itself, cause tumours to become invasive. This fundamental transition is instead reliant on additional and progressive molecular-genetic changes, leading to detachment from the primary epithelial tumour mass, invasion into the underlying tissue and dissemination around the body (reviewed in Woodhouse *et al.*, 1997).

It has long been known that one of the most important cellular changes observed during tumour invasion is loss of the differentiated cell phenotype (Gabbert *et al.*, 1985). As discussed previously, well differentiated colorectal adenocarcinomas more closely resemble the normal colonic epithelium than their poorly differentiated counterparts and are characterised by the persistence of many predominant crypt-like tubular structures. This is gradually lost with increasing tumour grade (Compton *et al.*, 2000; Hamilton and Aaltonen, 2000) and poorly differentiated adenocarcinomas are associated with a more invasive phenotype and poorer patient prognosis (Purdie and Piris, 2000). Nevertheless, by their nature well differentiated adenocarcinomas are still able to invade and disseminate into surrounding tissues. It is now widely accepted that this occurs via localised de-differentiation of tumour cells at the invasive front (Brabletz *et al.*, 2002) and malignant tumours are frequently composed of a heterogeneous and often concentric differentiation pattern, being well differentiated towards the tumour centre with graduated de-differentiation towards the host-tumour interface (Ono *et al.*, 1996). High grade adenocarcinomas are therefore believed to demonstrate a poorer patient prognosis since they require only a subtle increase in de-differentiation to undergo invasion (Gabbert *et al.*, 1985).

Although tumour progression to malignancy is understood to arise from the progressive accumulation of additional genetic lesions (Fearon and Vogelstein, 1990), one common observation when examining colorectal metastases at secondary tumour sites is that they often closely recapitulate the morphology and differentiation of the primary tumour from which they

derive. These findings suggest that tumour de-differentiation and metastasis arises largely from reversible and transient changes in cellular phenotype, rather than being fixed irrecoverably through genetic alteration (Brabletz *et al.*, 2002). Following extravasation at a secondary site in the body, metastatic cells demonstrate a converse series of events, undergoing re-differentiation to model the tissue architecture of the primary tumour. Colorectal tumour progression is therefore not uni-directional but is instead a complex, highly regulated and reversible morphogenic process which depends on dynamic changes in cell phenotype.

Histological de-differentiation is now known to be accompanied by changes at the cellular level, with epithelial cells undergoing considerable alterations to enable their invasive growth into the underlying tissue. Epithelial cells are characterised as tight layers of cells that are closely adjoined by a variety of specialised cell-cell adhesion junctions and demonstrate a distinctive apical-basolateral polarisation. The epithelial cadherin protein (E-cadherin; encoded by the *CDH1* gene) is the main adhesion molecule in the epithelium and is often used as a marker of epithelial cell differentiation (Pignatelli, 1993). Functional E-cadherin localises to the plasma membrane, where it mediates strong epithelial cell-cell adhesion with juxtaposed cells as an essential component of the cellular adherens junctions (*FIGURE 5-1A*). Therefore, loss of E-cadherin is one of the most common and critical alterations in human epithelial cancers and is considered to be an important prerequisite to invasion and metastasis (reviewed in Hirohashi, 1998).

Loss of E-cadherin expression is also known to be an integral feature of CRC invasion and has been shown to correlate directly with reduced patient survival (Gofuku *et al.*, 1999; Nigam *et al.*, 1993). Crucially, loss of E-cadherin has been shown to be a major causative factor in the transition from a benign to malignant phenotype in both animal models (Perl *et al.*, 1998) and various cell lines (Behrens *et al.*, 1989; Frixen *et al.*, 1991; Vleminckx *et al.*, 1991), where a direct correlation can be made between E-cadherin expression and the epithelial cell phenotype. E-cadherin is therefore a key tumour and invasion suppressor in epithelial tissues (reviewed in Christofori and Semb, 1999). *CDH1* mutations, however, are not a common feature of CRC (Schuhmacher *et al.*, 1999), inferring that loss of E-cadherin protein expression must instead be regulated by other dynamic and reversible mechanisms.

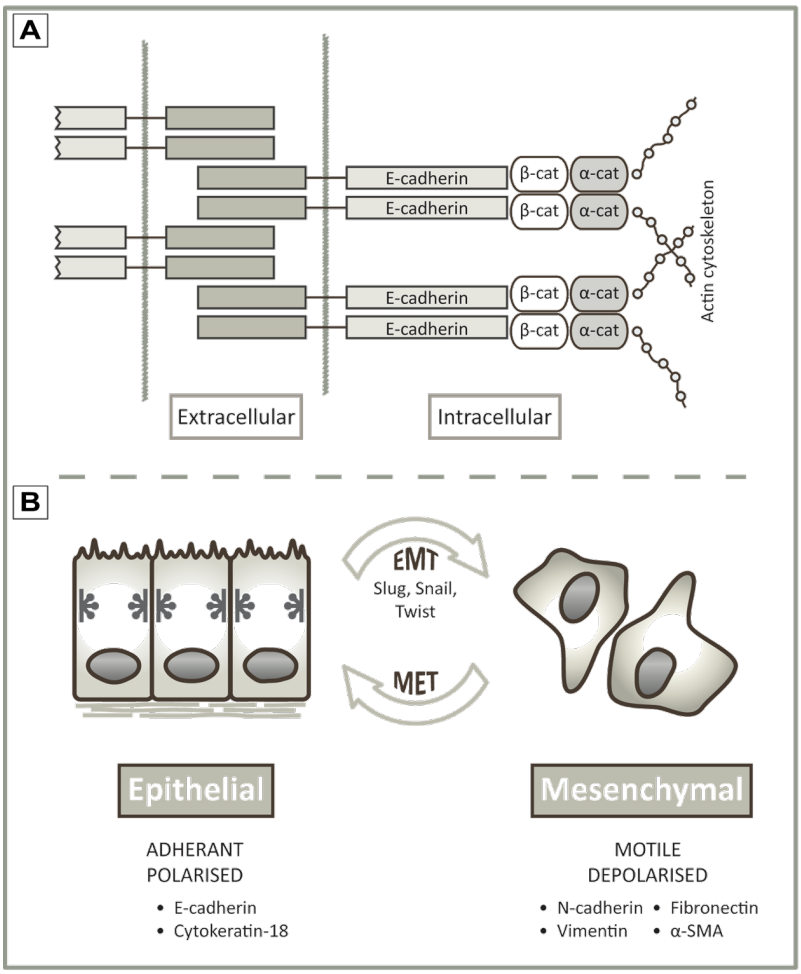


Figure 5-1| ***E-cadherin is an essential component of the epithelial cell adherens junctions, which is disrupted during EMT.*** [**A**] The transmembrane E-cadherin protein mediates extracellular, calcium-dependent interaction with E-cadherin expressed on the external surface of adjacent cells. The intracellular portion connects indirectly with components of the cell actin and microtubule cytoskeleton via several key intracellular partners, including α- and β-catenin. [**B**] Epithelial-mesenchymal transitions (EMT) comprise a wide spectrum of changes in epithelial plasticity, leading to loss of adhesion and increased cellular motility. During developmental and malignant EMT, epithelial cells lose their characteristic adherent and polarised structure and adopt a more mesenchymal phenotype, with loose or no cell-cell cohesion. In addition, cells undergoing EMT also adopt a mesenchymal expression profile, including the intermediate filament protein vimentin and the transient adhesion protein N-cadherin. EMT is largely transient and reversible, with the converse process of mesenchymal-epithelial transition (MET) being observed at sites of *de novo* organogenesis and secondary tumour formation.

5.1.2 TUMOUR INVASION AND THE EPITHELIAL TO MESENCHYMAL TRANSITION

Down-regulated E-cadherin expression is a fundamental physiological feature during normal embryonic morphogenesis, where epithelial cells adopt a more mesenchymal phenotype and invade into the underlying tissue for the formation of new tissue structures (reviewed in Hay, 2005). This epithelial to mesenchymal transition (EMT; *FIGURE 5-1B*) is an important process, since epithelial and mesenchymal cells differ greatly in their functional and phenotypic characteristics.

Despite their cohesive and ordered structure, epithelial cells demonstrate motility and migration within the epithelial layer, but do not detach and move away under normal conditions. In contrast, mesenchymal (stromal) cells do not form organised cellular layers and are not polarised, having distinct spindle-shaped fibroblast morphology in culture. They make only loose and transient contacts with neighbouring cells and are therefore considered to have greater cellular motility and plasticity compared to the epithelium.

EMT is regulated by a number of highly conserved mechanisms, but relies largely on transcriptional repression of *CDH1* by several highly conserved transcription factors, including Slug, Snail and Twist (Nieto, 2002; Yang *et al.*, 2004). As well as inhibiting E-cadherin expression, these factors induce a gene expression profile consistent with the mesenchymal phenotype, such as vimentin, N-cadherin, fibronectin and α-smooth muscle actin (α-SMA), where an up-regulated expression of these non-epithelial factors is associated with EMT. Following invasive growth and cellular translocation to the secondary site, the converse process of mesenchymal to epithelial transition (MET) occurs, enabling re-differentiation and the generation of new tissue structures to proceed (Hay, 2005). These cells down-regulate EMT associated factors and begin to re-express high levels of E-cadherin.

The de-differentiation and migration observed during tumour invasion and metastasis is therefore highly analogous to these dramatic changes in normal embryonic development and EMT is now widely accepted to be a central process governing malignancy in many cancers, including CRC (reviewed in Brabletz *et al.*, 2005a). Increased expression of central EMT mediators such as Slug, Snail and Twist, plus mesenchymal markers, such as Vimentin, are also frequently observed during colorectal tumour progression and, in many cases, are found to closely correlate with reduced patient survival (Ngan *et al.*, 2007; Roy *et al.*, 2005; Shioiri *et al.*, 2006; Valdes-Mora *et al.*, 2009). Taken together this highlights the paramount importance of E-cadherin expression and EMT in the invasion of epithelial cancers; however, at present many of the underlying molecular mechanisms governing these transitions remain unknown.

5.1.3 DEVELOPMENTAL SIGNALLING PATHWAYS ARE ESSENTIAL FOR EMT

It is clear that the EMT and MET are fundamental to embryonic morphogenesis and it should not be surprising that, at the top level, these processes are found to be controlled by the various developmental signalling pathways (reviewed in Thiery and Sleeman, 2006). In a similar fashion, it is now increasingly well understood that, in addition to tumour initiation, the morphogenetic code also forms an essential underlying basis in tumour invasion and metastasis (reviewed in

Brabletz *et al.*, 2005a). In particular, TGF-β has been shown to be a major inducer of EMT and invasive growth during normal development in the embryo and following treatment of many different cell types in culture (reviewed in Zavadil and Bottinger, 2005). In colorectal tumours, transition to the adenocarcinoma phenotype is known to coincide with loss of sensitivity to TGF-β-mediated growth inhibition (Engle *et al.*, 1999; Manning *et al.*, 1991), with TGF-β instead switching function to promote invasive tumour growth (Roman *et al.*, 2001; Schroy *et al.*, 1990), being associated with a poorer patient prognosis (Friedman *et al.*, 1995; Robson *et al.*, 1996).

The Wnt signalling pathway is also known to be a potent inducer of EMT in a range of physiological or aberrant contexts. Wnt activation and/or stabilisation of β-catenin has been shown to induce premature EMT induction during the early stages of embryogenesis (Kemler *et al.*, 2004; Mohamed *et al.*, 2004) and is heavily implicated in cancer invasion and metastasis (reviewed in Brabletz *et al.*, 2002; & Fodde and Brabletz, 2007). Nuclear β-catenin is increased with human colorectal tumour progression (Brabletz *et al.*, 2001; Kirchner and Brabletz, 2000) and is predominantly accumulated in cells at the invasive front (Brabletz *et al.*, 1998; Jung *et al.*, 2001). Nuclear β-catenin is therefore also inversely linked with both differentiation status and E-cadherin expression in colorectal tumours, and has been strongly implicated in EMT-associated changes both *in vivo* (Brabletz *et al.*, 2001) and *in vitro* (Conacci-Sorrell *et al.*, 2003). In CRCs, it is believed that aberrant Wnt activation undergoes yet a further transient and reversible increase to induce EMT at the invasive front, reducing again in established secondary tumours (Brabletz *et al.*, 2002) and has been shown to functionally induce EMT in CRC cells *in vitro* (Kim *et al.*, 2002a).

5.1.4 NOTCH SIGNALLING IS A POTENT INDUCER OF EMT

As discussed previously, Notch signalling is known to influence a diverse array of cellular processes in both embryonic and adult tissues. During mammalian embryogenesis, it is known that altered cellular adhesion and EMT are common features of processes in which Notch participates (Grego-Bessa *et al.*, 2004; Hay, 2005), as demonstrated by neural crest formation (Endo *et al.*, 2002), somitogenesis (Pourquie, 2000) and angiogenesis (Gridley, 2001). Notch has therefore been found to be a potent inducer of EMT in embryogenesis and this has been most well defined in the developing heart endocardium. This tissue is highly dependent on endothelial-mesenchymal transitions, a specialised form of EMT, for the proper formation of cardiac septa and valve structures (Eisenberg and Markwald, 1995). Various Notch ligands and receptors are expressed in the developing endocardium *in vivo* and pathway activation has been shown to functionally induce EMT in this tissue (Fischer *et al.*, 2007; Noseda *et al.*, 2004b; Timmerman *et*

al., 2004). Similarly, Notch activation has also been shown to induce changes consistent with mesenchymal trans-differentiation in vascular and cardiac endothelial cells *in vitro* (Niessen *et al.*, 2008; Noseda *et al.*, 2004b; Timmerman *et al.*, 2004). In these tissues, this occurs through the induction of Slug and Snail, leading to decreased expression of endothelial VE-cadherin and increased expression of mesenchymal markers, such as α-SMA. Moreover, *Slug* and *α-SMA* were also shown to be direct transcriptional targets of active Notch signalling and were found to contain numerous CSL putative binding sites in their promoter regions (Niessen *et al.*, 2008; Noseda *et al.*, 2006).

In a similar fashion, TGFβ related pathways are also known to play an essential role during proper endocardium formation (Nakajima *et al.*, 2000) and have been shown to synergise with Notch during EMT in the endothelium (Niessen *et al.*, 2008). Notch components have been shown to be up-regulated during TGF-β-mediated EMT induction in a range of tissues and Notch inhibition is sufficient to block this effect (Zavadil *et al.*, 2001; Zavadil *et al.*, 2004). Wnt signalling is also an essential feature of endocardial EMT (Liebner *et al.*, 2004) and multiple nodes of potential synergy are now known to exist between the Notch and Wnt signalling pathways in a variety of contexts (*SECTION 4.1.1, p125*). It is clear, therefore, that Notch, Wnt and TGF-β all have a central role in the induction of EMT in the embryo and it is now known that this role for both TGF-β and Wnt persists in the adult to promote tumour malignancy. A predictable invasion promoting role for Notch signalling, however, has continued to remain much less well defined.

Importantly, the expression of both N1 and Jag1 has been shown to strongly correlate with the poorly differentiated phenotype and a poorer patient prognosis in human breast cancer (Parr *et al.*, 2004; Reedijk *et al.*, 2005). Furthermore, mammary-specific over-expression of constitutively active forms of Notch receptors is also known to result in the formation of aggressive and metastatic breast tumours in mice (Gallahan *et al.*, 1996; Hu *et al.*, 2006). Reduced E-cadherin and Slug expression are associated with a poorer patient prognosis in these tissues (Come *et al.*, 2004; Martin *et al.*, 2005; Vincent-Salomon and Thiery, 2003), and *Slug* has been shown to up-regulated in *N1* and *Jag1* positive breast cancers (Leong *et al.*, 2007). Slug was also confirmed as a direct transcriptional target of Notch in this setting and pathway activation was shown to functionally induce EMT-like changes, leading to a motile and invasive phenotype (Leong *et al.*, 2007). Conversely, inhibition of Notch signalling results in reduced levels of *Slug*, increased E-cadherin (*CDH1*) and inhibition of growth and metastatic potential (Leong *et al.*, 2007).

Taken together these results strongly suggest that the negative regulation of the cadherin family of proteins is a central feature of the Notch signalling pathway, which is essential during embryonic development and becomes aberrantly re-activated during malignant tumour progression. Evidence for similar mechanisms in the intestinal epithelium is so far limited; however analysis in *Drosophila* has recently shown that the negative regulation of E-cadherin by active Notch signalling may be an integral and highly conserved feature of pathway activation in this tissue (Maeda *et al.*, 2008). During stem and progenitor cell segregation, E-cadherin-mediated cell-cell adhesion is essential to prolong cellular interaction long enough for Notch activation to occur, after which events downstream of Notch decrease the expression of E-cadherin and induce cellular separation. Although this remains to be tested in vertebrate models, it was shown previously in this study that increased N1 expression is strongly correlates with malignant tumour progression in the human colorectal epithelium (*SECTION 3.2.5, p103*). Given that N1 has now been shown to be a major contributory factor in the malignant progression of tumours from other tissues (Leong *et al.*, 2007), these results strongly suggest that active N1 signalling has a central role in the induction of EMT and tumour de-differentiation in the human colorectal tumours; however, despite an essential need to further understand the molecular mechanisms behind colorectal tumour progression, the potential role for Notch in this process has continued to remain untested.

5.1.5 HYPOTHESIS AND AIMS

The hypothesis guiding this chapter is that N1 is increased during CRC de-differentiation and EMT induction, where activated N1 is responsible for the functional modulation of EMT associated factors and down-regulation of E-cadherin expression.

The specific aims of this chapter were therefore;

1. To assess potential changes in N1 expression during de-differentiation and EMT-like changes in CRC cells and to determine a potential inverse relationship between endogenous N1 and E-cadherin expression in these cells.
2. To determine the effect of exogenous Notch pathway modulation on the expression of E-cadherin and other EMT associated factors.
3. To determine the effect of exogenous Notch activation and inhibition on the migratory potential of CRC cells.

5.2 RESULTS

5.2.1 NOTCH1 IS UP-REGULATED IN DE-DIFFERENTIATED CRC CELLS

It has previously been shown that loss of the epithelial cell phenotype and induction of EMT in CRC can be modelled *in vitro* using the SW480 and HCT116 adenocarcinoma cell lines (Brabletz *et al.*, 2001; Conacci-Sorrell *et al.*, 2003). Sparse cultures of both cell lines mimic cells undergoing EMT at the invasive front and demonstrate both nuclear β-catenin and low level E-cadherin expression. Conversely, cells seeded at higher density increasingly adopt a more epithelial phenotype and demonstrate characteristics reminiscent of MET, with β-catenin co-localising with high levels of E-cadherin at the cell membrane. These cell lines therefore provide an excellent *in vitro* model of spontaneous CRC de-differentiation and EMT induction, without requiring any treatment with additional exogenous growth factors.

As a prelude to functional analysis, this established model was used to investigate potential changes in the expression of endogenous N1 protein during loss of the epithelial cell phenotype and induction of EMT in CRC cells. The Notch pathway is well known for its dependency on cell-cell contact and in the previous chapter it was shown that N1 protein expression is dramatically reduced in colorectal tumour cell lines, including SW480 and HCT116, on reaching a state of total confluency (*SECTION 4.2.13, p170*). This result therefore further suggested a role for N1 in colorectal tumour de-differentiation; however, it was not entirely clear whether this resulted from increased cellular confluency or increasing time in culture. Therefore, consistent with previous studies (Conacci-Sorrell *et al.*, 2003), both the SW480 and HCT116 cell lines were seeded either sparsely (1 or 2 x 10^4 cells.cm^{-2}), moderately (4 or 8 x 10^4 cells.cm^{-2}) or densely (16 or 24 x 10^4 cells.cm^{-2}) and grown under standard conditions for 48 hours. The degree of confluency was assessed in both cell lines using phase contrast microscopy (*FIGURE 5-2A*) and only cells seeded densely became fully confluent within this time. Increasing adoption of the epithelial cell phenotype with confluency in these cells was confirmed by western blotting analysis of E-cadherin protein expression (*FIGURE 5-2B*) (*SECTION 2.5, p65*) and, in agreement with previous studies (Conacci-Sorrell *et al.*, 2003), changes in E-cadherin expression were found to be less dramatic in HCT116 cells compared to SW480. This was due to relatively higher levels of endogenous protein present in sparse culture. Furthermore, in direct support of a role for N1 during the de-differentiation of CRC cells, it was found that the N1 protein was inversely expressed to that of E-cadherin and was most highly expressed in sparsely seeded cells associated with a more mesenchymal and invasive cell phenotype (Conacci-Sorrell *et al.*, 2003).

In order to compare these changes in expression and sub-cellular localisation of both proteins, SW480 and HCT116 cells were seeded on glass cover slips, either sparsely (2 x 10^4 cells.cm^{-2}) or densely (16 x 10^4 cells.cm^{-2}) and grown for 48 hours under normal growth conditions. Fixed cells at both densities were fluorescently immunolabelled for endogenous N1 and E-cadherin expression (*SECTION 2.6, p68*). In line with previous studies (Conacci-Sorrell *et al.*, 2003), sparse cultures of the SW480 cell line were associated with low, mostly perinuclear E-cadherin expression, while dense cultures demonstrated increased levels of membranous protein, confirming transition to a more epithelial phenotype (*FIGURE 5-3*). Similar changes were also observed in the HCT116 cell line, although more endogenous E-cadherin remained present in sparse culture and the transition was much less pronounced (*FIGURE 5-4*). In contrast, N1 protein expression was readily detectable in sparse culture of both cell lines but was undetectable in densely cultured cells, further confirming that N1 expression is decreased in CRC cells following adoption of a more epithelial phenotype.

The HT29 colorectal adenocarcinoma cell line is known to be well differentiated (Fogh *et al.*, 1977) and was used here as a positive control for the epithelial cell phenotype and E-cadherin expression. Even in sparse culture, strong membranous E-cadherin expression was observed in the HT29 cell line (*FIGURE 5-5*). Furthermore, HT29 cells were previously shown to demonstrate the lowest levels of endogenous N1 protein expression of any of the adenocarcinoma cell line tested at 80% confluency (*SECTION 3.2.7, p111*) and N1 protein remained only marginally detectable by this technique, even in sparse culture of these cells. Taken together these results strongly suggest that N1 protein expression inversely correlates with that of E-cadherin in these *in vitro* cell lines and is most highly expressed in cells mimicking de-differentiation and EMT at the invasive front of malignant colorectal tumours.

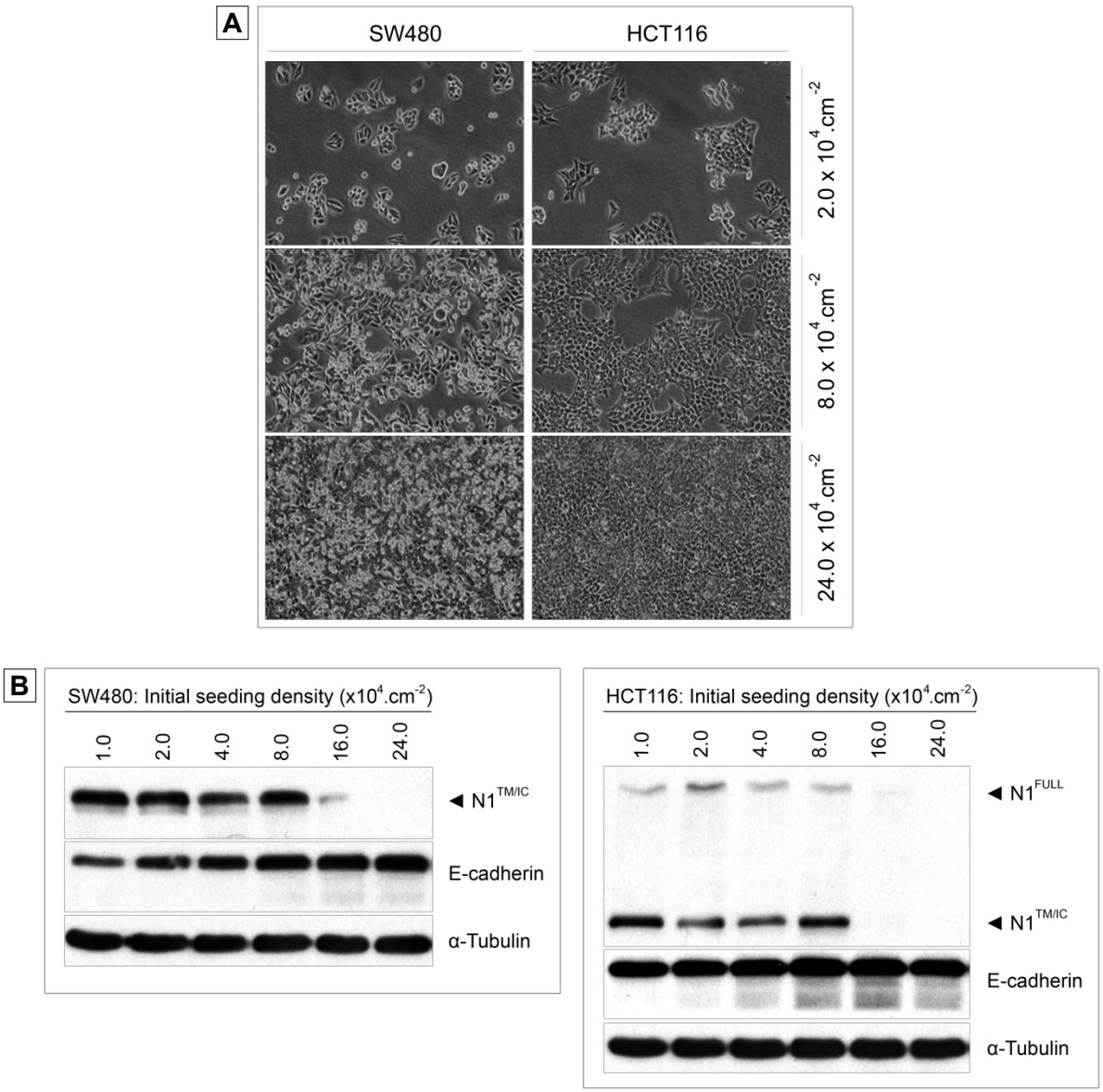


Figure 5-2| ***Notch1 protein expression is greatest in cells mimicking the invasive front of colorectal tumours.*** Phase contrast imaging [**A**] and western blotting analysis [**B**] of SW480 and HCT116 cells seeded SPARSELY (1 or 2 x 10^4 cells.cm^{-2}), MODERATELY (4 or 8 x 10^4 cells.cm^{-2}) or DENSELY (16 or 24 x 10^4 cells.cm^{-2}) and grown for 48 hours under normal growth conditions. Cell lysates were assessed for both Notch1 and E-cadherin protein expression and α-Tubulin is included as a loading control. These data are representative of three separate experiments. *(Magnification x10)*

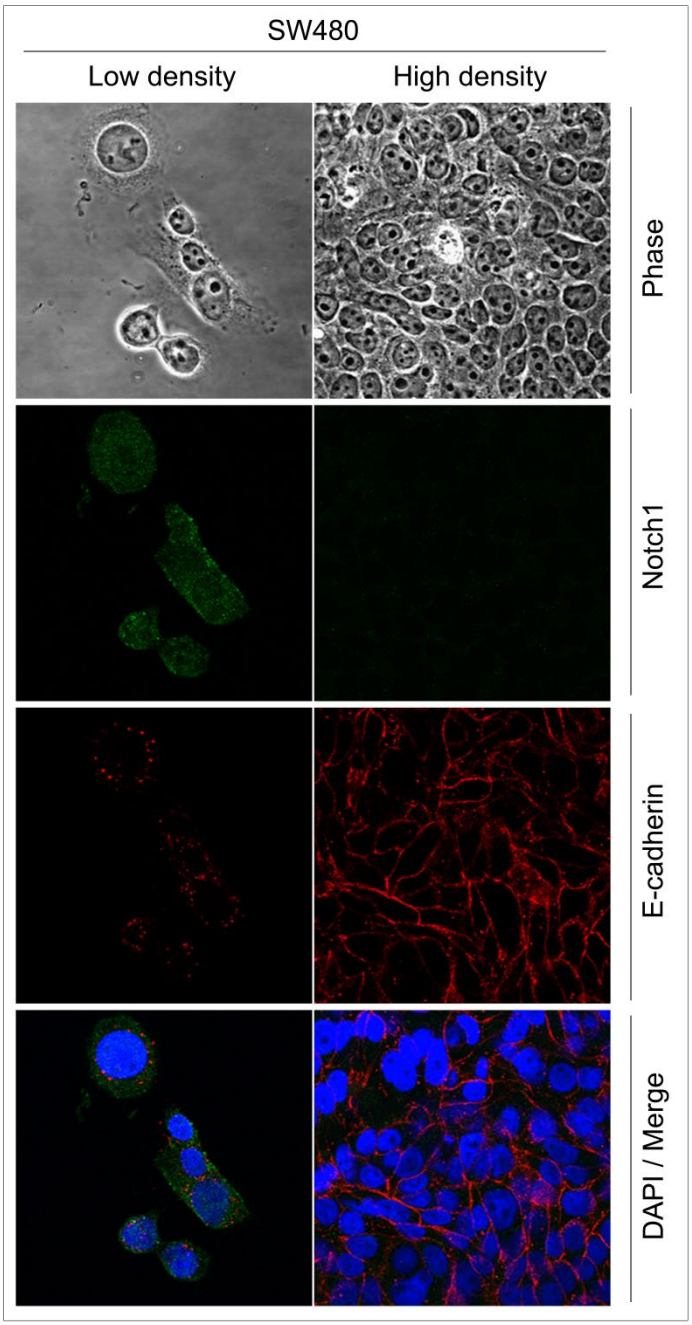


Figure 5-3| ***Notch1 expression is reduced in densely seeded SW480 cells adopting the epithelial phenotype.*** Immunocytochemical analysis of endogenous Notch1 and E-cadherin expression in SW480 cells seeded SPARSELY (2 x 10^4 cells.cm^{-2}) or DENSELY (16 x 10^4 cells.cm^{-2}). Cells were fluorescently dual-labelled for endogenous Notch1 (*green*) and E-cadherin (*red*) protein expression. Analysis was performed by confocal microscopy and detail of cell structure was provided by blue DAPI nuclear counter-stain plus phase contrast imaging. *(Magnification x40)*

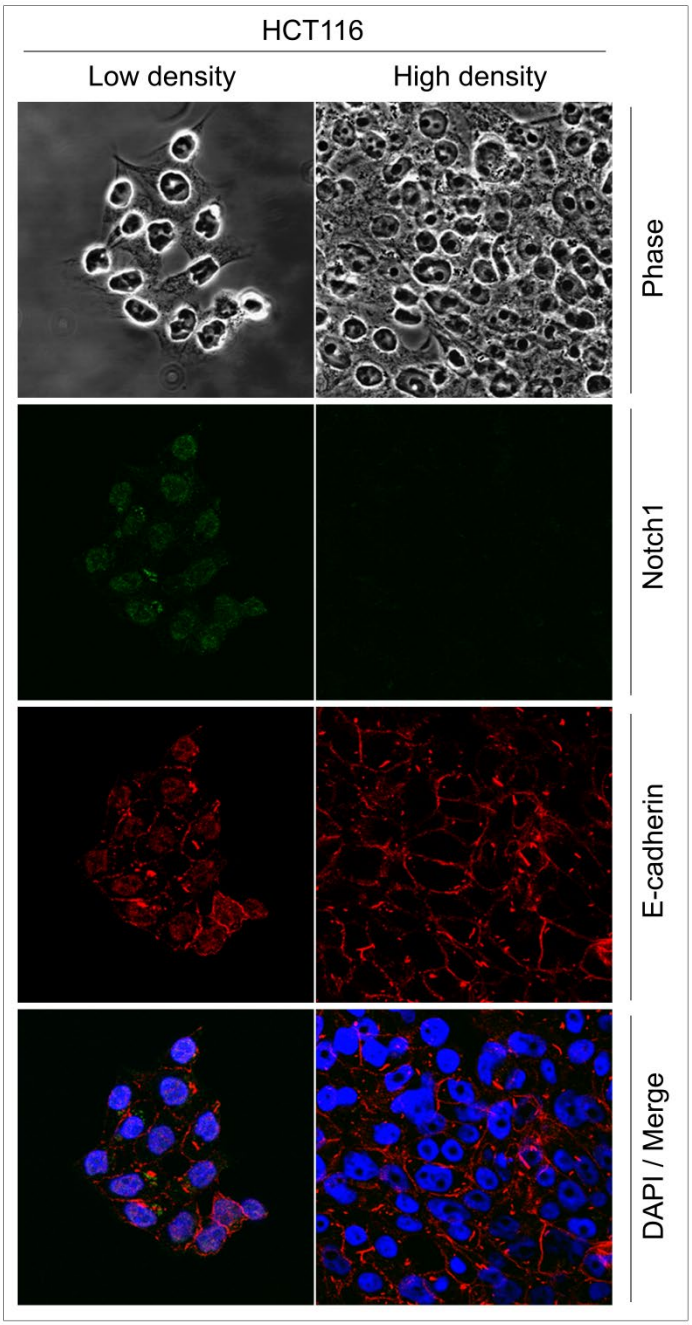


Figure 5-4| *Notch1 expression is reduced in densely seeded HCT116 cells adopting the epithelial phenotype.* Immunocytochemical analysis of endogenous Notch1 and E-cadherin expression in HCT116 cells seeded SPARSELY (2 x 10^4 cells.cm^{-2}) or DENSELY (16 x 10^4 cells.cm^{-2}). Cells were fluorescently dual-labelled for endogenous Notch1 (*green*) and E-cadherin (*red*) protein expression. Analysis was performed by confocal microscopy and detail of cell structure was provided by blue DAPI nuclear counter-stain plus phase contrast imaging. *(Magnification x40)*

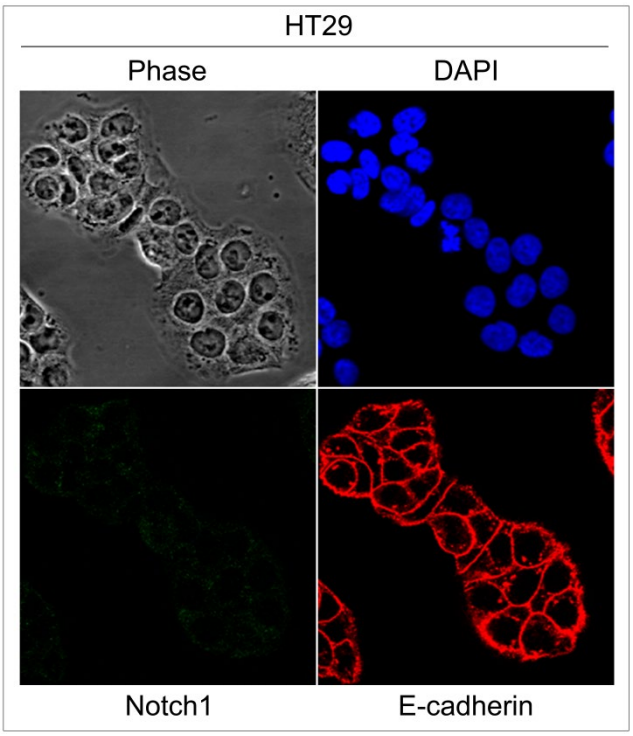


Figure 5-5| *Notch1 expression is undetectable in the epithelial-type positive control cell line HT29.* Immunocytochemical analysis of endogenous Notch1 and E-cadherin expression in HT29 cells (4 x 10^4 cells.cm^{-2}). Cells were fluorescently dual-labelled for endogenous Notch1 (*green*) and E-cadherin (*red*). Analysis was performed by confocal microscopy and detail of cell structure was provided by blue DAPI nuclear counter-stain, plus phase contrast imaging. *(Magnification x40)*

5.2.2 NOTCH1 ACTIVATION ALTERS COLORECTAL CANCER CELL MORPHOLOGY

It has previously been shown that a raised/rounded cellular morphology in other tissues is indicative of cells in a partial or transitional EMT state (Fischer *et al.*, 2007) and in CRC cells is closely associated with the disruption of functional adherens junctions (Watabe-Uchida *et al.*, 1998). The SW480 cell line has further been proposed as a model of tumour cell invasion on the basis of two distinct (but potentially interchangeable) sub-populations, being adherent (SW480-ADH) or rounded (SW480-R) (Palmer *et al.*, 2001; Tomita *et al.*, 1992). Of these, the SW480-R is believed to possess a greater malignant potential and is associated with both reduced E-cadherin expression and increased tumorigenicity when implanted in mice. Interestingly, in addition to the correlative evidence described above, it was shown here that forced expression of active intracellular N1 protein ($N1^{IC}$) (*SECTION 2.3, p61*) in SW480 cells consistently resulted in adoption of a notably lifted and rounded cell morphology (*FIGURE 5-6*). This was highly reminiscent of the SW480-R sub-population and further suggested that N1 activation may induce changes associated with EMT and increased malignant potential in colorectal cancer cells.

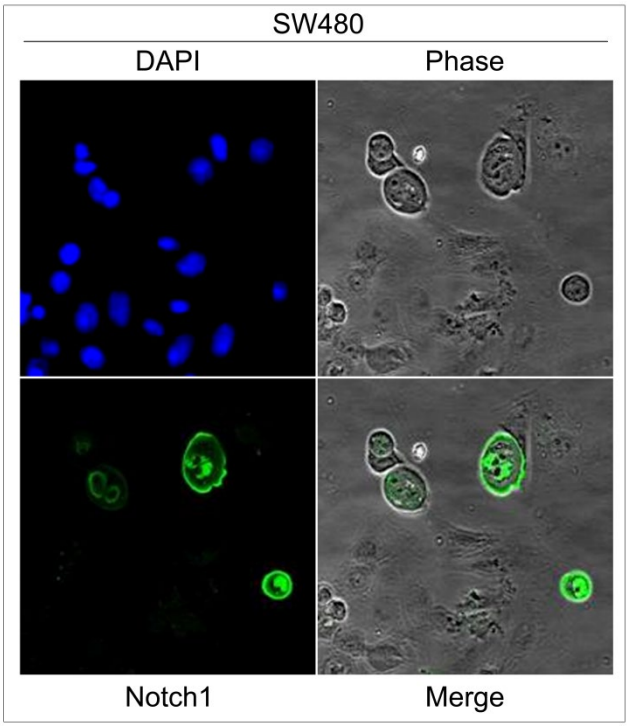


Figure 5-6| ***Exogenous Notch1 activation in SW480 cells results in adoption of a rounded cell phenotype.*** Immunocytochemical analysis of SW480 cells following 24h transient transfection with plasmid encoding active Notch1 ($N1^{IC}$). Cells were fluorescently labelled for N1 (*green*) and cell nuclei were highlighted using blue DAPI counter-stain. Analysis was performed by confocal microscopy and cell morphology was determined by corresponding phase contrast imaging. *(Magnification x 40)*

5.2.3 ACTIVATED NOTCH1 DOES NOT ALTER E-CADHERIN EXPRESSION IN CRC CELLS

In order to investigate a functional contribution for N1 signalling in de-differentiation and EMT induction of CRC cells, both the transcriptional activity and protein expression of E-cadherin (*CDH1*) were evaluated in SW480 and HCT116 cells following N1 activation. Crucially, both cell lines undergo spontaneous changes reminiscent of EMT in culture (*SECTION 5.2.1, p195*) and are highly amenable to exogenous N1 modulation (*SECTION 4.2.1, p132, SECTION 4.2.6, p148*). Cells were transiently co-transfected with either an expression plasmid encoding $N1^{IC}$ or empty vector control, plus a *CDH1* (Batlle *et al.*, 2000) or *Hes1* promoter reporter (Nishimura *et al.*, 1998) (*SECTION 2.3, p61*). N1 activation was confirmed by a significant increase in transcriptional activity of *Hes1* in both cell lines, compared to control (*FIGURE 5-7A,B*). In agreement with a functional role for N1 in suppressing E-cadherin in CRC, *CDH1* transcriptional activity was shown to significantly decrease in the presence of $N1^{IC}$ in the SW480 cell line, although no significant change was observed in HCT116. This analysis was also performed at 48 hours post-transfection and matching results were also observed at this time (*data not shown*). To investigate potential changes in expression at the protein level, corresponding cell lysates were analysed by western blotting and pathway activation confirmed by strong expression of the $N1^{IC}$ isoform in both cell lines. However, no change in E-cadherin protein expression was discernable by this technique following N1 activation in either SW480 or HCT116 cells (*FIGURE 5-7C*).

The closely related zinc-finger transcription factors Snail (*Snail1*) and Slug (*Snail2*) are both known to be key mediators of E-cadherin transcriptional repression (Nieto, 2002) and are up-regulated during Notch-induced EMT in other tissues (Leong *et al.*, 2007; Timmerman *et al.*, 2004). Twist1 is also known to promote tumour progression by down-regulating E-cadherin (Kang and Massague, 2004; Karreth and Tuveson, 2004) and has previously been shown to be up-regulated in CRC (Valdes-Mora *et al.*, 2009). Consistent with previous findings, Slug but not Snail was found to be prominently expressed in these colorectal cancer cell lines (Conacci-Sorrell *et al.*, 2003), in which Twist was also found to be strongly expressed. In agreement with findings for E-cadherin, the protein levels of Slug, Snail and Twist were also found to remain largely unchanged following N1 activation (*FIGURE 5-7C*), further suggesting that N1 does not induce EMT in these CRC cells.

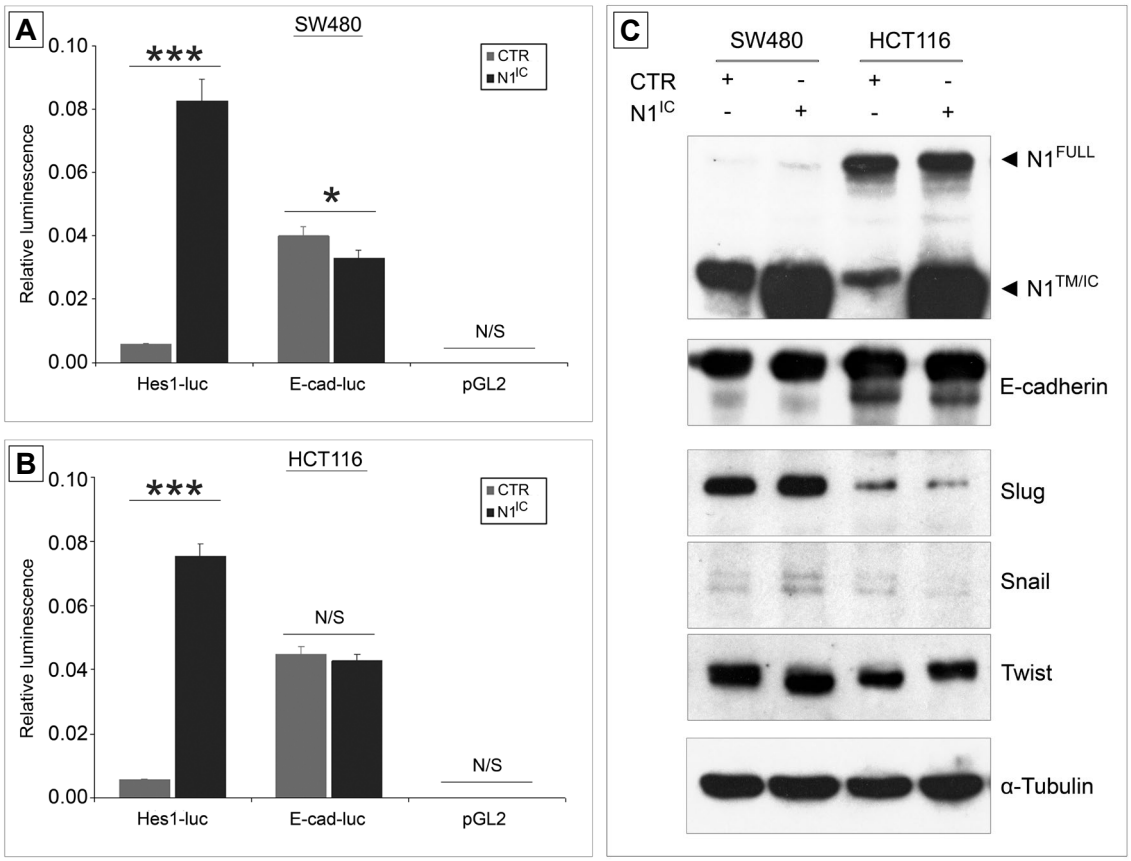


Figure 5-7| *Notch1 activation does not induce EMT in colorectal cancer cells.* Analysis of *CDH1* transcriptional activity in the SW480 [**A**] and HCT116 [**B**] colorectal cancer cell lines following exogenous N1 activation. Both cell lines were transiently co-transfected for 24 hours with expression plasmid encoding $N1^{IC}$ or empty vector control, plus *CDH1* or *Hes1* promoter reporter constructs. N1 activation was confirmed by increased *Hes1* promoter activity and the empty pGL2 plasmid was included as a vector control. Values are normalised to *Renilla* control and average values determined from three separate experiments performed in triplicate. (*Statistical analysis was performed using the Student's t-test, where degrees of significance are stated as *: p<0.05, **: p<0.01, ***: p<0.001 and error bars represent SEM*). [**C**] Western blotting analysis of E-cadherin and EMT associated transcriptional repressors Slug, Snail and Twist following N1 activation. Both the SW480 and HCT116 cell lines were transiently transfected with plasmid encoding $N1^{IC}$, vs. empty vector control, for 48 hours prior to analysis. α-Tubulin is included as a loading control and data are representative of three separate experiments.

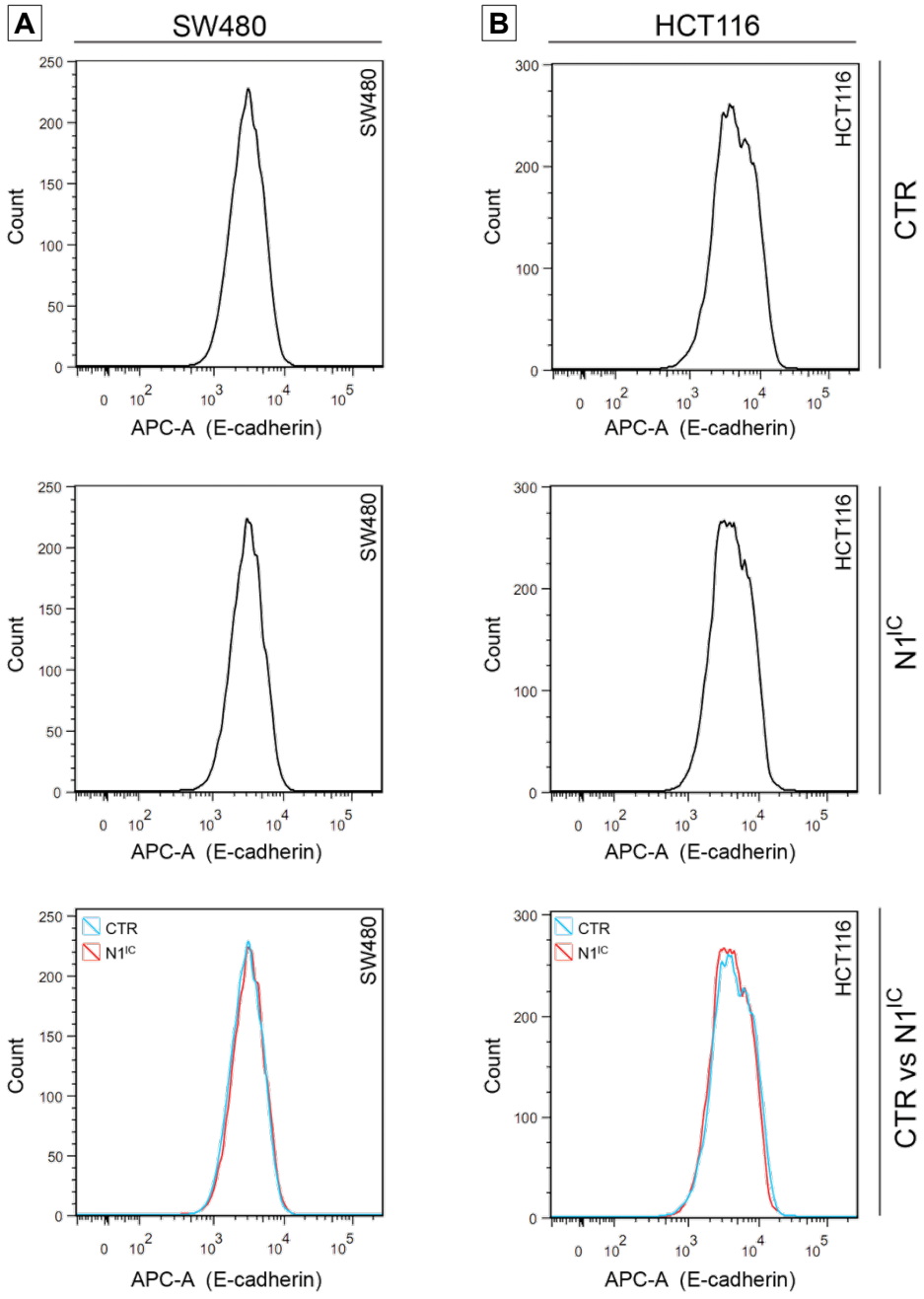


Figure 5-8| *Notch1 activation has no affect on the E-cadherin expression profile of CRC cells.* Flow cytometric analysis of E-cadherin protein expression in the SW480 [**A**] and HCT116 [**B**] cell lines following exogenous N1 activation. Both cell lines were transiently co-transfected for 48 hours with expression plasmid encoding $N1^{IC}$ or empty vector control and immunofluorescently labelled for E-cadherin (*Alexaluor647 - APC-A*). Cells were analysed by flow cytometry and expression profiles were determined using BD FACSDiva (*BD Biosciences, UK*) and FlowJo (*Tree Star, USA*) analysis software. Data are representative of three separate experiments.

In addition to western blotting analysis, transiently transfected cells were immunofluorescently labelled for E-cadherin protein expression and analysed by flow cytometry (*SECTION 2.7, p69*), enabling subtle changes in E-cadherin expression profile or the formation of cellular sub-populations to be detected. However, consistent with western blotting analysis, a highly overlapping distribution of E-cadherin expression was observed between N1 and control transfected cells in both cell lines (*FIGURE 5-8*). Taken together these results suggest that, although endogenous N1 expression inversely correlates with that of E-cadherin in CRC cells, increased N1 activation does not functionally down-regulate E-cadherin expression in these cells.

5.2.4 THE DOWN-MODULATION OF NOTCH DOES NOT ALTER E-CADHERIN EXPRESSION

Although increased N1 activation was found not to modulate E-cadherin expression in either SW480 or HCT116 cells, it was also important to consider that both cell lines express readily detectable levels of endogenous N1 and may be insensitive to further increases in N1 activation. Therefore, the effect of Notch inhibition was also assessed in these cells. Importantly, Presinilin-1 (PS-1) of the γ-secretase complex has previously been shown to have fundamental effects on the stability of both E-cadherin (Marambaud *et al.*, 2002) and β-catenin (Zhang *et al.*, 1998), two major determinants of CRC morphogenesis and EMT (Conacci-Sorrell *et al.*, 2003). For this reason, N1-specific and pan-Notch inhibition was instead achieved by siRNA-mediated knockdown of *N1* or *CSL* (*Dharmacon*) (*SECTION 2.3.3, p62*). Scrambled negative siRNA (*Dharmacon*) and mock transfectants were also included as controls. The HCT116 cell line was selected both for its comparatively low endogenous N1 expression and its amenability to siRNA treatment.

Successful protein knockdown was confirmed by western blotting analysis of N1 protein expression (*FIGURE 5-9*) and *Hes1* promoter activity (*concurrent to analysis in SECTION 4.2.4, p141*). However, in further agreement with analysis following pathway activation, no change in the expression of E-cadherin or of the transcriptional repressors Slug, Snail or Twist was detectable following treatment with siRNA (*FIGURE 5-9*). Accordingly, no change in the expression profile of E-cadherin was observed following analysis by flow cytometry either (*FIGURE 5-10*).

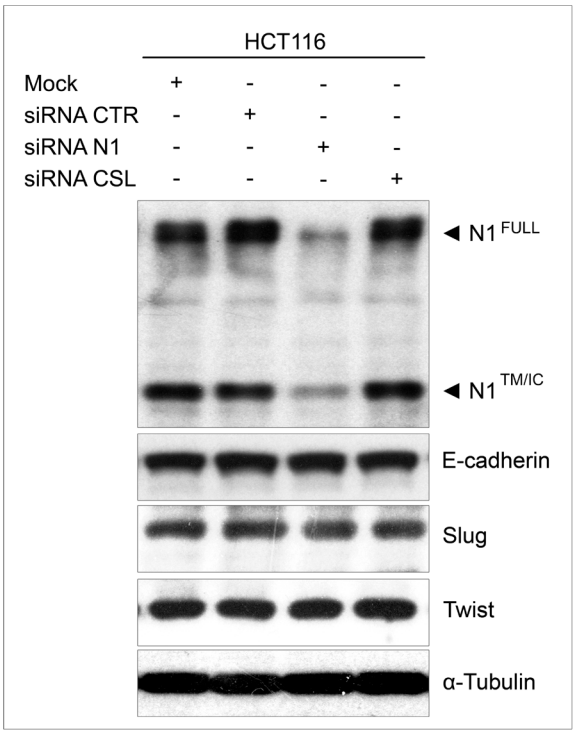


Figure 5-9| *Notch down-modulation does not increase expression of the E-cadherin protein.* Western blotting analysis of E-cadherin protein expression following N1 or pan-Notch inhibition in HCT116 cells. Cells were transiently transfected for 72 hours with siRNA sequences targeting either *N1* or *CSL* (*Dharmacon*) vs. scrambled negative control. Lysates were probed for N1 to confirm transfection efficacy. Expression of E-cadherin and the EMT-associated transcriptional repressors Slug, Snail and Twist were also assessed. These data are representative of three separate experiments and α-Tubulin is included as a loading control.

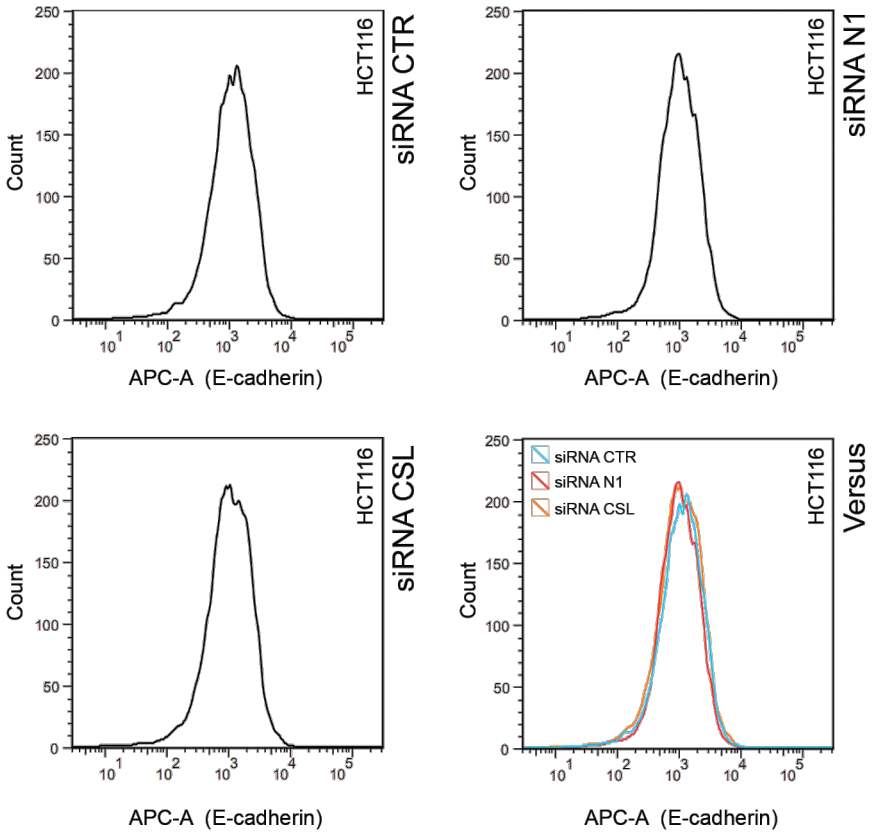


Figure 5-10| ***Notch down-modulation has no effect on the E-cadherin expression profile of CRC cells.*** Flow cytometric analysis of E-cadherin protein expression following N1 or pan-Notch inhibition in HCT116 cells. Cells were transiently transfected for 72 hours with siRNA sequences targeting either *N1* or *CSL* (*Dharmacon*) vs. scrambled negative control and immunofluorescently labelled for E-cadherin expression (Alexafluor647; *APC-A*). Cells were analysed by flow cytometry and expression profiles were determined using BD FACSDiva (*BD Biosciences, UK*) and FlowJo (*Tree Star, USA*) analysis software. These data are representative of three separate experiments.

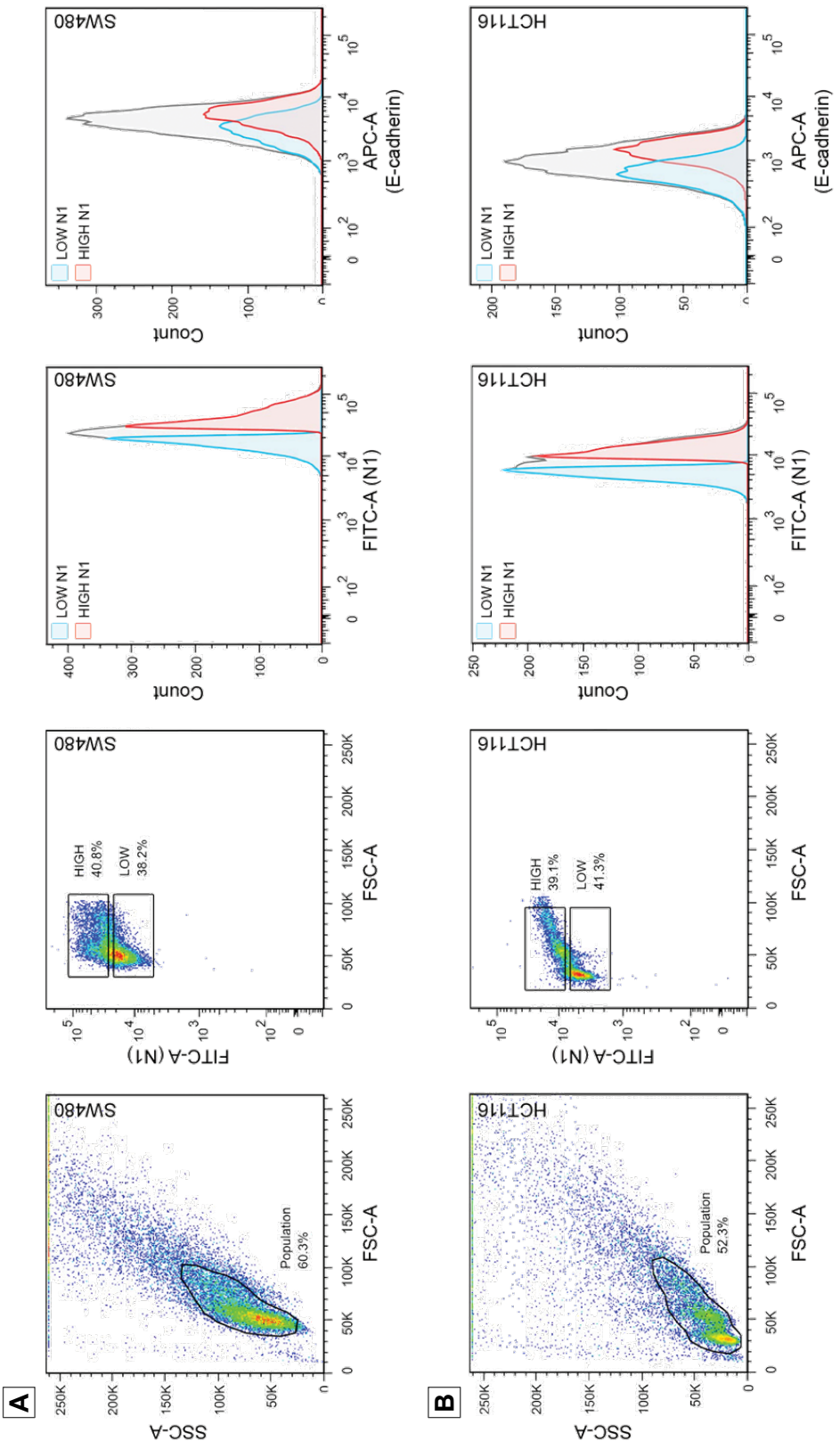
5.2.5 ENDOGENOUS E-CADHERIN AND NOTCH1 ARE NOT INVERSELY EXPRESSED IN CRC

Both the SW480 and HCT116 cell lines are known to contain heterogeneous populations of cells and both undergo spontaneous changes consistent with EMT induction (Conacci-Sorrell *et al.*, 2003). This suggests that a range of tumour differentiation states exist within these cell lines at any one time. Untreated SW480 and HCT116 cells were, therefore, immunofluorescently dual labelled for the expression of both N1 and E-cadherin proteins prior to analysis by flow cytometry (*SECTION 2.7.1, p69*). This enables direct cell by cell comparison to be made between the endogenous expressions of these proteins. Non-apoptotic cells were gated on the basis of either low (*bottom 40%*) or high (t*op 40%*) N1 protein comparison was made with E-cadherin. In both SW480 and HCT116 cells it was found that high N1 expressing cells do not demonstrate low levels of E-cadherin expression, or *vice versa* (*FIGURE 5-11A-B*). N1 and E-cadherin instead appear to be directly, rather than inversely, related in these cells (*TABLE 5-1*), although this likely relates to changes in cell surface area rather than any functional correlation between these proteins. These results therefore support functional analysis described above and further suggests that N1 does not down-regulate E-cadherin expression in these CRC cells.

CELL POPULATION (Based on Notch1)		NOTCH1 (Fluorescence)		E-CADHERIN (Fluorescence)	
		mean	% difference	mean	% difference
SW480	Total	*25802*	---	*3701*	---
	Low 40%	*15197*	↓ *219%*	*2810*	↓ *153%*
	High 40%	*33227*		*4295*	
HCT116	Total	*18380*	---	*2937*	---
	Low 40%	*9647*	↓ *243%*	*1941*	↓ *176%*
	High 40%	*23490*		*3422*	

Table 5-1| ***Direct comparison between Notch1 and E-cadherin expression by immunofluoresence.*** Untreated SW480 and HCT116 cells were immunofluorescently labelled for endogenous Notch1 (N1) and E-cadherin protein expression prior to analysis by flow cytometry. Cells were gated on the basis of high (40%) or low (40%) N1 expression to enable comparison with endogenous E-cadherin. Values represent average fluorescence values from three independent experiments. Analysis of percentage difference between the low and high N1 expressing populations demonstrates a direct relationship between N1 and E-cadherin.

Figure 5-11| ***High endogenous Notch1 expression does not correlate with low levels of E-cadherin.*** Flow cytometric analysis of untreated SW480 [**A**] and HCT116 [**B**] cells immunofluorescently dual-labelled for endogenous Notch1 (N1) and E-cadherin expression. Non-apoptotic cells were gated on the basis of either 'low 40%' (*blue*) or 'high 40%' (*red*) basal N1 expression (Alexafluor488*; FITC-A*) for direct comparison with E-cadherin (Alexafluor647*; APC-A*). Expression profiles were determined using BD FACSDiva (*BD Biosciences, UK*) and FlowJo (*Tree Star, USA*) analysis software. These data are representative of three separate experiments. ▶


A
SW480
SSC-A
FSC-A
Population
60.3%
FITC-A (N1)
HIGH
40.8%
LOW
38.2%
Count
LOW N1
HIGH N1
APC-A
(E-cadherin)
B
HCT116
Population
52.3%
HIGH
39.1%
LOW
41.3%

5.2.6 NOTCH ACTIVATION DECREASES THE MOTILITY OF HCT116 CELLS

Acquisition of a motile phenotype is a hallmark of colorectal tumour progression and is a pre-requisite to tumour cell invasion. Although decreased E-cadherin expression and EMT-like changes have been strongly implicated in CRC invasion (Brabletz *et al.*, 2005a), EMT in carcinogenesis remains controversial and a number of E-cadherin/EMT-independent mechanisms of tumour cell migration and invasion have been proposed (Tarin *et al.*, 2005; Wicki *et al.*, 2006). The motility of *in vitro* cell lines from various tissues, including colorectal cancer cells, is classically analysed using Boyden-chamber transwell filter migration assays (Efstathiou *et al.*, 1999). In order to study the effect of positive Notch modulation on CRC cell motility, HCT116 cells were transiently transfected with expression plasmids encoding either $N1^{IC}$ or constitutively active CSL (caCSL), for comparison with empty vector and un-transfected controls. 24 hours post-transfection, cells were prepared and loaded into Boyden-chamber transwell filters (*SECTION 2.8, p70*). Cells were incubated for a further 24 hours (*total of 48 hours post-transfection*) to allow cell migration through the collagen-coated filters towards a serum attractant. Analysis was also performed using HCT116 cells pre-treated for 48 hours with siRNA targeting *N1* or *CSL*, vs. scrambled and mock controls (*total of 72 hours post-transfection*). The degree of migration was measured as the average number of cells present on the lower filter surface in 10 fields of view (*x20 magnification*). Surprisingly, although siRNA-mediated inhibition of *N1* or *CSL* had no significant effect on HCT116 migration, both N1 and pan-Notch activation resulted in a significant decrease in cell migration across the transwell filter compared to empty vector control, suggesting that increased Notch activation inhibits rather than promotes CRC cell migration.

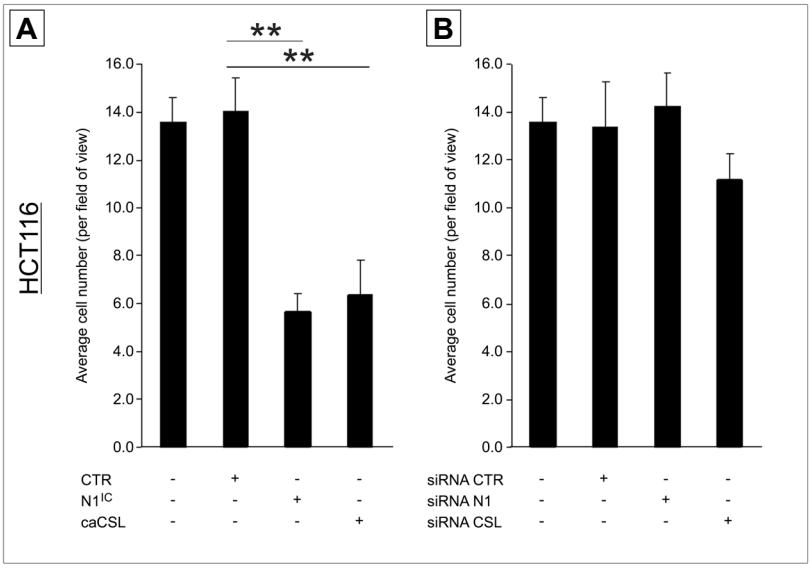


Figure 5-12| *Notch activation has an anti-migratory effect on HCT116 cells.* Analysis of HCT116 cell motility following N1 and pan-Notch modulation. Analysis was performed using the Boyden chamber transwell-filter migration assay system. For positive modulation, cells were transiently transfected with either activated N1 ($N1^{IC}$) or constitutively active CSL (caCSL), vs. empty vector or untransfected controls. After 24 hours, cells were loaded into the transwell filters and incubated for a further 24 hours. The degree of migration was measured as the average number of haematoxylin-stained cells on the lower filter surface in 10 fields of view (*x20 magnification*) [**A**]. For negative modulation, cells were instead transiently transfected with siRNA sequences targeting either *N1* or *CSL* (*Dharmacon*), vs. scrambled negative siRNA control or mock transfectants for 48 hours prior to analysis [**B**]. (*Statistical analysis was performed using the Student's t-test, where degrees of significance are stated as *: p<0.05, **: p<0.01, ***: p<0.001 and error bars represent SD*)

5.3 DISCUSSION

5.3.1 A CONTEXT-DEPENDENT BASIS FOR NOTCH-MEDIATED INVASIVE GROWTH

This study has shown that expression of the N1 protein is significantly increased between colorectal adenoma and adenocarcinoma tissues, both *in vivo* and *in vitro* (*SECTION 3.2.5, p103 – SECTION 3.2.7, p111*), suggesting a fundamental role for N1 in the malignant progression of colorectal tumours. Increasing evidence from other tissues strongly suggests that active Notch signalling is a potent inducer of EMT and invasion in both developmental and malignant contexts, although this had not previously been tested in CRC cells. The key aim of this chapter was therefore to determine possible changes in expression and/or the functional contribution of N1 signalling during EMT and loss of the epithelial cell phenotype in the CRC setting.

Several pieces of preliminary evidence from the present study strongly support a hypothesis that N1 suppresses E-cadherin expression and the differentiated tumour phenotype. Firstly, using an *in vitro* model of EMT induction in CRC cells, it was shown that N1 expression is converse to that of E-cadherin, being most highly expressed in cells mimicking the mesenchymal phenotype of cells at the invasive front (*Figure 5-2* - *Figure 5-4*). Moreover, exogenous N1 activation in the SW480 cell line resulted in a morphological transition consistent with increased tumorigenicity and malignant potential (*FIGURE 5-6*). In addition to *in vivo* and *in vitro* results from a previous chapter (*SECTION 3.2.5, p103 – SECTION 3.2.7, p111*), these correlative findings further suggest that increased N1 expression may be a central feature of CRC malignancy and that N1 activation may induce morphological changes associated with increased malignant potential. In contrast, however, E-cadherin expression was found to remain unchanged in response to either positive or negative Notch modulation in both the SW480 and HCT116 CRC cell lines. Similarly, the key EMT promoting factors Slug, Snail and Twist, which are known positive targets of active N1 in other tissue contexts, were also shown not to change following Notch modulation in these cells.

Although these findings were initially surprising, they in fact add further weight to a highly context-dependent role for N1 in the colorectal epithelium and CRC cells. Therefore, these results do not discount a prominent role for Notch in the promotion of colorectal malignancy but instead disprove the hypothesis in this context. Although these functional experiments were performed under the standard (sub-confluent) experimental conditions for these cell lines, it is clear that N1, E-cadherin and the epithelial phenotype are all highly dependent on cellular confluency and Notch-induced EMT may be reliant on other culture conditions, such as cells in dense culture. Furthermore, while the cell lines used here provide an excellent *in vitro* model of CRC de-

differentiation and EMT, it should be noted that both SW480 and HCT116 cell lines, like most colorectal cell lines, are of adenocarcinoma origin. Therefore they retain the malignant properties of their parent tumours from which they derive and are both anchorage-independent and tumorigenic. It is therefore possible that Notch activation only promotes a specific or initial phase in the EMT process, or may only be effective in previously non-malignant cells.

In agreement with this proposal, the induction of EMT by Notch has largely been demonstrated in non-tumour contexts, using immortalised endothelial cells (Niessen *et al.*, 2008; Noseda *et al.*, 2004a; Timmerman *et al.*, 2004). Although one paper has previously demonstrated that Notch can induce malignant EMT-like changes in the breast epithelium (Leong *et al.*, 2007), this was shown using MCF10A, an immortalised normal breast epithelial cell line that is both anchorage-dependent and non-tumorigenic (Soule *et al.*, 1990). Similarly, N1 activation has been strongly implicated in the malignant progression of melanomas but, again, induction of the invasive phenotype was shown to be limited to primary melanocytes and N1 had little effect on already metastatic melanoma cells (Balint *et al.*, 2005). In possible explanation, N1 induction has recently been demonstrated in bladder carcinoma cells undergoing EMT following fibroblast growth factor treatment, although it was shown that N1 is immediately and transiently up-regulated at a stage preceding EMT or loss of cellular adhesion (Billottet *et al.*, 2008). This suggests that N1 is important for the earliest events in EMT induction and, therefore, may not be effective in cells already undergoing different degrees of spontaneous mesenchymal transition.

As discussed previously, following analysis of proliferation in CRC cells (*SECTION 4.3.3, p179*), the multitude of functional Notch-mediated outcomes in development are known to be highly dependent on different threshold levels of pathway activation (Guentchev and McKay, 2006). It is also possible that similar aberrant threshold levels apply to colorectal tumorigenesis and malignant progression. For example, MCF-10A cells are known to possess extremely low levels of endogenous Notch activity due to high levels of the natural Notch repressor, Numb (Sahlgren *et al.*, 2008). Both SW480 and HCT116 cell lines express readily detectable levels of endogenous N1 and are growth unresponsive to altered Notch signalling (*SECTION 3.2.7, p111, SECTION 4.2.7, p150 – SECTION 4.2.8, p154*), while the HT29 adenocarcinoma cell line represents a well differentiated CRC cell phenotype, expresses relatively lower levels of endogenous N1/Hes1 and has been shown to demonstrate a significant change in cell number in response to Notch activation or inhibition (Ghaleb *et al.*, 2008). Indirect evidence suggests that active Notch signalling may promote the malignant potential of HT29 cells, since forced expression of the downstream

negative Notch target *Atoh1* results in the suppression of anchorage-independent growth in these cells (Leow *et al.*, 2004). In the present study, transient and stable transfection of HT29 cells with expression plasmids for $N1^{FULL}$ and $N1^{IC}$ was attempted but sufficient expression could not be achieved. Similar limitations were also encountered while attempting to modulate N1 in the various pre-malignant adenoma cell lines available to this study, precluding the study of E-cadherin expression or EMT induction in these cells. Therefore, interesting future work might aim to establish stable clones of the HT29 or adenoma cell lines containing inducible expression vectors for the activation or inhibition of Notch for analysis of EMT induction.

Alternatively, a lack of Notch-induced EMT in these cell lines may also be accounted for by a number of other features, such as tissue- or pathway-specific differences. For example, it is known that N1 has an essential tumour suppressor role in the mammalian epidermis (Nicolas *et al.*, 2003) where, in contrast to promoting invasiveness, Notch activation has been shown to promote keratinocyte cohesiveness (Lowell and Watt, 2001) and increased formation of cadherin-mediated intercellular junctions (Boyer-Di Ponio *et al.*, 2007). In the present study it was shown that, reminiscent of expression analysis in the normal epidermis, N1 protein expression is predominantly localised in the upper differentiating region of the colonic crypt (*SECTION 3.2.2, p92*), suggesting that active Notch signalling may be responsible for similar functions in these tissues. This may therefore begin to explain the fact that exogenous Notch activation does not induce EMT-like changes in CRC cells (*FIGURE 5-7*) and instead, actually reduces migration of the HCT116 cell line (*FIGURE 5-12*). Given that endogenous N1 expression was shown to demonstrate a direct relationship with that of E-cadherin in both the SW480 and HCT116 cell lines, it is also possible that, in a context-dependent fashion, Notch activation may actually induce E-cadherin expression and increase cellular cohesion in these CRC cell lines.

N1 is widely accepted as the principal Notch receptor expressed in the intestinal epithelium and CRC, therefore N1 has remained the prominent focus for this and other intestinal studies. In a similar fashion, much of the evidence for Notch-mediated EMT in other tissues has also focussed around N1. However, the expression of N4 has also been shown to be a central player during EMT induction in the developing endocardium and endothelial cells (Noseda *et al.*, 2004b). In addition to $N1^{IC}$, forced expression of $N2^{IC}$ and $N4^{IC}$ also leads to the formation of aggressive and metastatic breast tumours (Gallahan *et al.*, 1996; Hu *et al.*, 2006) and both $N1^{IC}$ and $N4^{IC}$ can functionally induce EMT in breast epithelial cells in culture (Leong *et al.*, 2007). The possible involvement of other Notch receptors in CRC EMT was partially accounted for in the present

study through the inclusion of both positive and negative CSL modulation, although it still remains possible the that EMT-like events may lie downstream of Notch receptors other than N1 in these tissues.

Similarly, *Hes1* remains widely accepted as the principal Notch target gene in many tissues, including the colonic epithelium, however *Hes1* expression has recently been shown not to be an indicator of prognosis in CRC patients (Reedijk *et al.*, 2008), suggesting that Hes1 may not have a key role in EMT induction or tumour invasion. Although poorly defined, several other transcriptional targets are also important effectors of Notch signalling (reveiwed in Iso *et al.*, 2003). Crucially, Notch-induced EMT in other tissues has been shown to be largely dependent on the Hes-related factors Hey1, Hey2 and HeyL. These alternative factors have been shown to be essential targets of Notch activation during endocardial development (Fischer *et al.*, 2007; Timmerman *et al.*, 2004) and during EMT induction in endothelial cells (Niessen *et al.*, 2008). These alternative factors are also up-regulated in invasive breast cancers *in vivo* (Parker *et al.*, 2004) and during Notch-mediated EMT in breast MCF10A cells *in vitro* (Leong *et al.*, 2007). It has also been shown that *Jag1* expression directly correlates with *Hey1/2/L* in breast cancer patients, further underscoring the potential significance of these alternative Notch target genes in the malignant progression of these tumours (Leong *et al.*, 2007). In contrast, however, it has previously been shown that N1 activation does not induce the expression of *Hey1* in the normal intestinal epithelium (Fre *et al.*, 2005) and, therefore, induction of these alternative factors may not occur downstream of N1 in human CRC. Furthermore, while *Jag1* expression was also found to directly correlate with the expression of *Slug* in cancers, this was not shown for cancers of the colorectal epithelium (Leong *et al.*, 2007), suggesting that Notch-induced EMT may not be a prominent feature of malignancy in these tissues.

5.3.2 NOTCH MAY BE INTERDEPENDENT ON OTHER PRO-INVASIVE FACTORS

Tumour invasion and metastasis is known to be a highly complex and polygenic process, involving many processes, factors and cell types. It is now well accepted that colorectal tumour progression to malignancy and beyond is heavily dependent on the integration of various key cell-cell signalling pathways and altered environmental conditions (Thiery and Sleeman, 2006). Induction of EMT in CRC by Notch may therefore be reliant on or driven by the presence or absence of these additional factors. For example, Wnt signalling is known to switch from promotion of CRC proliferation to invasion in a dose-dependent fashion (reviewed in Fodde and Brabletz, 2007). The Notch and Wnt pathways are known to be heavily integrated in many

contexts and may also synergise at multiple levels in CRC, including the co-regulation of immediate transcriptional target genes (*SECTION 4.2.4, p141 – SECTION 4.2.6, p148*). The functional outcome of altered Notch signalling in the normal intestine and during intestinal tumorigenesis has recently been shown to be heavily dependent on Wnt (Fre *et al.*, 2009; Rodilla *et al.*, 2009), although this remains to be tested in the context of colorectal malignancy. The Notch and TGF-β pathways are also known to converge on the expression of various transcriptional target genes and both are known to cooperate in the induction of EMT (Niimi *et al.*, 2007; Timmerman *et al.*, 2004; Zavadil *et al.*, 2004). In this context, it has recently been shown that N1 induces Slug (but not Snail) and TGFβ instead induces Snail (but not Slug) in endothelial cells (Niessen *et al.*, 2008), suggesting that N1 and TGF-β act in a distinct but complementary fashion in these tissues. It now also appears that N1 and TGF-β demonstrate an overlapping expression pattern in the normal intestinal crypt (*SECTION 3.2.2, p92*) (Avery *et al.*, 1993; Barnard *et al.*, 1989), where they might synergise to control normal intestinal differentiation, as is already known to occur in breast epithelial cells (Niimi *et al.*, 2007). Since TGF-β demonstrates a significant functional switch from tumour suppressor to malignant promoter with progression along the colorectal adenoma-adenocarcinoma sequence (*SECTION 1.3.5, p26*), a similar change in role might also be hypothesised for Notch signalling but may be dependent on aspects of TGF-β.

In addition, tumour initiation and progression are heavily influenced by the tumour microenvironment. This is especially evident since EMT occurs along the tumour-host interface and does not appear to be a clonal or entirely genetic process. Notch-induced EMT may therefore be reliant on the presence of specific environmental conditions which may not be present under the optimal growth conditions provided *in vitro*. For example, hypoxia is known to promote tumour malignancy (reviewed in Keith and Simon, 2007) and is known to alter colorectal tumour cell behaviour through the modulation of key molecular pathways, such as Wnt (Kaidi *et al.*, 2006). It was also recently shown that Notch signalling acts as a critical intermediary between the hypoxic response and induction of EMT in a range of different tumour cells, including the colorectal adenocarcinoma cell line HCT116 (Sahlgren *et al.*, 2008). In many cases, active Notch signalling was found to be essential for the induction of EMT by hypoxia and it could be suggested that the converse is also true in CRC.

5.3.3 NOTCH MAY PROMOTE INVASION AND METASTASIS INDEPENDENTLY OF EMT

It is also important to note that EMT is only one of many steps underlying tumour invasion and metastasis (Bogenrieder and Herlyn, 2003; Woodhouse *et al.*, 1997). Furthermore, any

involvement of EMT remains controversial and a number of EMT-independent events have now also been highlighted (Christiansen and Rajasekaran, 2006; Tarin *et al.*, 2005). For example, it is known that EMT does not always accompany epithelial cell delamination and there are many cases in which epithelial cells migrate as a polarized adhesive cell cluster, rather than as single mesenchymal-type cells (Rorth, 2007). This is exemplified in the intestinal epithelium during restitutive regeneration, a process which was shown to involve increased expression of N1 (*SECTION 3.2.4, p97*). It might therefore be proposed that Notch activity functionally contributes to colorectal malignancy independently of E-cadherin/EMT through the modulation of other invasion-associated factors, such as matrix metalloproteinases (MMP) (Fischer *et al.*, 2007; Wang *et al.*, 2006), α-smooth muscle actin (α-SMA) (Noseda *et al.*, 2006), integrins (Karsan, 2008), or Eph/ephrins (Koo *et al.*, 2009).

Alternatively, activated Notch might also confer other necessary properties to malignant colorectal tumour cells other than induction of invasion, including resistance to apoptosis or anoikis. N1 has long been appreciated as a key inhibitor of apoptosis in many cell types (reviewed in Miele and Osborne, 1999), but a role for Notch signalling in the specialised cell contact-dependent form of apoptosis, known as 'anoikis', is less well defined. Resistance to anoikis is especially important to invading tumour cells and, complementary to the invasive process, is integral to malignant progression by enabling cells to become anchorage-independent and detach from the primary tumour site (reviewed in Chiarugi and Giannoni, 2008). Notch activation has been shown to protect breast epithelial cells from apoptosis in general (Stylianou *et al.*, 2006) and protects against anoikis specifically when breast epithelial cells are cultured in soft agar (Leong *et al.*, 2007). Notch signalling has also been shown to activate additional targets involved in cellular survival such as PI3K/Akt, Bcl-Xl (Sade *et al.*, 2004) and Survivin (Wang *et al.*, 2006) in other cell contexts and these factors may now offer promising areas of future research in the contribution of Notch signalling to colorectal tumorigenesis and tumour progression.

This may be of clinical significance since Notch activation has been shown to increase chemoresistance in a wide range of tissues, including T-ALL (Sade *et al.*, 2004), breast cancer (Stylianou *et al.*, 2006), cervical cancer (Nair *et al.*, 2003), lung cancer (Mungamuri *et al.*, 2006) and more recently in colorectal cancer (Akiyoshi *et al.*, 2008). The EMT process itself is also increasingly associated with chemoresistance, as was also recently demonstrated in the context of colorectal cancer cells (Hoshino *et al.*, 2009). Furthermore, chemoresistance is frequently attributed to the presence of 'cancer stem cells' and the low frequency of cells that disseminate

at the invasive front plus the similarities between primary and secondary tumours may be hypothetically explained by adoption of the stem cell phenotype (reviewed in Brabletz *et al.*, 2005b). From analysis of other contexts, Jag1 is frequently associated with EMT induction but is also thought to be the Notch ligand responsible for maintenance of a progenitor/stem phenotype in other contexts (Jones *et al.*, 1998; Varnum-Finney *et al.*, 1998), suggestive of similar potential functions in CRC.

5.3.4 CONCLUDING REMARKS

Increasing evidence from embryonic development and carcinogenesis in other tissues strongly implies a fundamental role for Notch signalling in EMT and cellular invasion. Correlative evidence from previous chapters also suggests that N1 may be a central contributory feature during the malignant progression of colorectal tumours. While evidence presented in this chapter further supports this hypothesis, demonstrating that increased N1 expression is a significant feature of colorectal cancer de-differentiation, it was also shown that Notch signalling does not directly promote EMT in these cells. Therefore, this potentially adds yet further weight to a highly context-dependent role for N1 in these tissues, or may also allude to other important, but as yet untested, roles for N1 in colorectal malignancy. Given the prognostic significance of the malignant phenotype in patients presenting with colorectal tumours and the resurgent interest in Notch-mediated carcinogenesis in the colon, it is undoubtable that this topic will remain the continued focus of extensive research over coming years and, on the basis of results presented here, it is clear that this topic is likely to remain both complex and elusive in its foundation.

CHAPTER 6

GENERAL DISCUSSION

6 GENERAL DISCUSSION

Colorectal cancer undoubtedly remains one of the leading causes of death in the western world and, as westernised habits continue to spread, incidence and mortality levels look set to rise (*SECTION 1.1.1, p2 - SECTION 1.1.2, p2*). A clearer understanding of the molecular basis of this disease is now essential for the generation of much needed adjuvant therapies to improve patient survival. There is clearly an excellent rationale to suggest that Notch signalling plays a significant role in colorectal carcinogenesis (*SECTION 1.1.6, p7*); however, at the outset of this study, any role was poorly defined and analysis of N1 expression and/or function in the human colorectal epithelium and CRC had barely been addressed. Therefore, the over-riding aim of this investigation was to determine N1 protein expression in these tissues and to correlate potential alterations with various pre-malignant and malignant stages of human colorectal disease. Furthermore, this study aimed to evaluate the functional contribution of Notch in CRC cells, focussing particularly on proliferative and malignant potential, the two major determinants of virulence in this disease.

6.1 A DIFFERENTIAL ROLE FOR NOTCH IN THE COLORECTAL EPITHELIUM

Analysis in animal models has suggested a role for Notch signalling towards the base of the normal intestinal crypt, where it is believed to promote the proliferative progenitor phenotype and early intestinal tumorigenesis (Fre *et al.*, 2005; van Es *et al.*, 2005). However, from analysis of *in vivo* human colorectal tissues, two of the most interesting and novel findings presented here were that; i) The N1 protein is predominantly expressed towards the top of the colonic crypt, and ii) N1 expression is significantly increased between benign and malignant colorectal tumours. This instead suggests that N1 may have an important role in intestinal differentiation and late-stage colorectal tumorigenesis. Therefore, findings presented here initially appear to contrast with the aforementioned animal models.

However, confidence in these results comes from the fact that this IHC technique was rigorously validated and was supported by a corresponding pattern of Hes1 expression in these tissues (*SECTION 3.2.2, p92*). Analysis in the normal human SI and murine epithelium demonstrated this expression pattern to be conserved (*SECTION 3.2.3, p93*). The expression of these proteins towards the top of the crypt was also supported by the fact that (in a pathological setting) both proteins are readily detectable at the base of the crypt when present (*SECTION 3.2.4, p97*). Crucially, although N1 and Hes1 predominantly localise to the upper crypt region, low level and scattered

expression was also evident towards the base of the crypt (*SECTION 3.2.2, p92*) and sequential fractionation revealed that both proteins are, in fact, widely expressed along the crypt axis (*SECTION 3.2.3, p93*). This suggests N1 may carry out essential functions in both crypt compartments and, paradoxically, may govern both the progenitor and differentiating phenotype in these cells (*SECTION 4.1.3, p129*). Although complex, this is consistent with functional analysis from the normal murine intestine, in which N1 has independently been shown to promote both proliferation (Fre *et al.*, 2005) and differentiation (Zecchini *et al.*, 2005).

Furthermore, a dichotomous role for Notch in these normal tissues was also supported by analysis of *in vitro* colorectal tumour cell lines, where N1 was shown to be expressed in both actively dividing and spontaneously differentiating populations (*SECTION 4.2.13, p170*). Analyses in these cell lines also supports findings from tumour tissues and a role for N1 in malignant progression, demonstrating increased expression in adenocarcinomas compared to adenomas (*SECTION 3.2.7, p111*), as well as the de-differentiated CRC phenotype (*SECTION 5.2.1, p195*). Support for a N1 role in tumour malignancy was also derived from the finding that N1 is strongly expressed in the delaminated and migratory epithelium associated with restitutive repair (*SECTION 3.2.4, p97*). This tissue shares many analogies to tumour de-differentiation at the invasive front but also highlights yet another important physiological role for Notch in the intestinal epithelium.

In summary, these results suggest that N1 may, in fact, be responsible for multiple, context-dependent functions within both the normal and neoplastic epithelium. Therefore, rather than discrediting previously hypothesised roles, these findings extend current understanding and highlight several important, but largely overlooked, contributions for Notch signalling in the intestine. However, what now remains unclear is how N1 may be responsible for such contrasting functions within these tissues and how these differential roles may correlate with the pattern of N1 protein expression observed here.

6.2 A DYNAMIC BASIS FOR NOTCH IN THE COLORECTAL EPITHELIUM

The long withstanding hypothesis that Notch maintains cells in an un-differentiated state has largely been derived from analysis during neurogenesis and has remained a paradigm for Notch function in general (Fortini and Artavanis-Tsakonas, 1993). In this context, Notch signalling is also classically known for its ability to direct equivalent cells to differentiate along different cell lineages via 'lateral inhibition' and loss of Notch signalling therefore leads to precocious differentiation along the default neural lineage (*SECTION 1.4.4, p33*). The intestinal epithelium is now also known to be governed by similar Notch-mediated mechanisms and relies on the same

pro-neural factors (*SECTION 1.5.7, p43*). As a consequence, very recent insights from the neurogenic epithelium may offer possible explanation of the results presented here in the normal and neoplastic human colorectal epithelium.

In the classical model of lateral inhibition, stochastic differences between equipotent cells are amplified through positive Notch-mediated feedback to ensure the correct distribution of cell fate within these tissues (*FIGURE 6-1A*) (Lewis, 1996). In the developing nervous system, these mechanisms are thought to transiently elevate both Delta-like ligand (Dll) and pro-neural factor expression in post-mitotic progenitor cells undergoing neural differentiation (Dunwoodie *et al.*, 1997; Henrique *et al.*, 1995; Myat *et al.*, 1996). Ligand expression on these differentiating cells activates Notch signalling in their neighbours, causing them to remain as immature progenitors. However, recent evidence has now shown that far more dynamic and oscillatory mechanisms underlie lateral inhibition than previously appreciated and this classical model has now been challenged (reviewed in Kageyama *et al.*, 2008).

Although Notch activation and Hes1 expression maintains the immature neural progenitor phenotype, it is now known that Hes1 expression is highly variable within this cellular population (Baek *et al.*, 2006; Shimojo *et al.*, 2008). Crucially, low Hes1 expressing cells have been shown to express both Dll and pro-neural factors, indicating that their expression is not confined to post-mitotic neurons as previously believed. Sustained expression of the pro-neural factors is known to induce cell cycle arrest and differentiation but, surprisingly, it has recently been shown that sustained Notch activation also leads to mitotic arrest in these cells (Baek *et al.*, 2006; Shimojo *et al.*, 2008; Yoshiura *et al.*, 2007). Therefore, the expression of $N1^{IC}$/Hes1 must be highly dynamic and transient for efficient proliferation or differentiation to proceed. Dynamic control of Notch signalling is a known feature of other key morphogenic processes, such as somitogenesis, where negative auto-regulatory mechanisms cause *Hes* gene expression to oscillate with a period of only 2-3 hours (*SECTION 4.3.1, p175*). Hes oscillations have now been shown to govern lateral inhibition, causing neural progenitors to dynamically 'flip-flop' between the ligand or receptor expressing configuration until expression becomes sustained in the post-mitotic state (*FIGURE 6-1B*) (Kageyama *et al.*, 2008).

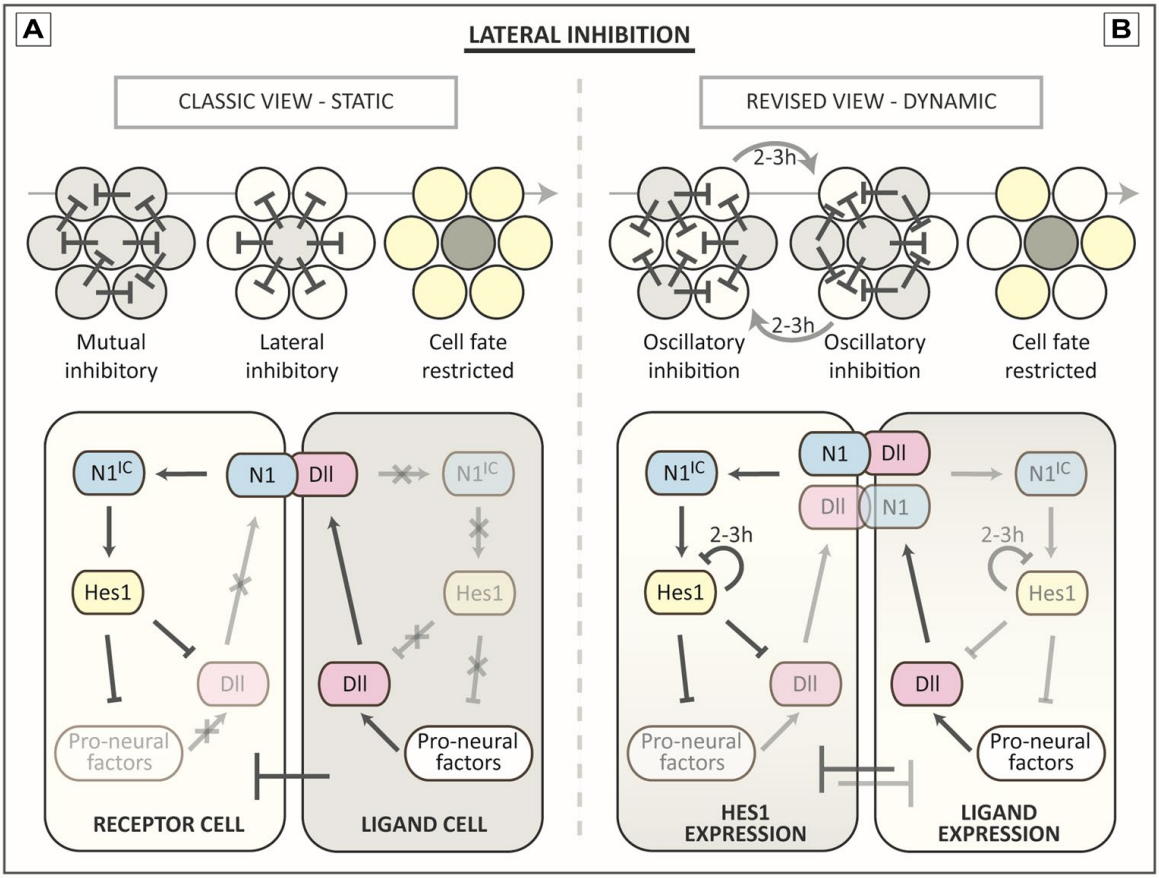


Figure 6-1| *A dynamic basis for lateral inhibition.* [**A**] In the classical model, cells inhibit their neighbours from adopting the same (neural or secretory) cell fate by up-regulating ligand expression and activating Notch signalling in adjacent cells. Hes1 suppresses the expression of downstream pro-neural factors and ligand expression is also suppressed. This mechanism amplifies stochastic differences arising between different cells, leading to a sustained 'salt and pepper' configuration (Lewis, 1996). [**B**] In the revised model, *Hes1* oscillates with a period of 2-3 hours as a result of potent autoregulation. This causes pro-neural factors and ligand expression to oscillate in an opposing fashion and cells therefore 'flip-flop' between ligand and receptor expressing phenotypes. This occurs with the same periodicity until expression becomes sustained in post-mitotic cells (Kageyama *et al.*, 2008).

Given that N1, Hes1 and pro-neural factors also govern normal intestinal homeostasis, it is probable that the same dynamic mechanisms also exist within this tissue. It is likely, therefore, that dynamic and transient $N1^{IC}$/Hes1 expression is essential in the proliferative intestinal progenitor pool and that signalling patterns become sustained in post-mitotic epithelial cells towards the lumenal surface. Assuming that (like neurogenesis) sustained protein expression is more readily detectable than dynamic oscillatory expression, this model may explain the distribution of N1 expression presented here (*SECTION 3.2.2, p92*). Notch signalling may therefore be highly active in the proliferative progenitor compartment, while remaining only variably and intermittently expressed. This suggests that sustained expression occurs in post-mitotic intestinal cells and is consistent with the uniform N1 and Hes1 expression in the upper crypt compartment.

Although neural cell differentiation is associated with sustained Notch suppression and pro-neural factor expression, it is important to note that sustained Notch activity is an essential feature of the surrounding post-mitotic cells which do not differentiate along the neural lineage (Baek *et al.*, 2006). In the intestinal epithelium, secretory differentiation is analogous to neural differentiation and, by lateral inhibition, the majority of cells (6/7) will adopt the non-secretory lineage, as confirmed by absorptive cells constituting approximately 80% of the epithelial cell population (Karam, 1999). This suggests that the majority of post-mitotic cells in the intestine should be associated with sustained N1 and Hes1 expression. Alternatively, N1 and Hes1 are known to be expressed throughout neurogenesis (Myat *et al.*, 1996) and, surprising, sustained $N1^{IC}$ expression is frequently observed in many terminally differentiated neural cells (Ahmad *et al.*, 1995; Berezovska *et al.*, 1998; Redmond *et al.*, 2000; Sestan *et al.*, 1999). Although the role of persistent N1 expression in these post-mitotic cells is unknown, it appears this is important for maintaining the fully differentiated state (Louvi and Artavanis-Tsakonas, 2006). Therefore, sustained Notch activation may also be a significant feature of terminally differentiated secretory cells in the intestinal epithelium, as speculated previously from analysis in animal models (Zecchini *et al.*, 2005).

In the neurogenic epithelium, sustained N1 activation has also been shown to be a defining feature of the neural stem cell (NSC) population (Kageyama *et al.*, 2008), where sustained Hes1 expression is essential for NSC maintenance (Bai *et al.*, 2007), and distinguishes these cells from their proliferative progenitor counterparts. This strongly suggests that sustained N1 activity may be an essential feature of stem cells in the intestinal epithelium and sustained vs. oscillatory expression may have significant implications for intestinal tumorigenesis. Importantly, this model may also explain the N1 expression patterns observed here in human colorectal tumour tissues and the apparent contrast with hypothesised roles in early intestinal tumorigenesis. Consistent with expansion of the proliferative progenitor phenotype as a source for these tumours (Boman *et al.*, 2004), N1 immunoreactivity in colorectal adenomas was reminiscent of staining observed in cells at the base of the normal crypt, being both low level and intermittently expressed (*SECTION 3.2.5, p103*). This suggests that N1 activation may also be dynamic and oscillatory in these adenomas and, in a similar vein to the normal epithelium, high levels of dynamic activation may be present without readily detectable N1 protein expression in these tissues. Therefore, this model does not discount an essential role for Notch signalling in early intestinal tumorigenesis, but instead suggests that N1 expression is dynamic in the early stages and may be either increased or become sustained with malignant tumour progression.

6.3 NOTCH ACTIVATION IS SUBORDINATE TO CELL CYCLE PROGRESSION

In addition to the dynamic oscillation changes outlined above, analysis in neural progenitors also demonstrates Notch activity to be highly dependent on cell cycle progression, with Hes1 being expressed through S and G2/M, but remaining low in G1 (Baek *et al.*, 2006; Shimojo *et al.*, 2008; Yoshiura *et al.*, 2007). This is important since Hes1 enhances G2/M progression but, when either sustained or inhibited it retards cells in G1 (Shimojo *et al.*, 2008; Yoshiura *et al.*, 2007). Hes1 expression during S/G2/M leads to rapid autoregulatory oscillations and therefore double-layered oscillatory mechanisms operate in these cells. In a similar fashion, both *Notch1* and *Dll1* have also been shown to oscillate with cell cycle progression in these cells (Cisneros *et al.*, 2008) and this is believed, in part, to prevent differentiation and senescence prior to G0/G1 (Latasa *et al.*, 2009). This capacity to differentially express Notch factors in a cell-cycle dependent fashion is now believed to be an intrinsic characteristic of neural precursors (Latasa *et al.*, 2009) and appears to be a highly conserved feature of the Notch pathway in general (Negre *et al.*, 2003).

These mechanisms have not yet been demonstrated in the normal colorectal epithelium but it is highly likely that they are also essential to the progenitor phenotype and suggest yet further levels of Notch dynamicity in the proliferative cells of the lower crypt compartment. This therefore gives rise to a theoretical model, whereby sustained Notch activation maintains the crypt stem cell population, but then expression becomes highly dynamic in the progenitors under the control of both the cell cycle and rapid Hes1 autoregulation. This enables efficient proliferation and cell fate determination to proceed (*FIGURE 6-2*), after which expression patterns become sustained in post-mitotic cells undergoing terminal differentiation towards the top of the crypt. In possible explanation of the differential outcome of sustained Notch activation, it important to note that in the stem cell context this occurs in the presence of high Wnt activation, while in the differentiating compartments this occurs in the absence of Wnt, but with high level TGFβ/BMP.

Furthermore, evidence presented here suggests that these mechanisms may be operational in CRC cells. Primarily, it was shown here that exogenous N1 activation increased *Hes1* promoter activity, but does not elicit an increase in Hes1 protein expression (*SECTION 4.2.3, p138*) and suggests that Hes1 autoregulatory mechanisms may persist in these cells. In direct agreement with findings from neurogenesis, it was also consistently found that N1 protein expression increases in actively cycling CRC cells and appears to be transiently up-regulated during later stages of S-phase (*SECTION 4.2.9, p157 – SECTION 4.2.12, p167*). The functional significance of this is

not yet clear, but the differential expression of cell fate-controlling genes during S-phase is now known to be a crucial feature of embryonic stem cells (Azuara *et al.*, 2006), sparking major interest into this evolving field and has now become the focus of novel and on-going research in CRC (*D Winton, personal correspondence*).

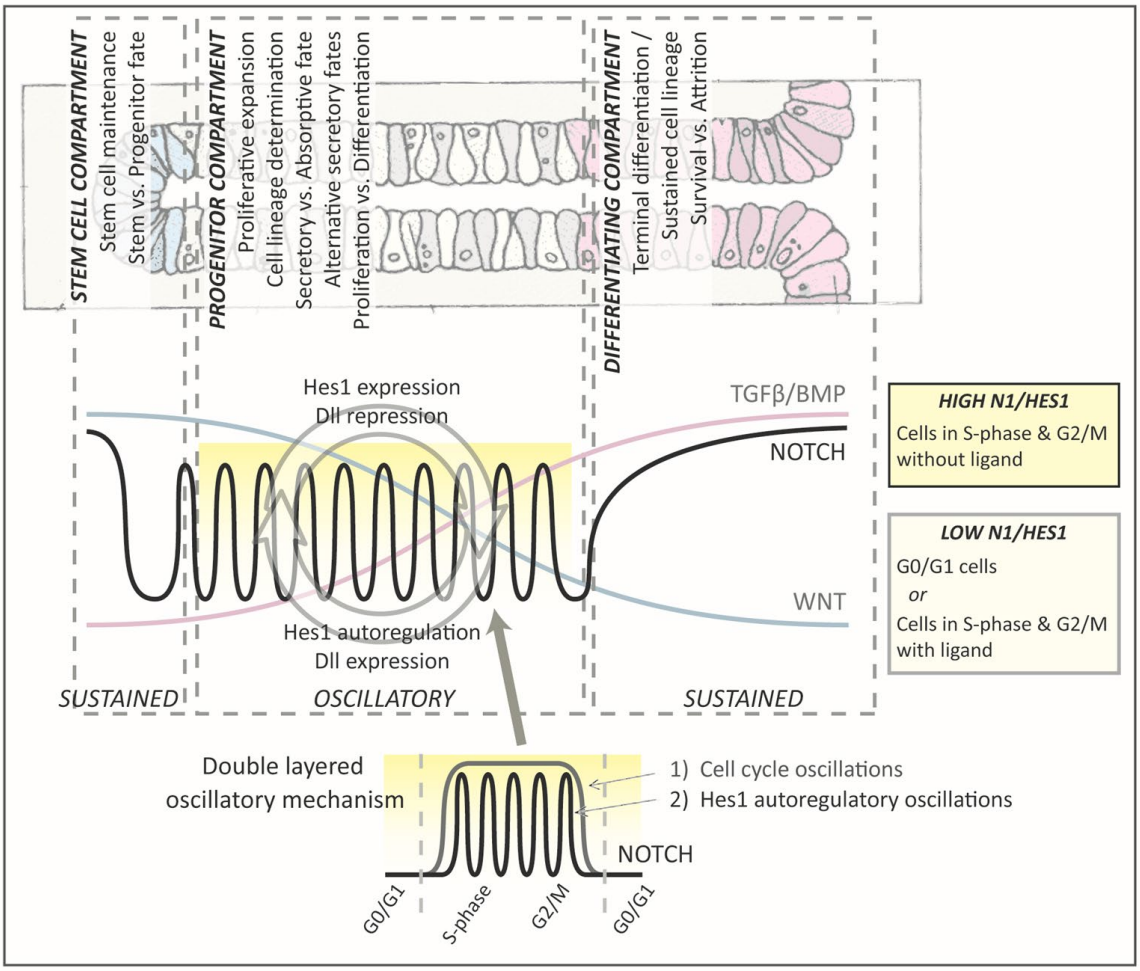


Figure 6-2| ***A dynamic basis for Notch in the colonic crypt.*** The dynamics of Notch pathway activation change along the length of the normal colonic crypt. Sustained N1 activation and Hes1 expression is required for maintenance of the stem cell compartment and may mediate binary cell fate decision between the stem and progenitor cell phenotype. Sustained N1 activation is inhibitory to proliferation and instead becomes dynamic and oscillatory in the progenitor pool, under the control of both the cell cycle and potent Hes1 autoregulatory mechanisms. Actively cycling cells therefore 'flip-flop' between receptor and ligand expressing conformation before expression becomes sustained in post-mitotic cells of the upper differentiating crypt compartment.

6.4 A MORPHOGENETIC BASIS TO NOTCH ACTIVITY

Although cell cycle progression may clearly activate or inactivate dynamic Notch oscillation, this does not explain how activation may become sustained. In possible explanation, the oscillation of Hes1 in neural progenitors is dependent on corresponding oscillations of the Jak-Stat pathway

and loss of Jak-Stat signalling results in sustained expression of the Hes1 protein, even when mRNA expression is suppressed (Yoshiura *et al.*, 2007). The function of Jak-Stat signalling in the colorectal epithelium is not well defined, but it has been shown that this pathway is predominantly active in progenitor cells of the colonic crypt and is absent from the upper crypt (Ferrand *et al.*, 2005), further suggesting that sustained vs. oscillatory Notch activation may demarcate the proliferative and differentiating crypt compartments.

Conversely, the Id (Inhibitor of DNA binding) family of transcription factors have emerged as positive regulators of sustained Hes1 expression in neurogenesis, where they prevent Hes1 autoregulation without affecting the repression of other downstream target genes (Bai *et al.*, 2007). Id1-3 proteins are also known to be expressed in the intestine and localise predominantly in the upper third of the normal colonic crypt (Wilson *et al.*, 2001). This is consistent with their positive regulation by BMP/TGFβ pathways in other tissues (reviewed in Perk *et al.*, 2005) and are therefore likely to elicit sustained Notch pathway activity in the upper crypt region. Importantly, these proteins are also known to become aberrantly expressed in CRC and crucially, only become significantly up-regulated following malignant progression (Rockman *et al.*, 2001; Wilson *et al.*, 2001). This is believed, at least in part, to be governed by p53 mutations (Wilson *et al.*, 2001), a known marker of the adenoma-adenocarcinoma transition (Baker *et al.*, 1990) and could explain how sustained Notch expression may, paradoxically, be a feature of both the normal differentiating crypt compartment and malignant tumour progression.

Activating mutations in the RAS-MAPK pathway have also been shown to have substantial effects on the kinetics of N1 expression in other tissues, causing the N1 protein to mature and be activated much more rapidly, increasing the steady state of $N1^{IC}$ expression (Weijzen *et al.*, 2002a). In breast tumours it was shown that RAS positive tissues demonstrate strong cytoplasmic positivity for N1 expression, while RAS negative tissues demonstrate a much weaker and heterogeneous staining pattern, consistent with the differences between colorectal adenoma and adenocarcinoma tissues observed here. This may be an important consideration given that RAS-MAPK associated mutations are frequently associated with the later stages of colorectal tumour progression (reviewed in Sebolt-Leopold and Herrera, 2004) and are highly prevalent in many other human malignancies (Bos *et al.*, 1987).

It is possible therefore that the increased N1 expression in colorectal adenocarcinoma tissues may arise from the expansion of tumour cell clones containing mutations in *TP53* and/or *RAS,* leading to both sustained expression and activation of N1 by two independent mechanisms.

Similar, as yet unknown, clonal alterations to Jak-Stat oscillations would be likely to impact on N1 expression in these tissues. It should also be noted that, in continued analogy to changes in neural precursor cells (Shimizu *et al.*, 2008), results presented here also demonstrated novel mechanisms by which the Wnt pathway may directly influence Hes1 expression dynamics in these tissues (*SECTION 4.2.4, p141 - SECTION 4.2.5, p145*). This may be important because further increases in Wnt pathway activation are a consistent feature of colorectal tumour progression and malignancy (Anderson *et al.*, 2002; Blaker *et al.*, 2003; Brabletz *et al.*, 1998). Of further consideration, sustained N1 expression in the presence of TGFβ may promote normal intestinal differentiation, as is already known in the breast epithelium (Niimi *et al.*, 2007); however, N1 cooperation with TGFβ also promotes EMT in other contexts (Zavadil *et al.*, 2004) and altered TGFβ is a major contributor to malignancy in the colorectal epithelium (Roman *et al.*, 2001), suggesting that TGFβ may also impact on N1 expression and/or function in the later stages of colorectal carcinogenesis. Although undefined, it is likely, therefore, that Notch meshes into functional networks with other signalling pathways in both normal and aberrant contexts, the organisation of which remains to be explored.

6.5 THERAPEUTIC IMPLICATIONS FOR THE NOTCH PATHWAY

The clinical potential for Notch-targeted therapy has been widely speculated in many cancers (Miele *et al.*, 2006) and Notch inhibitors have now undergone phase I clinical trials in patients with refractory T-ALL (*http://www.clinicaltrials.gov/ct/show/NCT00100152*). Evidence from animal models has suggested a prevailing role for Notch in intestinal tumorigenesis and highlight pathway inhibition as a potential means of treating these tumours. Most importantly, results presented here from analysis in human tissues support this notion, and show for the first time that N1 expression is significantly increased with malignant colorectal tumour progression. Targeting of the N1 pathway may therefore provide a desperately needed avenue for adjuvant therapy in these patients; however, despite the attractive simplicity of the core pathway, findings of this study also begin to highlight a number of important considerations for Notch-mediated therapy in this tissue. Profound alterations to normal intestinal homeostasis are a known side-effect of Notch pathway inhibition (*SECTION 1.5.3, p39*) and findings presented here further these concerns by suggesting additional tumour suppressor and reparative functions for Notch in the colorectal epithelium. Nevertheless, while these factors are of significant concern, it is likely that, with greater understanding, any deleterious effects may be avoided.

For example, this and other recent studies highlight the prominent expression of both N1 and Jag1 in CRC (Chu *et al.*, 2009; Rodilla *et al.*, 2009) and it has been proposed that selectively targeting these components might avoid compromising the normal tissue (Guilmeau *et al.*, 2009; Riccio *et al.*, 2008). Alternatively, it was also recently demonstrated that combined therapy with glucocorticoids reduces Notch inhibitor gut cytotoxic effects (Real and Ferrando, 2009), greatly furthering their potential for clinical application. Combination therapy may have further significance since Notch inhibition was shown here not to affect CRC growth (*SECTION 4.2.8 p154*) but has now been shown to greatly enhance the effectiveness of other chemotherapeutics (Akiyoshi *et al.*, 2008; Meng *et al.*, 2009). In summary, Notch signalling appears to be a highly promising target for future therapies in many cancers, including CRC; however, it is also increasingly obvious that a refined understanding of the multiple context-dependent outcomes and interdependence on other key signalling factors is essential before these inhibitors can be widely used.

6.6 CONCLUDING REMARKS

In the first ever review of Notch signalling by Ted Wright it was stated that "If one was asked to choose the single, most important genetic variation concerned with expression of the genome during embryogenesis in *Drosophila*, the answer would have to be the *Notch* locus" (Wright, 1970). Therefore, the highly pleiotropic action of the Notch signalling pathway was recognised early on and, given the highly conserved nature of the pathway, it is likely that this statement is equally pertinent to virtually all metazoans, including man. Given its biological importance, involvement of the Notch pathway in many human diseases should not be surprising; however, despite an explosive interest in recent years, many elemental questions still remain unanswered. This study is one of the first of its kind to assess N1 expression in human colorectal tissues and is unique in demonstrating significant correlations with malignant tumour progression. Importantly, this study also accentuates a number of largely unappreciated facets of N1 in the colorectal epithelium, many of which are likely to feature heavily in intestinal Notch research over coming years. Although it may not be possible to ascribe a 'unitary' function to Notch in these tissues, it is already apparent that Notch-mediated therapy may provide a powerful tool for the management of neoplastic disease and, hopefully, improved patient survival.

LITERATURE REFERENCES

LITERATURE REFERENCES

Adams, P. D. (2001). Regulation of the retinoblastoma tumor suppressor protein by cyclin/cdks. *Biochim Biophys Acta* **1471**, M123-33.

Ahimou, F., Mok, L. P., Bardot, B. and Wesley, C. (2004). The adhesion force of Notch with Delta and the rate of Notch signaling. *J Cell Biol* **167**, 1217-29.

Ahmad, I., Zaqouras, P. and Artavanis-Tsakonas, S. (1995). Involvement of Notch-1 in mammalian retinal neurogenesis: association of Notch-1 activity with both immature and terminally differentiated cells. *Mech Dev* **53**, 73-85.

Akazawa, C., Ishibashi, M., Shimizu, C., Nakanishi, S. and Kageyama, R. (1995). A mammalian helix-loop-helix factor structurally related to the product of Drosophila proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system. *J Biol Chem* **270**, 8730-8.

Akiyoshi, T., Nakamura, M., Yanai, K., Nagai, S., Wada, J., Koga, K., Nakashima, H., Sato, N., Tanaka, M. and Katano, M. (2008). Gamma-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells. *Gastroenterology* **134**, 131-44.

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. and Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* **100**, 3983-8.

Allenspach, E. J., Maillard, I., Aster, J. C. and Pear, W. S. (2002). Notch signaling in cancer. *Cancer Biol Ther* **1**, 466-76.

Alves-Guerra, M. C., Ronchini, C. and Capobianco, A. J. (2007). Mastermind-like 1 Is a specific coactivator of beta-catenin transcription activation and is essential for colon carcinoma cell survival. *Cancer Res* **67**, 8690-8.

Amos-Landgraf, J. M., Kwong, L. N., Kendziorski, C. M., Reichelderfer, M., Torrealba, J., Weichert, J., Haag, J. D., Chen, K. S., Waller, J. L., Gould, M. N. *et al.* (2007). A target-selected Apc-mutant rat kindred enhances the modeling of familial human colon cancer. *Proc Natl Acad Sci U S A* **104**, 4036-41.

Anderson, C. B., Neufeld, K. L. and White, R. L. (2002). Subcellular distribution of Wnt pathway proteins in normal and neoplastic colon. *Proc Natl Acad Sci U S A* **99**, 8683-8.

Ando, K., Kanazawa, S., Tetsuka, T., Ohta, S., Jiang, X., Tada, T., Kobayashi, M., Matsui, N. and Okamoto, T. (2003). Induction of Notch signaling by tumor necrosis factor in rheumatoid synovial fibroblasts. *Oncogene* **22**, 7796-803.

Andoh, A., Bamba, S., Fujiyama, Y., Brittan, M. and Wright, N. A. (2005). Colonic subepithelial myofibroblasts in mucosal inflammation and repair: contribution of bone marrow-derived stem cells to the gut regenerative response. *J Gastroenterol* **40**, 1089-99.

Andreu, P., Colnot, S., Godard, C., Gad, S., Chafey, P., Niwa-Kawakita, M., Laurent-Puig, P., Kahn, A., Robine, S., Perret, C. *et al.* (2005). Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. *Development* **132**, 1443-51.

Apelqvist, A., Ahlgren, U. and Edlund, H. (1997). Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Curr Biol* **7**, 801-4.

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D. J., Honjo, T., Hrabe de Angelis, M., Lendahl, U. and Edlund, H. (1999). Notch signalling controls pancreatic cell differentiation. *Nature* **400**, 877-81.

Aragaki, M., Tsuchiya, K., Okamoto, R., Yoshioka, S., Nakamura, T., Sakamoto, N., Kanai, T. and Watanabe, M. (2008). Proteasomal degradation of Atoh1 by aberrant Wnt signaling maintains the undifferentiated state of colon cancer. *Biochem Biophys Res Commun* **368**, 923-9.

Archer, S. Y., Meng, S., Shei, A. and Hodin, R. A. (1998). p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. *Proc Natl Acad Sci U S A* **95**, 6791-6.

Armitage, P. and Doll, R. (1954). The age distribution of cancer and a multi-stage theory of carcinogenesis. *Br J Cancer* **8**, 1-12.

Armstrong, B. and Doll, R. (1975). Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer* **15**, 617-31.

Armstrong, F., Brunet de la Grange, P., Gerby, B., Rouyez, M. C., Calvo, J., Fontenay, M., Boissel, N., Dombret, H., Baruchel, A., Landman-Parker, J. *et al.* (2009). NOTCH is a key regulator of human T-cell acute leukemia initiating cell activity. *Blood* **113**, 1730-40.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6.

Aster, J. C., Robertson, E. S., Hasserjian, R. P., Turner, J. R., Kieff, E. and Sklar, J. (1997). Oncogenic forms of NOTCH1 lacking either the primary binding site for RBP-Jkappa or nuclear localization sequences retain the ability to associate with RBP-Jkappa and activate transcription. *J Biol Chem* **272**, 11336-43.

Aster, J. C., Xu, L., Karnell, F. G., Patriub, V., Pui, J. C. and Pear, W. S. (2000). Essential roles for ankyrin repeat and transactivation domains in induction of T-cell leukemia by notch1. *Mol Cell Biol* **20**, 7505-15.

Atcha, F. A., Munguia, J. E., Li, T. W., Hovanes, K. and Waterman, M. L. (2003). A new beta-catenin-dependent activation domain in T cell factor. *J Biol Chem* **278**, 16169-75.

Atcha, F. A., Syed, A., Wu, B., Hoverter, N. P., Yokoyama, N. N., Ting, J. H., Munguia, J. E., Mangalam, H. J., Marsh, J. L. and Waterman, M. L. (2007). A unique DNA binding domain converts T-cell factors into strong Wnt effectors. *Mol Cell Biol* **27**, 8352-63.

Auclair, B. A., Benoit, Y. D., Rivard, N., Mishina, Y. and Perreault, N. (2007). Bone morphogenetic protein signaling is essential for terminal differentiation of the intestinal secretory cell lineage. *Gastroenterology* **133**, 887-96.

Augenlicht, L., Velcich, A. and Heerdt, B. G. (1995). Short-chain fatty acids and molecular and cellular mechanisms of colonic cell differentiation and transformation. *Adv Exp Med Biol* **375**, 137-48.

Augenlicht, L., Velcich, A., Mariadason, J., Bordonaro, M. and Heerdt, B. (1999). Colonic cell proliferation, differentiation, and apoptosis. *Adv Exp Med Biol* **470**, 15-22.

Avery, A., Paraskeva, C., Hall, P., Flanders, K. C., Sporn, M. and Moorghen, M. (1993). TGF-beta expression in the human colon: differential immunostaining along crypt epithelium. *Br J Cancer* **68**, 137-9.

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996). Interaction between Wingless and Notch signaling pathways mediated by dishevelled. *Science* **271**, 1826-32.

Azuara, V., Perry, P., Sauer, S., Spivakov, M., Jorgensen, H. F., John, R. M., Gouti, M., Casanova, M., Warnes, G., Merkenschlager, M. *et al.* (2006). Chromatin signatures of pluripotent cell lines. *Nat Cell Biol* **8**, 532-8.

Baek, J. H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T. and Kageyama, R. (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. *Development* **133**, 2467-76.

Bai, G., Sheng, N., Xie, Z., Bian, W., Yokota, Y., Benezra, R., Kageyama, R., Guillemot, F. and Jing, N. (2007). Id sustains Hes1 expression to inhibit precocious neurogenesis by releasing negative autoregulation of Hes1. *Dev Cell* **13**, 283-97.

Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K., Hamilton, S. and Vogelstein, B. (1990). p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res* **50**, 7717-22.

Baldi, A., De Falco, M., De Luca, L., Cottone, G., Paggi, M. G., Nickoloff, B. J., Miele, L. and De Luca, A. (2004). Characterization of tissue specific expression of Notch-1 in human tissues. *Biol Cell* **96**, 303-11.

Balint, K., Xiao, M., Pinnix, C. C., Soma, A., Veres, I., Juhasz, I., Brown, E. J., Capobianco, A. J., Herlyn, M. and Liu, Z. J. (2005). Activation of Notch1 signaling is required for beta-catenin-mediated human primary melanoma progression. *J Clin Invest* **115**, 3166-76.

Balkwill, F., Charles, K. A. and Mantovani, A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7**, 211-7.

Bao, S., Wu, Q., Sathornsumetee, S., Hao, Y., Li, Z., Hjelmeland, A. B., Shi, Q., McLendon, R. E., Bigner, D. D. and Rich, J. N. (2006). Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res* **66**, 7843-8.

Baonza, A. and Freeman, M. (2005). Control of cell proliferation in the Drosophila eye by Notch signaling. *Dev Cell* **8**, 529-39.

Barker, N. and Clevers, H. (2007). Tracking down the stem cells of the intestine: strategies to identify adult stem cells. *Gastroenterology* **133**, 1755-60.

Barker, N., Ridgway, R. A., van Es, J. H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A. R., Sansom, O. J. and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* **457**, 608-11.

Barker, N., van Es, J. H., Jaks, V., Kasper, M., Snippert, H., Toftgard, R. and Clevers, H. (2008). Very Long-term Self-renewal of Small Intestine, Colon, and Hair Follicles from Cycling Lgr5+ve Stem Cells. *Cold Spring Harb Symp Quant Biol*.

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J. *et al.* (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* **449**, 1003-7.

Barlogie, B. and Drewinko, B. (1980). Lethal and cytokinetic effects of mitomycin C on cultured human colon cancer cells. *Cancer Res* **40**, 1973-80.

Barnard, J. A., Beauchamp, R. D., Coffey, R. J. and Moses, H. L. (1989). Regulation of intestinal epithelial cell growth by transforming growth factor type beta. *Proc Natl Acad Sci U S A* **86**, 1578-82.

Barnard, J. A. and Warwick, G. (1993). Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. *Cell Growth Differ* **4**, 495-501.

Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev* **16**, 1167-81.

Barolo, S., Stone, T., Bang, A. G. and Posakony, J. W. (2002). Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes Dev* **16**, 1964-76.

Baron, M. (2003). An overview of the Notch signalling pathway. *Semin Cell Dev Biol* **14**, 113-9.

Barshishat, M., Polak-Charcon, S. and Schwartz, B. (2000). Butyrate regulates E-cadherin transcription, isoform expression and intracellular position in colon cancer cells. *Br J Cancer* **82**, 195-203.

Batlle, E., Bacani, J., Begthel, H., Jonkheer, S., Gregorieff, A., van de Born, M., Malats, N., Sancho, E., Boon, E., Pawson, T. *et al.* (2005). EphB receptor activity suppresses colorectal cancer progression. *Nature* **435**, 1126-30.

Batlle, E., Henderson, J. T., Beghtel, H., van den Born, M. M., Sancho, E., Huls, G., Meeldijk, J., Robertson, J., van de Wetering, M., Pawson, T. *et al.* (2002). Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* **111**, 251-63.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J. and Garcia De Herreros, A. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* **2**, 84-9.

Becker, L., Huang, Q. and Mashimo, H. (2008). Immunostaining of Lgr5, an intestinal stem cell marker, in normal and premalignant human gastrointestinal tissue. *ScientificWorldJournal* **8**, 1168-76.

Behrens, I. and Kissel, T. (2003). Do cell culture conditions influence the carrier-mediated transport of peptides in Caco-2 cell monolayers? *Eur J Pharm Sci* **19**, 433-42.

Behrens, J., Mareel, M. M., Van Roy, F. M. and Birchmeier, W. (1989). Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J Cell Biol* **108**, 2435-47.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638-42.

Beier, D., Hau, P., Proescholdt, M., Lohmeier, A., Wischhusen, J., Oefner, P. J., Aigner, L., Brawanski, A., Bogdahn, U. and Beier, C. P. (2007). CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. *Cancer Res* **67**, 4010-5.

Ben-Arie, N., Bellen, H. J., Armstrong, D. L., McCall, A. E., Gordadze, P. R., Guo, Q., Matzuk, M. M. and Zoghbi, H. Y. (1997). Math1 is essential for genesis of cerebellar granule neurons. *Nature* **390**, 169-72.

Berezovska, O., Xia, M. Q. and Hyman, B. T. (1998). Notch is expressed in adult brain, is coexpressed with presenilin-1, and is altered in Alzheimer disease. *J Neuropathol Exp Neurol* **57**, 738-45.

Berman, D. M., Karhadkar, S. S., Maitra, A., Montes De Oca, R., Gerstenblith, M. R., Briggs, K., Parker, A. R., Shimada, Y., Eshleman, J. R., Watkins, D. N. *et al.* (2003). Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* **425**, 846-51.

Bermingham, N. A., Hassan, B. A., Price, S. D., Vollrath, M. A., Ben-Arie, N., Eatock, R. A., Bellen, H. J., Lysakowski, A. and Zoghbi, H. Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. *Science* **284**, 1837-41.

Bessho, Y. and Kageyama, R. (2003). Oscillations, clocks and segmentation. *Curr Opin Genet Dev* **13**, 379-84.

Bettenhausen, B., Hrabe de Angelis, M., Simon, D., Guenet, J. L. and Gossler, A. (1995). Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to Drosophila Delta. *Development* **121**, 2407-18.

Bezrookove, V., Smits, R., Moeslein, G., Fodde, R., Tanke, H. J., Raap, A. K. and Darroudi, F. (2003). Premature chromosome condensation revisited: a novel chemical approach permits efficient cytogenetic analysis of cancers. *Genes Chromosomes Cancer* **38**, 177-86.

Billottet, C., Tuefferd, M., Gentien, D., Rapinat, A., Thiery, J. P., Broet, P. and Jouanneau, J. (2008). Modulation of several waves of gene expression during FGF-1 induced epithelial-mesenchymal transition of carcinoma cells. *J Cell Biochem*.

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev Biol* **172**, 126-38.

Bjerknes, M., Cheng, H., Hay, K. and Gallinger, S. (1997). APC mutation and the crypt cycle in murine and human intestine. *Am J Pathol* **150**, 833-9.

Blaker, H., Scholten, M., Sutter, C., Otto, H. F. and Penzel, R. (2003). Somatic mutations in familial adenomatous polyps. Nuclear translocation of beta-catenin requires more than biallelic APC inactivation. *Am J Clin Pathol* **120**, 418-23.

Blaumueller, C. M., Qi, H., Zagouras, P. and Artavanis-Tsakonas, S. (1997). Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell* **90**, 281-91.

Bocchetta, M., Miele, L., Pass, H. I. and Carbone, M. (2003). Notch-1 induction, a novel activity of SV40 required for growth of SV40-transformed human mesothelial cells. *Oncogene* **22**, 81-9.

Bogenrieder, T. and Herlyn, M. (2003). Axis of evil: molecular mechanisms of cancer metastasis. *Oncogene* **22**, 6524-36.

Boivin, G. P., Washington, K., Yang, K., Ward, J. M., Pretlow, T. P., Russell, R., Besselsen, D. G., Godfrey, V. L., Doetschman, T., Dove, W. F. *et al.* (2003). Pathology of mouse models of intestinal cancer: consensus report and recommendations. *Gastroenterology* **124**, 762-77.

Boland, C. R. (2000). Molecular genetics of hereditary nonpolyposis colorectal cancer. *Ann N Y Acad Sci* **910**, 50-9; discussion 59-61.

Bolin, R. W., Robinson, W. A., Sutherland, J. and Hamman, R. F. (1982). Busulfan versus hydroxyurea in long-term therapy of chronic myelogenous leukemia. *Cancer* **50**, 1683-6.

Boller, K., Arpin, M., Pringault, E., Mangeat, P. and Reggio, H. (1988). Differential distribution of villin and villin MRNA in mouse intestinal epithelial cells. *Differentiation* **39**, 51-7.

Bolos, V., Blanco, M., Medina, V., Aparicio, G., Diaz-Prado, S. and Grande, E. (2009). Notch signalling in cancer stem cells. *Clin Transl Oncol* **11**, 11-9.

Boman, B. M., Fields, J. Z., Bonham-Carter, O. and Runquist, O. A. (2001). Computer modeling implicates stem cell overproduction in colon cancer initiation. *Cancer Res* **61**, 8408-11.

Boman, B. M., Walters, R., Fields, J. Z., Kovatich, A. J., Zhang, T., Isenberg, G. A., Goldstein, S. D. and Palazzo, J. P. (2004). Colonic crypt changes during adenoma development in familial adenomatous polyposis: immunohistochemical evidence for expansion of the crypt base cell population. *Am J Pathol* **165**, 1489-98.

Bonnet, D. and Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3**, 730-7.

Bordonaro, M., Mariadason, J. M., Aslam, F., Heerdt, B. G. and Augenlicht, L. H. (1999). Butyrate-induced apoptotic cascade in colonic carcinoma cells: modulation of the beta-catenin-Tcf pathway and concordance with effects of sulindac and trichostatin A but not curcumin. *Cell Growth Differ* **10**, 713-20.

Bos, J. L. (1989). ras oncogenes in human cancer: a review. *Cancer Res* **49**, 4682-9.

Bos, J. L., Fearon, E. R., Hamilton, S. R., Verlaan-de Vries, M., van Boom, J. H., van der Eb, A. J. and Vogelstein, B. (1987). Prevalence of ras gene mutations in human colorectal cancers. *Nature* **327**, 293-7.

Bourdon, J. C. (2007). p53 and its isoforms in cancer. *Br J Cancer* **97**, 277-82.

Boyer-Di Ponio, J., Wright-Crosnier, C., Groyer-Picard, M. T., Driancourt, C., Beau, I., Hadchouel, M. and Meunier-Rotival, M. (2007). Biological function of mutant forms of JAGGED1 proteins in Alagille syndrome: inhibitory effect on Notch signaling. *Hum Mol Genet* **16**, 2683-92.

Boyle, P. and Langman, J. S. (2000). ABC of colorectal cancer: Epidemiology. *Bmj* **321**, 805-8.

Boyle, P. and Leon, M. E. (2002). Epidemiology of colorectal cancer. *Br Med Bull* **64**, 1-25.

Brabletz, T., Hlubek, F., Spaderna, S., Schmalhofer, O., Hiendlmeyer, E., Jung, A. and Kirchner, T. (2005a). Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. *Cells Tissues Organs* **179**, 56-65.

Brabletz, T., Jung, A., Hermann, K., Gunther, K., Hohenberger, W. and Kirchner, T. (1998). Nuclear overexpression of the oncoprotein beta-catenin in colorectal cancer is localized predominantly at the invasion front. *Pathol Res Pract* **194**, 701-4.

Brabletz, T., Jung, A. and Kirchner, T. (2002). Beta-catenin and the morphogenesis of colorectal cancer. *Virchows Arch* **441**, 1-11.

Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L. A., Knuechel, R. and Kirchner, T. (2001). Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci U S A* **98**, 10356-61.

Brabletz, T., Jung, A., Spaderna, S., Hlubek, F. and Kirchner, T. (2005b). Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer* **5**, 744-9.

Bracey, T. S., Miller, J. C., Preece, A. and Paraskeva, C. (1995). Gamma-radiation-induced apoptosis in human colorectal adenoma and carcinoma cell lines can occur in the absence of wild type p53. *Oncogene* **10**, 2391-6.

Brattain, M. G., Fine, W. D., Khaled, F. M., Thompson, J. and Brattain, D. E. (1981). Heterogeneity of malignant cells from a human colonic carcinoma. *Cancer Res* **41**, 1751-6.

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* **7**, 678-89.

Breeden, L. and Nasmyth, K. (1987). Similarity between cell-cycle genes of budding yeast and fission yeast and the Notch gene of Drosophila. *Nature* **329**, 651-4.

Bronner, C. E., Baker, S. M., Morrison, P. T., Warren, G., Smith, L. G., Lescoe, M. K., Kane, M., Earabino, C., Lipford, J., Lindblom, A. *et al.* (1994). Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature* **368**, 258-61.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A. and Israel, A. (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* **5**, 207-16.

Brown, M. S., Ye, J., Rawson, R. B. and Goldstein, J. L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* **100**, 391-8.

Browne, S. J., Williams, A. C., Hague, A., Butt, A. J. and Paraskeva, C. (1994). Loss of APC protein expressed by human colonic epithelial cells and the appearance of a specific low-molecular-weight form is associated with apoptosis in vitro. *Int J Cancer* **59**, 56-64.

Bulman, M. P., Kusumi, K., Frayling, T. M., McKeown, C., Garrett, C., Lander, E. S., Krumlauf, R., Hattersley, A. T., Ellard, S. and Turnpenny, P. D. (2000). Mutations in the human delta homologue, DLL3, cause axial skeletal defects in spondylocostal dysostosis. *Nat Genet* **24**, 438-41.

Butt, A. J., Hague, A. and Paraskeva, C. (1997). Butyrate- but not TGFbeta1-induced apoptosis of colorectal adenoma cells is associated with increased expression of the differentiation markers E-cadherin and alkaline phosphatase. *Cell Death Differ* **4**, 725-32.

Callahan, R. and Egan, S. E. (2004). Notch signaling in mammary development and oncogenesis. *J Mammary Gland Biol Neoplasia* **9**, 145-63.

Capobianco, A. J., Zagouras, P., Blaumueller, C. M., Artavanis-Tsakonas, S. and Bishop, J. M. (1997). Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. *Mol Cell Biol* **17**, 6265-73.

Carlesso, N., Aster, J. C., Sklar, J. and Scadden, D. T. (1999). Notch1-induced delay of human hematopoietic progenitor cell differentiation is associated with altered cell cycle kinetics. *Blood* **93**, 838-48.

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**, 604-8.

Chan, S. S., Zheng, H., Su, M. W., Wilk, R., Killeen, M. T., Hedgecock, E. M. and Culotti, J. G. (1996). UNC-40, a C. elegans homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. *Cell* **87**, 187-95.

Chen, G. and Courey, A. J. (2000). Groucho/TLE family proteins and transcriptional repression. *Gene* **249**, 1-16.

Cheng, H. and Leblond, C. P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* **141**, 537-61.

Chiarugi, P. and Giannoni, E. (2008). Anoikis: a necessary death program for anchorage-dependent cells. *Biochem Pharmacol* **76**, 1352-64.

Chigurupati, S., Arumugam, T. V., Son, T. G., Lathia, J. D., Jameel, S., Mughal, M. R., Tang, S. C., Jo, D. G., Camandola, S., Giunta, M. *et al.* (2007). Involvement of notch signaling in wound healing. *PLoS One* **2**, e1167.

Christiansen, J. J. and Rajasekaran, A. K. (2006). Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis. *Cancer Res* **66**, 8319-26.

Christofori, G. and Semb, H. (1999). The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem Sci* **24**, 73-6.

Chu, D., Wang, W., Xie, H., Li, Y., Dong, G., Xu, C., Chen, D., Zheng, J., Li, M., Lu, Z. *et al.* (2009). Notch1 expression in colorectal carcinoma determines tumor differentiation status. *J Gastrointest Surg* **13**, 253-60.

Chwalinski, S. and Potten, C. S. (1989). Crypt base columnar cells in ileum of BDF1 male mice--their numbers and some features of their proliferation. *Am J Anat* **186**, 397-406.

Cinquin, O. (2006). Fast-tracking morphogen diffusion. *J Theor Biol* **238**, 532-40.

Cisneros, E., Latasa, M. J., Garcia-Flores, M. and Frade, J. M. (2008). Instability of Notch1 and Delta1 mRNAs and reduced Notch activity in vertebrate neuroepithelial cells undergoing S-phase. *Mol Cell Neurosci* **37**, 820-31.

Citron, M. (2004). Strategies for disease modification in Alzheimer's disease. *Nat Rev Neurosci* **5**, 677-85.

Clarke, A. R. (2006). Wnt signalling in the mouse intestine. *Oncogene* **25**, 7512-21.

Cohen, B., Bashirullah, A., Dagnino, L., Campbell, C., Fisher, W. W., Leow, C. C., Whiting, E., Ryan, D., Zinyk, D., Boulianne, G. *et al.* (1997). Fringe boundaries coincide with Notch-dependent patterning centres in mammals and alter Notch-dependent development in Drosophila. *Nat Genet* **16**, 283-8.

Collu, G. M. and Brennan, K. (2007). Cooperation between Wnt and Notch signalling in human breast cancer. *Breast Cancer Res* **9**, 105.

Come, C., Arnoux, V., Bibeau, F. and Savagner, P. (2004). Roles of the transcription factors snail and slug during mammary morphogenesis and breast carcinoma progression. *J Mammary Gland Biol Neoplasia* **9**, 183-93.

Compton, C. C. (2003). Colorectal carcinoma: diagnostic, prognostic, and molecular features. *Mod Pathol* **16**, 376-88.

Compton, C. C., Fielding, L. P., Burgart, L. J., Conley, B., Cooper, H. S., Hamilton, S. R., Hammond, M. E., Henson, D. E., Hutter, R. V., Nagle, R. B. *et al.* (2000). Prognostic factors in colorectal cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med* **124**, 979-94.

Conacci-Sorrell, M., Simcha, I., Ben-Yedidia, T., Blechman, J., Savagner, P. and Ben-Ze'ev, A. (2003). Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. *J Cell Biol* **163**, 847-57.

Conboy, I. M., Conboy, M. J., Wagers, A. J., Girma, E. R., Weissman, I. L. and Rando, T. A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* **433**, 760-4.

Conlon, R. A., Reaume, A. G. and Rossant, J. (1995). Notch1 is required for the coordinate segmentation of somites. *Development* **121**, 1533-45.

Cooper, M. T., Tyler, D. M., Furriols, M., Chalkiadaki, A., Delidakis, C. and Bray, S. (2000). Spatially restricted factors cooperate with notch in the regulation of Enhancer of split genes. *Dev Biol* **221**, 390-403.

Cortina, C., Palomo-Ponce, S., Iglesias, M., Fernandez-Masip, J. L., Vivancos, A., Whissell, G., Huma, M., Peiro, N., Gallego, L., Jonkheer, S. *et al.* (2007). EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells. *Nat Genet* **39**, 1376-83.

Couso, J. P., Knust, E. and Martinez Arias, A. (1995). Serrate and wingless cooperate to induce vestigial gene expression and wing formation in Drosophila. *Curr Biol* **5**, 1437-48.

Cregger, M., Berger, A. J. and Rimm, D. L. (2006). Immunohistochemistry and quantitative analysis of protein expression. *Arch Pathol Lab Med* **130**, 1026-30.

Crissman, H. A. and Steinkamp, J. A. (1973). Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. *J Cell Biol* **59**, 766-71.

Croquelois, A., Blindenbacher, A., Terracciano, L., Wang, X., Langer, I., Radtke, F. and Heim, M. H. (2005). Inducible inactivation of Notch1 causes nodular regenerative hyperplasia in mice. *Hepatology* **41**, 487-96.

Crosnier, C., Stamataki, D. and Lewis, J. (2006). Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet* **7**, 349-59.

Crosnier, C., Vargesson, N., Gschmeissner, S., Ariza-McNaughton, L., Morrison, A. and Lewis, J. (2005). Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine. *Development* **132**, 1093-104.

CRUK. (2008). Incidence report / Mortality report / Bowel cancer factsheet. *Cancer Research UK website; http://info.cancerresearchuk.org/cancerstats*.

Cully, M., You, H., Levine, A. J. and Mak, T. W. (2006). Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* **6**, 184-92.

Cummings, J. H., Pomare, E. W., Branch, W. J., Naylor, C. P. and Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut* **28**, 1221-7.

Curry, C. L., Reed, L. L., Golde, T. E., Miele, L., Nickoloff, B. J. and Foreman, K. E. (2005). Gamma secretase inhibitor blocks Notch activation and induces apoptosis in Kaposi's sarcoma tumor cells. *Oncogene* **24**, 6333-44.

Daly, K., Cuff, M. A., Fung, F. and Shirazi-Beechey, S. P. (2005). The importance of colonic butyrate transport to the regulation of genes associated with colonic tissue homoeostasis. *Biochem Soc Trans* **33**, 733-5.

Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W. *et al.* (2002). Mutations of the BRAF gene in human cancer. *Nature* **417**, 949-54.

Davis, R. L., Turner, D. L., Evans, L. M. and Kirschner, M. W. (2001). Molecular targets of vertebrate segmentation: two mechanisms control segmental expression of Xenopus hairy2 during somite formation. *Dev Cell* **1**, 553-65.

Dawson, S. R., Turner, D. L., Weintraub, H. and Parkhurst, S. M. (1995). Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. *Mol Cell Biol* **15**, 6923-31.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. *et al.* (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-48.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J. *et al.* (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-22.

de Visser, K. E., Eichten, A. and Coussens, L. M. (2006). Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* **6**, 24-37.

del Amo, F. F., Gendron-Maguire, M., Swiatek, P. J., Jenkins, N. A., Copeland, N. G. and Gridley, T. (1993). Cloning, analysis, and chromosomal localization of Notch-1, a mouse homolog of Drosophila Notch. *Genomics* **15**, 259-64.

Devgan, V., Mammucari, C., Millar, S. E., Brisken, C. and Dotto, G. P. (2005). p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation. *Genes Dev* **19**, 1485-95.

Deysson, G. (1968). Autimitotic substances. *Int Rev Cytol* **24**, 99-148.

Dievart, A., Beaulieu, N. and Jolicoeur, P. (1999). Involvement of Notch1 in the development of mouse mammary tumors. *Oncogene* **18**, 5973-81.

Dohda, T., Maljukova, A., Liu, L., Heyman, M., Grander, D., Brodin, D., Sangfelt, O. and Lendahl, U. (2007). Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines. *Exp Cell Res* **313**, 3141-52.

Donehower, R. C. (1992). An overview of the clinical experience with hydroxyurea. *Semin Oncol* **19**, 11-9.

Dosik, G. M., Barlogie, B., White, R. A., Gohde, W. and Drewinko, B. (1981). A rapid automated stathmokinetic method for determination of in vitro cell cycle transit times. *Cell Tissue Kinet* **14**, 121-34.

Dotto, G. P. (2008). Notch tumor suppressor function. *Oncogene* **27**, 5115-23.

Dou, S., Zeng, X., Cortes, P., Erdjument-Bromage, H., Tempst, P., Honjo, T. and Vales, L. D. (1994). The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. *Mol Cell Biol* **14**, 3310-9.

Dunwoodie, S. L., Clements, M., Sparrow, D. B., Sa, X., Conlon, R. A. and Beddington, R. S. (2002). Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene Dll3 are associated with disruption of the segmentation clock within the presomitic mesoderm. *Development* **129**, 1795-806.

Dunwoodie, S. L., Henrique, D., Harrison, S. M. and Beddington, R. S. (1997). Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* **124**, 3065-76.

Dvorak, H. F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**, 1650-9.

Dylla, S. J., Beviglia, L., Park, I. K., Chartier, C., Raval, J., Ngan, L., Pickell, K., Aguilar, J., Lazetic, S., Smith-Berdan, S. *et al.* (2008). Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS ONE* **3**, e2428.

Efstathiou, J. A., Liu, D., Wheeler, J. M., Kim, H. C., Beck, N. E., Ilyas, M., Karayiannakis, A. J., Mortensen, N. J., Kmiot, W., Playford, R. J. *et al.* (1999). Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells. *Proc Natl Acad Sci U S A* **96**, 2316-21.

Efstratiadis, A., Szabolcs, M. and Klinakis, A. (2007). Notch, Myc and breast cancer. *Cell Cycle* **6**, 418-29.

Eisenberg, L. M. and Markwald, R. R. (1995). Molecular regulation of atrioventricular valvuloseptal morphogenesis. *Circ Res* **77**, 1-6.

Elder, D. J., Hague, A., Hicks, D. J. and Paraskeva, C. (1996). Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: enhanced apoptosis in carcinoma and in vitro-transformed adenoma relative to adenoma relative to adenoma cell lines. *Cancer Res* **56**, 2273-6.

Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. (1991). TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649-61.

Endo, Y., Osumi, N. and Wakamatsu, Y. (2002). Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development. *Development* **129**, 863-73.

Engle, S. J., Hoying, J. B., Boivin, G. P., Ormsby, I., Gartside, P. S. and Doetschman, T. (1999). Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res* **59**, 3379-86.

Engstrom, P. F., MacIntyre, J. M., Mittelman, A. and Klaassen, D. J. (1984). Chemotherapy of advanced colorectal carcinoma: fluorouracil alone vs. two drug combinations using fluorouracil, hydroxyurea, semustine, dacarbazine, razoxane, and mitomycin. A phase III trial by the Eastern Cooperative Oncology Group (EST: 1278). *Am J Clin Oncol* **7**, 313-8.

Eppert, K., Scherer, S. W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L. C., Bapat, B., Gallinger, S., Andrulis, I. L. *et al.* (1996). MADR2 maps to 18q21 and encodes a TGFbeta-

regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* **86**, 543-52.

Eshleman, J. R., Lang, E. Z., Bowerfind, G. K., Parsons, R., Vogelstein, B., Willson, J. K., Veigl, M. L., Sedwick, W. D. and Markowitz, S. D. (1995). Increased mutation rate at the hprt locus accompanies microsatellite instability in colon cancer. *Oncogene* **10**, 33-7.

Espinosa, L., Ingles-Esteve, J., Aguilera, C. and Bigas, A. (2003). Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. *J Biol Chem* **278**, 32227-35.

Estrach, S., Ambler, C. A., Lo Celso, C., Hozumi, K. and Watt, F. M. (2006). Jagged 1 is a beta-catenin target gene required for ectopic hair follicle formation in adult epidermis. *Development* **133**, 4427-38.

Evan, G. I. and Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature* **411**, 342-8.

Evers, B. M., Ko, T. C., Li, J. and Thompson, E. A. (1996). Cell cycle protein suppression and p21 induction in differentiating Caco-2 cells. *Am J Physiol* **271**, G722-7.

Evrard, Y. A., Lun, Y., Aulehla, A., Gan, L. and Johnson, R. L. (1998). lunatic fringe is an essential mediator of somite segmentation and patterning. *Nature* **394**, 377-81.

Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J. and Livingston, D. M. (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73**, 487-97.

Fan, X., Mikolaenko, I., Elhassan, I., Ni, X., Wang, Y., Ball, D., Brat, D. J., Perry, A. and Eberhart, C. G. (2004). Notch1 and notch2 have opposite effects on embryonal brain tumor growth. *Cancer Res* **64**, 7787-93.

Fantini, J., Abadie, B., Tirard, A., Remy, L., Ripert, J. P., el Battari, A. and Marvaldi, J. (1986). Spontaneous and induced dome formation by two clonal cell populations derived from a human adenocarcinoma cell line, HT29. *J Cell Sci* **83**, 235-49.

Farnie, G., Clarke, R. B., Spence, K., Pinnock, N., Brennan, K., Anderson, N. G. and Bundred, N. J. (2007). Novel cell culture technique for primary ductal carcinoma in situ: role of Notch and epidermal growth factor receptor signaling pathways. *J Natl Cancer Inst* **99**, 616-27.

Farre, D., Roset, R., Huerta, M., Adsuara, J. E., Rosello, L., Alba, M. M. and Messeguer, X. (2003). Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. *Nucleic Acids Res* **31**, 3651-3.

Fearnhead, N. S., Wilding, J. L. and Bodmer, W. F. (2002). Genetics of colorectal cancer: hereditary aspects and overview of colorectal tumorigenesis. *Br Med Bull* **64**, 27-43.

Fearon, E. R. and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* **61**, 759-67.

Feder, J. N., Jan, L. Y. and Jan, Y. N. (1993). A rat gene with sequence homology to the Drosophila gene hairy is rapidly induced by growth factors known to influence neuronal differentiation. *Mol Cell Biol* **13**, 105-13.

Feinberg, A. P., Ohlsson, R. and Henikoff, S. (2006). The epigenetic progenitor origin of human cancer. *Nat Rev Genet* **7**, 21-33.

Femia, A. P., Giannini, A., Fazi, M., Tarquini, E., Salvadori, M., Roncucci, L., Tonelli, F., Dolara, P. and Caderni, G. (2008). Identification of mucin depleted foci in the human colon. *Cancer Prev Res (Phila Pa)* **1**, 562-7.

Feolo, M., Helmberg, W., Sherry, S. and Maglott, D. R. (2000). NCBI genetic resources supporting immunogenetic research. *Rev Immunogenet* **2**, 461-7.

Fernandez-Majada, V., Aguilera, C., Villanueva, A., Vilardell, F., Robert-Moreno, A., Aytes, A., Real, F. X., Capella, G., Mayo, M. W., Espinosa, L. *et al.* (2007). Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. *Proc Natl Acad Sci U S A* **104**, 276-81.

Ferrand, A., Bertrand, C., Portolan, G., Cui, G., Carlson, J., Pradayrol, L., Fourmy, D., Dufresne, M., Wang, T. C. and Seva, C. (2005). Signaling pathways associated with colonic mucosa hyperproliferation in mice overexpressing gastrin precursors. *Cancer Res* **65**, 2770-7.

Ferraris, R. P., Villenas, S. A. and Diamond, J. (1992). Regulation of brush-border enzyme activities and enterocyte migration rates in mouse small intestine. *Am J Physiol* **262**, G1047-59.

Fevr, T., Robine, S., Louvard, D. and Huelsken, J. (2007). Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. *Mol Cell Biol* **27**, 7551-9.

Fidler, I. J. (1991). Cancer metastasis. *Br Med Bull* **47**, 157-77.

Fior, R. and Henrique, D. (2005). A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. *Dev Biol* **281**, 318-33.

Fior, R. and Henrique, D. (2008). "Notch-Off": a perspective on the termination of Notch signalling. *Int J Dev Biol*.

Firth, L. C. and Baker, N. E. (2005). Extracellular signals responsible for spatially regulated proliferation in the differentiating Drosophila eye. *Dev Cell* **8**, 541-51.

Fischer, A., Steidl, C., Wagner, T. U., Lang, E., Jakob, P. M., Friedl, P., Knobeloch, K. P. and Gessler, M. (2007). Combined loss of Hey1 and HeyL causes congenital heart defects because of impaired epithelial to mesenchymal transition. *Circ Res* **100**, 856-63.

Fisher, A. and Caudy, M. (1998a). The function of hairy-related bHLH repressor proteins in cell fate decisions. *Bioessays* **20**, 298-306.

Fisher, A. L. and Caudy, M. (1998b). Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev* **12**, 1931-40.

Fitzgerald, K., Harrington, A. and Leder, P. (2000). Ras pathway signals are required for notch-mediated oncogenesis. *Oncogene* **19**, 4191-8.

Fiuza, U. M. and Arias, A. M. (2007). Cell and molecular biology of Notch. *J Endocrinol* **194**, 459-74.

Flandez, M., Guilmeau, S., Blache, P. and Augenlicht, L. H. (2008). KLF4 regulation in intestinal epithelial cell maturation. *Exp Cell Res* **314**, 3712-23.

Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997). Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. *Development* **124**, 2973-81.

Fodde, R. (2002). The APC gene in colorectal cancer. *Eur J Cancer* **38**, 867-71.

Fodde, R. and Brabletz, T. (2007). Wnt/beta-catenin signaling in cancer stemness and malignant behavior. *Curr Opin Cell Biol* **19**, 150-8.

Fodde, R., Edelmann, W., Yang, K., van Leeuwen, C., Carlson, C., Renault, B., Breukel, C., Alt, E., Lipkin, M., Khan, P. M. *et al.* (1994). A targeted chain-termination mutation in the mouse Apc gene results in multiple intestinal tumors. *Proc Natl Acad Sci U S A* **91**, 8969-73.

Foe, V. E. (1989). Mitotic domains reveal early commitment of cells in Drosophila embryos. *Development* **107**, 1-22.

Fogh, J., Anderson, H. C., Allen, B., Petursson, G., Saunders, E. L. and Dalldorf, G. (1964). Cultivation and Characterization of Cells from a Malignant Lymphoma in an African Child. *Cancer Res* **24**, 416-31.

Fogh, J., Fogh, J. M. and Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* **59**, 221-6.

Foltz, D. R., Santiago, M. C., Berechid, B. E. and Nye, J. S. (2002). Glycogen synthase kinase-3beta modulates notch signaling and stability. *Curr Biol* **12**, 1006-11.

Forrester, K., Almoguera, C., Han, K., Grizzle, W. E. and Perucho, M. (1987). Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature* **327**, 298-303.

Fortini, M. E. and Artavanis-Tsakonas, S. (1993). Notch: neurogenesis is only part of the picture. *Cell* **75**, 1245-7.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D. and Artavanis-Tsakonas, S. (2005). Notch signals control the fate of immature progenitor cells in the intestine. *Nature* **435**, 964-8.

Fre, S., Pallavi, S. K., Huyghe, M., Lae, M., Janssen, K. P., Robine, S., Artavanis-Tsakonas, S. and Louvard, D. (2009). Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. *Proc Natl Acad Sci U S A*.

Friedman, E., Gold, L. I., Klimstra, D., Zeng, Z. S., Winawer, S. and Cohen, A. (1995). High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. *Cancer Epidemiol Biomarkers Prev* **4**, 549-54.

Frixen, U. H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D. and Birchmeier, W. (1991). E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* **113**, 173-85.

Fryer, C. J., Lamar, E., Turbachova, I., Kintner, C. and Jones, K. A. (2002). Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes Dev* **16**, 1397-411.

Fryer, C. J., White, J. B. and Jones, K. A. (2004). Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell* **16**, 509-20.

Fuchs, E. (1998). Beauty is skin deep: the fascinating biology of the epidermis and its appendages. *Harvey Lect* **94**, 47-77.

Fusse, B. and Hoch, M. (2002). Notch signaling controls cell fate specification along the dorsoventral axis of the Drosophila gut. *Curr Biol* **12**, 171-9.

Gabbert, H., Wagner, R., Moll, R. and Gerharz, C. D. (1985). Tumor dedifferentiation: an important step in tumor invasion. *Clin Exp Metastasis* **3**, 257-79.

Galceran, J., Sustmann, C., Hsu, S. C., Folberth, S. and Grosschedl, R. (2004). LEF1-mediated regulation of Delta-like1 links Wnt and Notch signaling in somitogenesis. *Genes Dev* **18**, 2718-23.

Gallahan, D., Jhappan, C., Robinson, G., Hennighausen, L., Sharp, R., Kordon, E., Callahan, R., Merlino, G. and Smith, G. H. (1996). Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. *Cancer Res* **56**, 1775-85.

Garber, J. E., Goldstein, A. M., Kantor, A. F., Dreyfus, M. G., Fraumeni, J. F., Jr. and Li, F. P. (1991). Follow-up study of twenty-four families with Li-Fraumeni syndrome. *Cancer Res* **51**, 6094-7.

Garces, C., Ruiz-Hidalgo, M. J., Font de Mora, J., Park, C., Miele, L., Goldstein, J., Bonvini, E., Porras, A. and Laborda, J. (1997). Notch-1 controls the expression of fatty acid-activated transcription factors and is required for adipogenesis. *J Biol Chem* **272**, 29729-34.

Garrett-Sinha, L. A., Eberspaecher, H., Seldin, M. F. and de Crombrugghe, B. (1996). A gene for a novel zinc-finger protein expressed in differentiated epithelial cells and transiently in certain mesenchymal cells. *J Biol Chem* **271**, 31384-90.

Gartner, L. P. and Hiatt, J. L. (1997). Digestive system II - Alimentary canal. *In Colour Textbook of Histology*, pp.333-337: Saunders company.

Gaspar, C., Cardoso, J., Franken, P., Molenaar, L., Morreau, H., Moslein, G., Sampson, J., Boer, J. M., de Menezes, R. X. and Fodde, R. (2008). Cross-species comparison of human and mouse intestinal polyps reveals conserved mechanisms in adenomatous polyposis coli (APC)-driven tumorigenesis. *Am J Pathol* **172**, 1363-80.

Gayet, J., Zhou, X. P., Duval, A., Rolland, S., Hoang, J. M., Cottu, P. and Hamelin, R. (2001). Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. *Oncogene* **20**, 5025-32.

Gebauer, F. and Hentze, M. W. (2004). Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol* **5**, 827-35.

Gersemann, M., Becker, S., Kubler, I., Koslowski, M., Wang, G., Herrlinger, K. R., Griger, J., Fritz, P., Fellermann, K., Schwab, M. *et al.* (2009). Differences in goblet cell differentiation between Crohn's disease and ulcerative colitis. *Differentiation* **77**, 84-94.

Ghaleb, A. M., Aggarwal, G., Bialkowska, A. B., Nandan, M. O. and Yang, V. W. (2008). Notch inhibits expression of the Kruppel-like factor 4 tumor suppressor in the intestinal epithelium. *Mol Cancer Res* **6**, 1920-7.

Gillespie, D. A. and Henriques, C. (2006). Centrifugal elutriation as a means of cell cycle phase separation and synchronisation. *Subcell Biochem* **40**, 359-61.

Girard, L., Hanna, Z., Beaulieu, N., Hoemann, C. D., Simard, C., Kozak, C. A. and Jolicoeur, P. (1996). Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. *Genes Dev* **10**, 1930-44.

Go, M. J. and Artavanis-Tsakonas, S. (1998). A genetic screen for novel components of the notch signaling pathway during Drosophila bristle development. *Genetics* **150**, 211-20.

Gofuku, J., Shiozaki, H., Tsujinaka, T., Inoue, M., Tamura, S., Doki, Y., Matsui, S., Tsukita, S., Kikkawa, N. and Monden, M. (1999). Expression of E-cadherin and alpha-catenin in patients with colorectal carcinoma. Correlation with cancer invasion and metastasis. *Am J Clin Pathol* **111**, 29-37.

Gordon, W. R., Vardar-Ulu, D., Histen, G., Sanchez-Irizarry, C., Aster, J. C. and Blacklow, S. C. (2007). Structural basis for autoinhibition of Notch. *Nat Struct Mol Biol* **14**, 295-300.

Goumans, M. J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P. and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* **21**, 1743-53.

Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E. (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-32.

Gradwohl, G., Dierich, A., LeMeur, M. and Guillemot, F. (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. *Proc Natl Acad Sci U S A* **97**, 1607-11.

Grady, W. M., Myeroff, L. L., Swinler, S. E., Rajput, A., Thiagalingam, S., Lutterbaugh, J. D., Neumann, A., Brattain, M. G., Chang, J., Kim, S. J. *et al.* (1999). Mutational inactivation of

transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Res* **59**, 320-4.

Grasset, E., Bernabeu, J. and Pinto, M. (1985). Epithelial properties of human colonic carcinoma cell line Caco-2: effect of secretagogues. *Am J Physiol* **248**, C410-8.

Grasset, E., Pinto, M., Dussaulx, E., Zweibaum, A. and Desjeux, J. F. (1984). Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am J Physiol* **247**, C260-7.

Gray, G. E., Mann, R. S., Mitsiadis, E., Henrique, D., Carcangiu, M. L., Banks, A., Leiman, J., Ward, D., Ish-Horowitz, D. and Artavanis-Tsakonas, S. (1999). Human ligands of the Notch receptor. *Am J Pathol* **154**, 785-94.

Gray, J. W. and Darzynkiewicz, Z. (1987). Techniques in cell cycle analysis: Humana Press.

Greaves, L. C., Preston, S. L., Tadrous, P. J., Taylor, R. W., Barron, M. J., Oukrif, D., Leedham, S. J., Deheragoda, M., Sasieni, P., Novelli, M. R. *et al.* (2006). Mitochondrial DNA mutations are established in human colonic stem cells, and mutated clones expand by crypt fission. *Proc Natl Acad Sci U S A* **103**, 714-9.

Greenwald, I. (1998). LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev* **12**, 1751-62.

Grego-Bessa, J., Diez, J., Timmerman, L. and de la Pompa, J. L. (2004). Notch and epithelial-mesenchyme transition in development and tumor progression: another turn of the screw. *Cell Cycle* **3**, 718-21.

Gregorieff, A., Pinto, D., Begthel, H., Destree, O., Kielman, M. and Clevers, H. (2005). Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology* **129**, 626-38.

Gridley, T. (2001). Notch signaling during vascular development. *Proc Natl Acad Sci U S A* **98**, 5377-8.

Gridley, T. (2003). Notch signaling and inherited disease syndromes. *Hum Mol Genet* **12 Spec No 1**, R9-13.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M. *et al.* (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66**, 589-600.

Grosveld, G. C. (2009). Gamma-secretase inhibitors: Notch so bad. *Nat Med* **15**, 20-1.

Grunert, S., Jechlinger, M. and Beug, H. (2003). Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. *Nat Rev Mol Cell Biol* **4**, 657-65.

Guentchev, M. and McKay, R. D. (2006). Notch controls proliferation and differentiation of stem cells in a dose-dependent manner. *Eur J Neurosci* **23**, 2289-96.

Guilmeau, S., Flandez, M., Bancroft, L., Sellers, R. S., Tear, B., Stanley, P. and Augenlicht, L. H. (2008). Intestinal deletion of Pofut1 in the mouse inactivates notch signaling and causes enterocolitis. *Gastroenterology* **135**, 849-60, 860 e1-6.

Guilmeau, S., Flandez, M., Mariadason, J. M. and Augenlicht, L. H. (2009). Heterogeneity of Jagged1 expression in human and mouse intestinal tumors: implications for targeting Notch signaling. *Oncogene*.

Gum, J. R., Kam, W. K., Byrd, J. C., Hicks, J. W., Sleisenger, M. H. and Kim, Y. S. (1987). Effects of sodium butyrate on human colonic adenocarcinoma cells. Induction of placental-like alkaline phosphatase. *J Biol Chem* **262**, 1092-7.

Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.

Gupta, S. K. and Singh, J. P. (1994). Inhibition of endothelial cell proliferation by platelet factor-4 involves a unique action on S phase progression. *J Cell Biol* **127**, 1121-7.

Guy, M., Moorghen, M., Bond, J. A., Collard, T. J., Paraskeva, C. and Williams, A. C. (2001). Transcriptional down-regulation of the retinoblastoma protein is associated with differentiation and apoptosis in human colorectal epithelial cells. *Br J Cancer* **84**, 520-8.

Haddon, C., Mowbray, C., Whitfield, T., Jones, D., Gschmeissner, S. and Lewis, J. (1999). Hair cells without supporting cells: further studies in the ear of the zebrafish mind bomb mutant. *J Neurocytol* **28**, 837-50.

Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D. and Lewis, J. (1998). Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125**, 359-70.

Haddow, A. (1972). Molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing? *Adv Cancer Res* **16**, 181-234.

Haggitt, R. C. and Reid, B. J. (1986). Hereditary gastrointestinal polyposis syndromes. *Am J Surg Pathol* **10**, 871-87.

Hague, A., Elder, D. J., Hicks, D. J. and Paraskeva, C. (1995). Apoptosis in colorectal tumour cells: induction by the short chain fatty acids butyrate, propionate and acetate and by the bile salt deoxycholate. *Int J Cancer* **60**, 400-6.

Hague, A., Manning, A. M., Hanlon, K. A., Huschtscha, L. I., Hart, D. and Paraskeva, C. (1993). Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int J Cancer* **55**, 498-505.

Hahn, W. C. and Weinberg, R. A. (2002). Rules for making human tumor cells. *N Engl J Med* **347**, 1593-603.

Haines, N. and Irvine, K. D. (2003). Glycosylation regulates Notch signalling. *Nat Rev Mol Cell Biol* **4**, 786-97.

Hallikas, O., Palin, K., Sinjushina, N., Rautiainen, R., Partanen, J., Ukkonen, E. and Taipale, J. (2006). Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. *Cell* **124**, 47-59.

Hamada, Y., Kadokawa, Y., Okabe, M., Ikawa, M., Coleman, J. R. and Tsujimoto, Y. (1999). Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development* **126**, 3415-24.

Hamilton, S. and Aaltonen, L. (2000). International Agency for Research on Cancer (IARC). Tumours of the colon and rectum Lyon: IARC press.

Hanahan, D. and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* **100**, 57-70.

Hanby, A. M., Chinery, R., Poulsom, R., Playford, R. J. and Pignatelli, M. (1996). Downregulation of E-cadherin in the reparative epithelium of the human gastrointestinal tract. *Am J Pathol* **148**, 723-9.

Hara, A., Hibi, T., Yoshioka, M., Toda, K., Watanabe, N., Hayashi, A., Iwao, Y., Saito, H., Watanabe, T. and Tsuchiya, M. (1993). Changes of proliferative activity and phenotypes in spontaneous differentiation of a colon cancer cell line. *Jpn J Cancer Res* **84**, 625-32.

Harada, N., Tamai, Y., Ishikawa, T., Sauer, B., Takaku, K., Oshima, M. and Taketo, M. M. (1999). Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. *EMBO J* **18**, 5931-42.

Haramis, A. P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G. J. and Clevers, H. (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* **303**, 1684-6.

Hardwick, J. C., Van Den Brink, G. R., Bleuming, S. A., Ballester, I., Van Den Brande, J. M., Keller, J. J., Offerhaus, G. J., Van Deventer, S. J. and Peppelenbosch, M. P. (2004). Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. *Gastroenterology* **126**, 111-21.

Harper, J. A., Yuan, J. S., Tan, J. B., Visan, I. and Guidos, C. J. (2003). Notch signaling in development and disease. *Clin Genet* **64**, 461-72.

Hart, M. J., de los Santos, R., Albert, I. N., Rubinfeld, B. and Polakis, P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. *Curr Biol* **8**, 573-81.

Hartley, D. A., Preiss, A. and Artavanis-Tsakonas, S. (1988). A deduced gene product from the Drosophila neurogenic locus, enhancer of split, shows homology to mammalian G-protein beta subunit. *Cell* **55**, 785-95.

Hartwell, L. H. and Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science* **246**, 629-34.

Hay, E. D. (2005). The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. *Dev Dyn* **233**, 706-20.

Hayward, P., Balayo, T. and Martinez Arias, A. (2006). Notch synergizes with axin to regulate the activity of armadillo in Drosophila. *Dev Dyn* **235**, 2656-66.

Hayward, P., Brennan, K., Sanders, P., Balayo, T., DasGupta, R., Perrimon, N. and Martinez Arias, A. (2005). Notch modulates Wnt signalling by associating with Armadillo/beta-catenin and regulating its transcriptional activity. *Development* **132**, 1819-30.

Hayward, P., Kalmar, T. and Arias, A. M. (2008). Wnt/Notch signalling and information processing during development. *Development* **135**, 411-24.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-12.

He, X. C., Yin, T., Grindley, J. C., Tian, Q., Sato, T., Tao, W. A., Dirisina, R., Porter-Westpfahl, K. S., Hembree, M., Johnson, T. *et al.* (2007). PTEN-deficient intestinal stem cells initiate intestinal polyposis. *Nat Genet* **39**, 189-98.

He, X. C., Zhang, J., Tong, W. G., Tawfik, O., Ross, J., Scoville, D. H., Tian, Q., Zeng, X., He, X., Wiedemann, L. M. *et al.* (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* **36**, 1117-21.

Heath, J. P. (1996). Epithelial cell migration in the intestine. *Cell Biol Int* **20**, 139-46.

Hebbes, T. R., Thorne, A. W. and Crane-Robinson, C. (1988). A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J* **7**, 1395-402.

Hebert, J., Cayuela, J. M., Berkeley, J. and Sigaux, F. (1994). Candidate tumor-suppressor genes MTS1 (p16INK4A) and MTS2 (p15INK4B) display frequent homozygous deletions in primary cells from T- but not from B-cell lineage acute lymphoblastic leukemias. *Blood* **84**, 4038-44.

Hecht, A. and Stemmler, M. P. (2003). Identification of a promoter-specific transcriptional activation domain at the C terminus of the Wnt effector protein T-cell factor 4. *J Biol Chem* **278**, 3776-85.

Heerdt, B. G., Houston, M. A. and Augenlicht, L. H. (1994). Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Res* **54**, 3288-93.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. *Development* **122**, 161-71.

Henkel, T., Ling, P. D., Hayward, S. D. and Peterson, M. G. (1994). Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa. *Science* **265**, 92-5.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-90.

Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M., Bruns, C. J. and Heeschen, C. (2007). Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* **1**, 313-23.

Hermiston, M. L., Wong, M. H. and Gordon, J. I. (1996). Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. *Genes Dev* **10**, 985-96.

Herpin, A. and Cunningham, C. (2007). Cross-talk between the bone morphogenetic protein pathway and other major signaling pathways results in tightly regulated cell-specific outcomes. *FEBS J* **274**, 2977-85.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat Cell Biol* **2**, 515-20.

Hidalgo, I. J., Raub, T. J. and Borchardt, R. T. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**, 736-49.

Higaki, J., Quon, D., Zhong, Z. and Cordell, B. (1995). Inhibition of beta-amyloid formation identifies proteolytic precursors and subcellular site of catabolism. *Neuron* **14**, 651-9.

Hinoi, T., Akyol, A., Theisen, B. K., Ferguson, D. O., Greenson, J. K., Williams, B. O., Cho, K. R. and Fearon, E. R. (2007). Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. *Cancer Res* **67**, 9721-30.

Hirata, H., Bessho, Y., Kokubu, H., Masamizu, Y., Yamada, S., Lewis, J. and Kageyama, R. (2004). Instability of Hes7 protein is crucial for the somite segmentation clock. *Nat Genet* **36**, 750-4.

Hirata, H., Yoshiura, S., Ohtsuka, T., Bessho, Y., Harada, T., Yoshikawa, K. and Kageyama, R. (2002). Oscillatory expression of the bHLH factor Hes1 regulated by a negative feedback loop. *Science* **298**, 840-3.

Hirohashi, S. (1998). Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* **153**, 333-9.

Hlsken, J. and Behrens, J. (2000). The Wnt signalling pathway. *J Cell Sci* **113 (Pt 20)**, 3545.

Hocker, M. and Wiedenmann, B. (1998). Molecular mechanisms of enteroendocrine differentiation. *Ann N Y Acad Sci* **859**, 160-74.

Hoffman, J., Kuhnert, F., Davis, C. R. and Kuo, C. J. (2004). Wnts as essential growth factors for the adult small intestine and colon. *Cell Cycle* **3**, 554-7.

Hofmann, M., Schuster-Gossler, K., Watabe-Rudolph, M., Aulehla, A., Herrmann, B. G. and Gossler, A. (2004). WNT signaling, in synergy with T/TBX6, controls Notch signaling by regulating Dll1 expression in the presomitic mesoderm of mouse embryos. *Genes Dev* **18**, 2712-7.

Hogan, B. L. (1999). Morphogenesis. *Cell* **96**, 225-33.

Holmberg, J., Genander, M., Halford, M. M., Anneren, C., Sondell, M., Chumley, M. J., Silvany, R. E., Henkemeyer, M. and Frisen, J. (2006). EphB receptors coordinate migration and proliferation in the intestinal stem cell niche. *Cell* **125**, 1151-63.

Holtzer, H., Rubinstein, N., Fellini, S., Yeoh, G., Chi, J., Birnbaum, J. and Okayama, M. (1975). Lineages, quantal cell cycles, and the generation of cell diversity. *Q Rev Biophys* **8**, 523-57.

Hopfer, O., Zwahlen, D., Fey, M. F. and Aebi, S. (2005). The Notch pathway in ovarian carcinomas and adenomas. *Br J Cancer* **93**, 709-18.

Hoshino, H., Miyoshi, N., Nagai, K., Tomimaru, Y., Nagano, H., Sekimoto, M., Doki, Y., Mori, M. and Ishii, H. (2009). Epithelial-mesenchymal transition with expression of SNAI1-induced chemoresistance in colorectal cancer. *Biochem Biophys Res Commun* **390**, 1061-5.

Houlston, R., Bevan, S., Williams, A., Young, J., Dunlop, M., Rozen, P., Eng, C., Markie, D., Woodford-Richens, K., Rodriguez-Bigas, M. A. *et al.* (1998). Mutations in DPC4 (SMAD4) cause juvenile polyposis syndrome, but only account for a minority of cases. *Hum Mol Genet* **7**, 1907-12.

Houlston, R. S. (2001). What we could do now: molecular pathology of colorectal cancer. *Mol Pathol* **54**, 206-14.

Hovanes, K., Li, T. W., Munguia, J. E., Truong, T., Milovanovic, T., Lawrence Marsh, J., Holcombe, R. F. and Waterman, M. L. (2001). Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. *Nat Genet* **28**, 53-7.

Howe, J. R., Bair, J. L., Sayed, M. G., Anderson, M. E., Mitros, F. A., Petersen, G. M., Velculescu, V. E., Traverso, G. and Vogelstein, B. (2001). Germline mutations of the gene encoding bone morphogenetic protein receptor 1A in juvenile polyposis. *Nat Genet* **28**, 184-7.

Howe, J. R., Roth, S., Ringold, J. C., Summers, R. W., Jarvinen, H. J., Sistonen, P., Tomlinson, I. P., Houlston, R. S., Bevan, S., Mitros, F. A. *et al.* (1998). Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* **280**, 1086-8.

Hrabe de Angelis, M., McIntyre, J., 2nd and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue DII1. *Nature* **386**, 717-21.

Hu, C., Dievart, A., Lupien, M., Calvo, E., Tremblay, G. and Jolicoeur, P. (2006). Overexpression of activated murine Notch1 and Notch3 in transgenic mice blocks mammary gland development and induces mammary tumors. *Am J Pathol* **168**, 973-90.

Huang, F., Hsu, S., Yan, Z., Winawer, S. and Friedman, E. (1994). The capacity for growth stimulation by TGF beta 1 seen only in advanced colon cancers cannot be ascribed to mutations in APC, DCC, p53 or ras. *Oncogene* **9**, 3701-6.

Huang, H. P., Liu, M., El-Hodiri, H. M., Chu, K., Jamrich, M. and Tsai, M. J. (2000). Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. *Mol Cell Biol* **20**, 3292-307.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G. and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech Dev* **59**, 3-10.

Hurlbut, G. D., Kankel, M. W., Lake, R. J. and Artavanis-Tsakonas, S. (2007). Crossing paths with Notch in the hyper-network. *Curr Opin Cell Biol* **19**, 166-75.

Ichii, S., Horii, A., Nakatsuru, S., Furuyama, J., Utsunomiya, J. and Nakamura, Y. (1992). Inactivation of both APC alleles in an early stage of colon adenomas in a patient with familial adenomatous polyposis (FAP). *Hum Mol Genet* **1**, 387-90.

Ilyas, M. (2005). Wnt signalling and the mechanistic basis of tumour development. *J Pathol* **205**, 130-44.

Ilyas, M., Tomlinson, I. P., Rowan, A., Pignatelli, M. and Bodmer, W. F. (1997). Beta-catenin mutations in cell lines established from human colorectal cancers. *Proc Natl Acad Sci U S A* **94**, 10330-4.

Imai, T., Tokunaga, A., Yoshida, T., Hashimoto, M., Mikoshiba, K., Weinmaster, G., Nakafuku, M. and Okano, H. (2001). The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. *Mol Cell Biol* **21**, 3888-900.

Imatani, A. and Callahan, R. (2000). Identification of a novel NOTCH-4/INT-3 RNA species encoding an activated gene product in certain human tumor cell lines. *Oncogene* **19**, 223-31.

Iravani, M., Dhat, R. and Price, C. M. (1997). Methylation of the multi tumor suppressor gene-2 (MTS2, CDKN1, p15INK4B) in childhood acute lymphoblastic leukemia. *Oncogene* **15**, 2609-14.

Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A. R., Sansom, O. J. and Winton, D. J. (2004). Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin. *Gastroenterology* **126**, 1236-46.

Irvine, K. D. and Wieschaus, E. (1994). fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development. *Cell* **79**, 595-606.

Ishibashi, M., Ang, S. L., Shiota, K., Nakanishi, S., Kageyama, R. and Guillemot, F. (1995). Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. *Genes Dev* **9**, 3136-48.

Ishibashi, M., Moriyoshi, K., Sasai, Y., Shiota, K., Nakanishi, S. and Kageyama, R. (1994). Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. *Embo J* **13**, 1799-805.

Iso, T., Kedes, L. and Hamamori, Y. (2003). HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol* **194**, 237-55.

Iso, T., Sartorelli, V., Chung, G., Shichinohe, T., Kedes, L. and Hamamori, Y. (2001a). HERP, a new primary target of Notch regulated by ligand binding. *Mol Cell Biol* **21**, 6071-9.

Iso, T., Sartorelli, V., Poizat, C., Iezzi, S., Wu, H. Y., Chung, G., Kedes, L. and Hamamori, Y. (2001b). HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Mol Cell Biol* **21**, 6080-9.

Ito, T., Udaka, N., Yazawa, T., Okudela, K., Hayashi, H., Sudo, T., Guillemot, F., Kageyama, R. and Kitamura, H. (2000). Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. *Development* **127**, 3913-21.

Ivanova, N. B., Dimos, J. T., Schaniel, C., Hackney, J. A., Moore, K. A. and Lemischka, I. R. (2002). A stem cell molecular signature. *Science* **298**, 601-4.

Iwao, K., Nakamori, S., Kameyama, M., Imaoka, S., Kinoshita, M., Fukui, T., Ishiguro, S., Nakamura, Y. and Miyoshi, Y. (1998). Activation of the beta-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. *Cancer Res* **58**, 1021-6.

Jackson, P. A., Green, M. A., Pouli, A., Hubbard, R., Marks, C. G. and Cook, M. G. (1995). Relation between stage, grade, proliferation, and expression of p53 and CD44 in adenomas and carcinomas of the colorectum. *J Clin Pathol* **48**, 1098-101.

Jaime, L., Xuanmao, J., Toshiyuki, S., Mathew, C. C., Lawrence, A. S., Thai, H. T., Xiaoming, J., Manran, L., Zhiping, L., Chenguang, W. *et al.* (2008). ErbB2 Induces Notch1 Activity and Function in Breast Cancer Cells. *Clinical and Translational Science* **1**, 107-115.

Jaks, V., Barker, N., Kasper, M., van Es, J. H., Snippert, H. J., Clevers, H. and Toftgard, R. (2008). Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nat Genet* **40**, 1291-9.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-8.

Jass, J. R. and Sobin, L. H. (1989). Histological Typing of Intestinal Tumours: Springer-Verlag.

Jeffries, S., Robbins, D. J. and Capobianco, A. J. (2002). Characterization of a high-molecular-weight Notch complex in the nucleus of Notch(ic)-transformed RKE cells and in a human T-cell leukemia cell line. *Mol Cell Biol* **22**, 3927-41.

Jen, J., Powell, S. M., Papadopoulos, N., Smith, K. J., Hamilton, S. R., Vogelstein, B. and Kinzler, K. W. (1994). Molecular determinants of dysplasia in colorectal lesions. *Cancer Res* **54**, 5523-6.

Jenny, M., Uhl, C., Roche, C., Duluc, I., Guillermin, V., Guillemot, F., Jensen, J., Kedinger, M. and Gradwohl, G. (2002). Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. *Embo J* **21**, 6338-47.

Jensen, J., Pedersen, E. E., Galante, P., Hald, J., Heller, R. S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P. and Madsen, O. D. (2000). Control of endodermal endocrine development by Hes-1. *Nat Genet* **24**, 36-44.

Jha, M. N., Bamburg, J. R. and Bedford, J. S. (1994). Cell cycle arrest by Colcemid differs in human normal and tumor cells. *Cancer Res* **54**, 5011-5.

Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G. H., Merlino, G. and Callahan, R. (1992). Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. *Genes Dev* **6**, 345-55.

Jiang, Y. J., Aerne, B. L., Smithers, L., Haddon, C., Ish-Horowicz, D. and Lewis, J. (2000). Notch signalling and the synchronization of the somite segmentation clock. *Nature* **408**, 475-9.

Jiang, Y. J., Brand, M., Heisenberg, C. P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt, M. *et al.* (1996). Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. *Development* **123**, 205-16.

Jiang, Y. J., Smithers, L. and Lewis, J. (1998). Vertebrate segmentation: the clock is linked to Notch signalling. *Curr Biol* **8**, R868-71.

Jin, Y. H., Kim, H., Ki, H., Yang, I., Yang, N., Lee, K. Y., Kim, N., Park, H. S. and Kim, K. (2009a). Beta-catenin modulates the level and transcriptional activity of Notch1/NICD through its direct interaction. *Biochim Biophys Acta* **1793**, 290-9.

Jin, Y. H., Kim, H., Oh, M., Ki, H. and Kim, K. (2009b). Regulation of Notch1/NICD and Hes1 expressions by GSK-3alpha/beta. *Mol Cells* **27**, 15-9.

Johnson, J. E., Birren, S. J. and Anderson, D. J. (1990). Two rat homologues of Drosophila achaete-scute specifically expressed in neuronal precursors. *Nature* **346**, 858-61.

Johnston, L. A. and Edgar, B. A. (1998). Wingless and Notch regulate cell-cycle arrest in the developing Drosophila wing. *Nature* **394**, 82-4.

Johnston, S. H., Rauskolb, C., Wilson, R., Prabhakaran, B., Irvine, K. D. and Vogt, T. F. (1997). A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway. *Development* **124**, 2245-54.

Jones, P., May, G., Healy, L., Brown, J., Hoyne, G., Delassus, S. and Enver, T. (1998). Stromal expression of Jagged 1 promotes colony formation by fetal hematopoietic progenitor cells. *Blood* **92**, 1505-11.

Jones, R. G., Li, X., Gray, P. D., Kuang, J., Clayton, F., Samowitz, W. S., Madison, B. B., Gumucio, D. L. and Kuwada, S. K. (2006). Conditional deletion of beta1 integrins in the intestinal epithelium

causes a loss of Hedgehog expression, intestinal hyperplasia, and early postnatal lethality. *J Cell Biol* **175**, 505-14.

Jones, S., Chen, W. D., Parmigiani, G., Diehl, F., Beerenwinkel, N., Antal, T., Traulsen, A., Nowak, M. A., Siegel, C., Velculescu, V. E. *et al.* (2008). Comparative lesion sequencing provides insights into tumor evolution. *Proc Natl Acad Sci U S A* **105**, 4283-8.

Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M. *et al.* (1991). Identification of deletion mutations and three new genes at the familial polyposis locus. *Cell* **66**, 601-13.

Joutel, A., Dodick, D. D., Parisi, J. E., Cecillon, M., Tournier-Lasserve, E. and Bousser, M. G. (2000). De novo mutation in the Notch3 gene causing CADASIL. *Ann Neurol* **47**, 388-91.

Jubb, A. M., Zhong, F., Bheddah, S., Grabsch, H. I., Frantz, G. D., Mueller, W., Kavi, V., Quirke, P., Polakis, P. and Koeppen, H. (2005). EphB2 is a prognostic factor in colorectal cancer. *Clin Cancer Res* **11**, 5181-7.

Jung, A., Schrauder, M., Oswald, U., Knoll, C., Sellberg, P., Palmqvist, R., Niedobitek, G., Brabletz, T. and Kirchner, T. (2001). The invasion front of human colorectal adenocarcinomas shows co-localization of nuclear beta-catenin, cyclin D1, and p16INK4A and is a region of low proliferation. *Am J Pathol* **159**, 1613-7.

Kadesch, T. (2004). Notch signaling: the demise of elegant simplicity. *Curr Opin Genet Dev* **14**, 506-12.

Kageyama, R. and Nakanishi, S. (1997). Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. *Curr Opin Genet Dev* **7**, 659-65.

Kageyama, R., Ohtsuka, T., Shimojo, H. and Imayoshi, I. (2008). Dynamic Notch signaling in neural progenitor cells and a revised view of lateral inhibition. *Nat Neurosci* **11**, 1247-51.

Kaidi, A., Qualtrough, D., Williams, A. C. and Paraskeva, C. (2006). Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res* **66**, 6683-91.

Kaneko, Y., Sakakibara, S., Imai, T., Suzuki, A., Nakamura, Y., Sawamoto, K., Ogawa, Y., Toyama, Y., Miyata, T. and Okano, H. (2000). Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. *Dev Neurosci* **22**, 139-53.

Kang, Y. and Massague, J. (2004). Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell* **118**, 277-9.

Kao, H. Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C. R., Evans, R. M. and Kadesch, T. (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev* **12**, 2269-77.

Kapeller, R. and Cantley, L. C. (1994). Phosphatidylinositol 3-kinase. *Bioessays* **16**, 565-76.

Karam, S. M. (1999). Lineage commitment and maturation of epithelial cells in the gut. *Front Biosci* **4**, D286-98.

Karayiannakis, A. J., Syrigos, K. N., Efstathiou, J., Valizadeh, A., Noda, M., Playford, R. J., Kmiot, W. and Pignatelli, M. (1998). Expression of catenins and E-cadherin during epithelial restitution in inflammatory bowel disease. *J Pathol* **185**, 413-8.

Karreth, F. and Tuveson, D. A. (2004). Twist induces an epithelial-mesenchymal transition to facilitate tumor metastasis. *Cancer Biol Ther* **3**, 1058-9.

Karsan, A. (2008). Notch and integrin affinity: a sticky situation. *Sci Signal* **1**, pe2.

Karsunky, H., Zeng, H., Schmidt, T., Zevnik, B., Kluge, R., Schmid, K. W., Duhrsen, U. and Moroy, T. (2002). Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. *Nat Genet* **30**, 295-300.

Katz, J. P., Perreault, N., Goldstein, B. G., Lee, C. S., Labosky, P. A., Yang, V. W. and Kaestner, K. H. (2002). The zinc-finger transcription factor Klf4 is required for terminal differentiation of goblet cells in the colon. *Development* **129**, 2619-28.

Kayahara, T., Sawada, M., Takaishi, S., Fukui, H., Seno, H., Fukuzawa, H., Suzuki, K., Hiai, H., Kageyama, R., Okano, H. *et al.* (2003). Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine. *FEBS Lett* **535**, 131-5.

Kazanjian, A., Wallis, D., Au, N., Nigam, R., Venken, K. J., Cagle, P. T., Dickey, B. F., Bellen, H. J., Gilks, C. B. and Grimes, H. L. (2004). Growth factor independence-1 is expressed in primary human neuroendocrine lung carcinomas and mediates the differentiation of murine pulmonary neuroendocrine cells. *Cancer Res* **64**, 6874-82.

Kedinger, M., Simon-Assmann, P. M., Lacroix, B., Marxer, A., Hauri, H. P. and Haffen, K. (1986). Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. *Dev Biol* **113**, 474-83.

Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S., Culotti, J. G. and Tessier-Lavigne, M. (1996). Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* **87**, 175-85.

Keith, B. and Simon, M. C. (2007). Hypoxia-inducible factors, stem cells, and cancer. *Cell* **129**, 465-72.

Kelleher, F. C., Fennelly, D. and Rafferty, M. (2006). Common critical pathways in embryogenesis and cancer. *Acta Oncol* **45**, 375-88.

Kemler, R., Hierholzer, A., Kanzler, B., Kuppig, S., Hansen, K., Taketo, M. M., de Vries, W. N., Knowles, B. B. and Solter, D. (2004). Stabilization of beta-catenin in the mouse zygote leads to premature epithelial-mesenchymal transition in the epiblast. *Development* **131**, 5817-24.

Kennedy, B. J. (1992). The evolution of hydroxyurea therapy in chronic myelogenous leukemia. *Semin Oncol* **19**, 21-6.

Kern, S. E. and Shibata, D. (2007). The fuzzy math of solid tumor stem cells: a perspective. *Cancer Res* **67**, 8985-8.

Kim, B. M., Mao, J., Taketo, M. M. and Shivdasani, R. A. (2007). Phases of canonical Wnt signaling during the development of mouse intestinal epithelium. *Gastroenterology* **133**, 529-38.

Kim, J., Sebring, A., Esch, J. J., Kraus, M. E., Vorwerk, K., Magee, J. and Carroll, S. B. (1996). Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. *Nature* **382**, 133-8.

Kim, K., Lu, Z. and Hay, E. D. (2002a). Direct evidence for a role of beta-catenin/LEF-1 signaling pathway in induction of EMT. *Cell Biol Int* **26**, 463-76.

Kim, K. A., Kakitani, M., Zhao, J., Oshima, T., Tang, T., Binnerts, M., Liu, Y., Boyle, B., Park, E., Emtage, P. *et al.* (2005). Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* **309**, 1256-9.

Kim, S., Domon-Dell, C., Wang, Q., Chung, D. H., Di Cristofano, A., Pandolfi, P. P., Freund, J. N. and Evers, B. M. (2002b). PTEN and TNF-alpha regulation of the intestinal-specific Cdx-2 homeobox gene through a PI3K, PKB/Akt, and NF-kappaB-dependent pathway. *Gastroenterology* **123**, 1163-78.

Kim, S. K. and Hebrok, M. (2001). Intercellular signals regulating pancreas development and function. *Genes Dev* **15**, 111-27.

Kimura, K., Satoh, K., Kanno, A., Hamada, S., Hirota, M., Endoh, M., Masamune, A. and Shimosegawa, T. (2007). Activation of Notch signaling in tumorigenesis of experimental pancreatic cancer induced by dimethylbenzanthracene in mice. *Cancer Sci* **98**, 155-62.

Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogelstein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Preisinger, A. C., Hedge, P., McKechnie, D. *et al.* (1991). Identification of FAP locus genes from chromosome 5q21. *Science* **253**, 661-5.

Kinzler, K. W. and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell* **87**, 159-70.

Kirchner, T. and Brabletz, T. (2000). Patterning and nuclear beta-catenin expression in the colonic adenoma-carcinoma sequence. Analogies with embryonic gastrulation. *Am J Pathol* **157**, 1113-21.

Kirkland, S. C. (1985). Dome formation by a human colonic adenocarcinoma cell line (HCA-7). *Cancer Res* **45**, 3790-5.

Kirsner, J. B. (2001). Historical origins of current IBD concepts. *World J Gastroenterol* **7**, 175-84.

Klafki, H. W., Paganetti, P. A., Sommer, B. and Staufenbiel, M. (1995). Calpain inhibitor I decreases beta A4 secretion from human embryonal kidney cells expressing beta-amyloid precursor protein carrying the APP670/671 double mutation. *Neurosci Lett* **201**, 29-32.

Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* **68**, 820-3.

Kobayashi, K., Karran, P., Oda, S. and Yanaga, K. (2005). Involvement of mismatch repair in transcription-coupled nucleotide excision repair. *Hum Cell* **18**, 103-15.

Koch, U. and Radtke, F. (2007). Notch and cancer: a double-edged sword. *Cell Mol Life Sci*.

Koo, B. K., Lim, H. S., Chang, H. J., Yoon, M. J., Choi, Y., Kong, M. P., Kim, C. H., Kim, J. M., Park, J. G. and Kong, Y. Y. (2009). Notch signaling promotes the generation of EphrinB1-positive intestinal epithelial cells. *Gastroenterology* **137**, 145-55, 155 e1-3.

Kopan, R. and Goate, A. (2000). A common enzyme connects notch signaling and Alzheimer's disease. *Genes Dev* **14**, 2799-806.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J. and Clevers, H. (1998a). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* **19**, 379-83.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**, 1784-7.

Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O. and Clevers, H. (1998b). Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse. *Mol Cell Biol* **18**, 1248-56.

Kosinski, C., Li, V. S., Chan, A. S., Zhang, J., Ho, C., Tsui, W. Y., Chan, T. L., Mifflin, R. C., Powell, D. W., Yuen, S. T. *et al.* (2007). Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *Proc Natl Acad Sci U S A* **104**, 15418-23.

Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R. *et al.* (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* **14**, 1343-52.

Krebs, L. T., Xue, Y., Norton, C. R., Sundberg, J. P., Beatus, P., Lendahl, U., Joutel, A. and Gridley, T. (2003). Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis* **37**, 139-43.

Kruh, J. (1982). Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol Cell Biochem* **42**, 65-82.

Kucerova, D., Sloncova, E., Tuhackova, Z., Uhlirova, M., Kos, M. and Sovova, V. (1999). Changes of E-cadherin and beta-catenin levels during induced differentiation of colorectal carcinoma cells. *Int J Mol Med* **4**, 541-4.

Kucherlapati, R., Lin, D. P. and Edelmann, W. (2001). Mouse models for human familial adenomatous polyposis. *Semin Cancer Biol* **11**, 219-25.

Kuhnert, F., Davis, C. R., Wang, H. T., Chu, P., Lee, M., Yuan, J., Nusse, R. and Kuo, C. J. (2004). Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A* **101**, 266-71.

Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H. and Honjo, T. (1999). Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J Biol Chem* **274**, 7238-44.

Kurooka, H. and Honjo, T. (2000). Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. *J Biol Chem* **275**, 17211-20.

Kusumi, K., Sun, E. S., Kerrebrock, A. W., Bronson, R. T., Chi, D. C., Bulotsky, M. S., Spencer, J. B., Birren, B. W., Frankel, W. N. and Lander, E. S. (1998). The mouse pudgy mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. *Nat Genet* **19**, 274-8.

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-73.

Lai, E. C., Burks, C. and Posakony, J. W. (1998). The K box, a conserved 3' UTR sequence motif, negatively regulates accumulation of enhancer of split complex transcripts. *Development* **125**, 4077-88.

Lammi, L., Arte, S., Somer, M., Jarvinen, H., Lahermo, P., Thesleff, I., Pirinen, S. and Nieminen, P. (2004). Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. *Am J Hum Genet* **74**, 1043-50.

Land, H., Parada, L. F. and Weinberg, R. A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304**, 596-602.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. *Nature* **358**, 15-6.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M. A. and Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645-8.

Lardelli, M., Dahlstrand, J. and Lendahl, U. (1994). The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech Dev* **46**, 123-36.

Latasa, M. J., Cisneros, E. and Frade, J. M. (2009). Cell cycle control of Notch signaling and the functional regionalization of the neuroepithelium during vertebrate neurogenesis. *Int J Dev Biol* **53**, 895-908.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? *Cell* **85**, 951-61.

Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A. and Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128**, 3675-83.

Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L. A., Nystrom-Lahti, M. *et al.* (1993). Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell* **75**, 1215-25.

Lee, J., Basak, J. M., Demehri, S. and Kopan, R. (2007a). Bi-compartmental communication contributes to the opposite proliferative behavior of Notch1-deficient hair follicle and epidermal keratinocytes. *Development* **134**, 2795-806.

Lee, J. C., Maa, M. C., Yu, H. S., Wang, J. H., Yen, C. K., Wang, S. T., Chen, Y. J., Liu, Y., Jin, Y. T. and Leu, T. H. (2005a). Butyrate regulates the expression of c-Src and focal adhesion kinase and inhibits cell invasion of human colon cancer cells. *Mol Carcinog* **43**, 207-14.

Lee, J. E. (1997). Basic helix-loop-helix genes in neural development. *Curr Opin Neurobiol* **7**, 13-20.

Lee, J. E., Hollenberg, S. M., Snider, L., Turner, D. L., Lipnick, N. and Weintraub, H. (1995). Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* **268**, 836-44.

Lee, S. H., Jeong, E. G., Yoo, N. J. and Lee, S. H. (2007b). Mutational analysis of NOTCH1, 2, 3 and 4 genes in common solid cancers and acute leukemias. *Apmis* **115**, 1357-63.

Lee, S. Y., Kumano, K., Masuda, S., Hangaishi, A., Takita, J., Nakazaki, K., Kurokawa, M., Hayashi, Y., Ogawa, S. and Chiba, S. (2005b). Mutations of the Notch1 gene in T-cell acute lymphoblastic leukemia: analysis in adults and children. *Leukemia* **19**, 1841-3.

Lefort, K., Mandinova, A., Ostano, P., Kolev, V., Calpini, V., Kolfschoten, I., Devgan, V., Lieb, J., Raffoul, W., Hohl, D. *et al.* (2007). Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKalpha kinases. *Genes Dev* **21**, 562-77.

Leibovitz, A., Stinson, J. C., McCombs, W. B., 3rd, McCoy, C. E., Mazur, K. C. and Mabry, N. D. (1976). Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* **36**, 4562-9.

Leonard, J. H., Cook, A. L., Van Gele, M., Boyle, G. M., Inglis, K. J., Speleman, F. and Sturm, R. A. (2002). Proneural and proneuroendocrine transcription factor expression in cutaneous mechanoreceptor (Merkel) cells and Merkel cell carcinoma. *Int J Cancer* **101**, 103-10.

Leong, K. G. and Karsan, A. (2006). Recent insights into the role of Notch signaling in tumorigenesis. *Blood* **107**, 2223-33.

Leong, K. G., Niessen, K., Kulic, I., Raouf, A., Eaves, C., Pollet, I. and Karsan, A. (2007). Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. *J Exp Med*.

Leow, C. C., Romero, M. S., Ross, S., Polakis, P. and Gao, W. Q. (2004). Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. *Cancer Res* **64**, 6050-7.

Lever, J. E. (1982). Cell differentiation and dome formation in polarized epithelial cell mono-

layers. New York: Cold Spring Harbor Laboratory Press.

Lewis, H. D., Leveridge, M., Strack, P. R., Haldon, C. D., O'Neil, J., Kim, H., Madin, A., Hannam, J. C., Look, A. T., Kohl, N. *et al.* (2007). Apoptosis in T cell acute lymphoblastic leukemia cells after cell cycle arrest induced by pharmacological inhibition of notch signaling. *Chem Biol* **14**, 209-19.

Lewis, J. (1996). Neurogenic genes and vertebrate neurogenesis. *Curr Opin Neurobiol* **6**, 3-10.

Lewis, J. (2008). From signals to patterns: space, time, and mathematics in developmental biology. *Science* **322**, 399-403.

Li, L., Krantz, I. D., Deng, Y., Genin, A., Banta, A. B., Collins, C. C., Qi, M., Trask, B. J., Kuo, W. L., Cochran, J. *et al.* (1997). Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* **16**, 243-51.

Li, Y., Rao, P. K., Wen, R., Song, Y., Muir, D., Wallace, P., van Horne, S. J., Tennekoon, G. I. and Kadesch, T. (2004). Notch and Schwann cell transformation. *Oncogene* **23**, 1146-52.

Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M. *et al.* (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat Genet* **16**, 64-7.

Lickert, H., Kispert, A., Kutsch, S. and Kemler, R. (2001). Expression patterns of Wnt genes in mouse gut development. *Mech Dev* **105**, 181-4.

Lieber, T., Kidd, S. and Young, M. W. (2002). kuzbanian-mediated cleavage of Drosophila Notch. *Genes Dev* **16**, 209-21.

Liebner, S., Cattelino, A., Gallini, R., Rudini, N., Iurlaro, M., Piccolo, S. and Dejana, E. (2004). Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. *J Cell Biol* **166**, 359-67.

Lin, G., Xu, N. and Xi, R. (2008). Paracrine Wingless signalling controls self-renewal of Drosophila intestinal stem cells. *Nature* **455**, 1119-23.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-17.

Lissemore, J. L. and Starmer, W. T. (1999). Phylogenetic analysis of vertebrate and invertebrate Delta/Serrate/LAG-2 (DSL) proteins. *Mol Phylogenet Evol* **11**, 308-19.

Litvak, D. A., Evers, B. M., Hwang, K. O., Hellmich, M. R., Ko, T. C. and Townsend, C. M., Jr. (1998). Butyrate-induced differentiation of Caco-2 cells is associated with apoptosis and early induction of p21Waf1/Cip1 and p27Kip1. *Surgery* **124**, 161-9; discussion 169-70.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G. H., Tan, Y., Zhang, Z., Lin, X. and He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108**, 837-47.

Liu, W., Dong, X., Mai, M., Seelan, R. S., Taniguchi, K., Krishnadath, K. K., Halling, K. C., Cunningham, J. M., Boardman, L. A., Qian, C. *et al.* (2000). Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. *Nat Genet* **26**, 146-7.

Liu, Y. and Bodmer, W. F. (2006). Analysis of P53 mutations and their expression in 56 colorectal cancer cell lines. *Proc Natl Acad Sci U S A* **103**, 976-81.

Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Draetta, G. F., Jessup, J. M. and Pagano, M. (1997). Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* **3**, 231-4.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G. and Israel, A. (1998). The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci U S A* **95**, 8108-12.

Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat Rev Neurosci* **7**, 93-102.

Lowell, S., Jones, P., Le Roux, I., Dunne, J. and Watt, F. M. (2000). Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. *Curr Biol* **10**, 491-500.

Lowell, S. and Watt, F. M. (2001). Delta regulates keratinocyte spreading and motility independently of differentiation. *Mech Dev* **107**, 133-40.

Luebeck, E. G. and Moolgavkar, S. H. (2002). Multistage carcinogenesis and the incidence of colorectal cancer. *Proc Natl Acad Sci U S A* **99**, 15095-100.

Lugli, A., Zlobec, I., Minoo, P., Baker, K., Tornillo, L., Terracciano, L. and Jass, J. R. (2007). Prognostic significance of the wnt signalling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: a tissue microarray-based analysis. *Histopathology* **50**, 453-64.

Luongo, C., Moser, A. R., Gledhill, S. and Dove, W. F. (1994). Loss of Apc+ in intestinal adenomas from Min mice. *Cancer Res* **54**, 5947-52.

Lynch, H. T. and de la Chapelle, A. (1999). Genetic susceptibility to non-polyposis colorectal cancer. *J Med Genet* **36**, 801-18.

Ma, S. K., Wan, T. S. and Chan, L. C. (1999). Cytogenetics and molecular genetics of childhood leukemia. *Hematol Oncol* **17**, 91-105.

Madison, B. B., Braunstein, K., Kuizon, E., Portman, K., Qiao, X. T. and Gumucio, D. L. (2005). Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development* **132**, 279-89.

Maeda, K., Takemura, M., Umemori, M. and Adachi-Yamada, T. (2008). E-cadherin prolongs the moment for interaction between intestinal stem cell and its progenitor cell to ensure Notch signaling in adult Drosophila midgut. *Genes Cells* **13**, 1219-27.

Mahyar-Roemer, M. and Roemer, K. (2001). p21 Waf1/Cip1 can protect human colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. *Oncogene* **20**, 3387-98.

Mailhos, C., Modlich, U., Lewis, J., Harris, A., Bicknell, R. and Ish-Horowicz, D. (2001). Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation* **69**, 135-44.

Maillard, I. and Pear, W. S. (2003). Notch and cancer: best to avoid the ups and downs. *Cancer Cell* **3**, 203-5.

Manning, A. M., Williams, A. C., Game, S. M. and Paraskeva, C. (1991). Differential sensitivity of human colonic adenoma and carcinoma cells to transforming growth factor beta (TGF-beta): conversion of an adenoma cell line to a tumorigenic phenotype is accompanied by a reduced response to the inhibitory effects of TGF-beta. *Oncogene* **6**, 1471-6.

Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z. *et al.* (2002). A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J* **21**, 1948-56.

Mariadason, J. M., Arango, D., Corner, G. A., Aranes, M. J., Hotchkiss, K. A., Yang, W. and Augenlicht, L. H. (2002). A gene expression profile that defines colon cell maturation in vitro. *Cancer Res* **62**, 4791-804.

Mariadason, J. M., Bordonaro, M., Aslam, F., Shi, L., Kuraguchi, M., Velcich, A. and Augenlicht, L. H. (2001). Down-regulation of beta-catenin TCF signaling is linked to colonic epithelial cell differentiation. *Cancer Res* **61**, 3465-71.

Mariadason, J. M., Nicholas, C., L'Italien, K. E., Zhuang, M., Smartt, H. J., Heerdt, B. G., Yang, W., Corner, G. A., Wilson, A. J., Klampfer, L. *et al.* (2005). Gene expression profiling of intestinal epithelial cell maturation along the crypt-villus axis. *Gastroenterology* **128**, 1081-8.

Mariadason, J. M., Rickard, K. L., Barkla, D. H., Augenlicht, L. H. and Gibson, P. R. (2000). Divergent phenotypic patterns and commitment to apoptosis of Caco-2 cells during spontaneous and butyrate-induced differentiation. *J Cell Physiol* **183**, 347-54.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R. S., Zborowska, E., Kinzler, K. W., Vogelstein, B. *et al.* (1995). Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* **268**, 1336-8.

Markowitz, S. D., Dawson, D. M., Willis, J. and Willson, J. K. (2002). Focus on colon cancer. *Cancer Cell* **1**, 233-6.

Marshman, E., Booth, C. and Potten, C. S. (2002). The intestinal epithelial stem cell. *Bioessays* **24**, 91-8.

Martin, T. A., Goyal, A., Watkins, G. and Jiang, W. G. (2005). Expression of the transcription factors snail, slug, and twist and their clinical significance in human breast cancer. *Ann Surg Oncol* **12**, 488-96.

Martinez Arias, A., Zecchini, V. and Brennan, K. (2002). CSL-independent Notch signalling: a checkpoint in cell fate decisions during development? *Curr Opin Genet Dev* **12**, 524-33.

Massari, M. E. and Murre, C. (2000). Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol* **20**, 429-40.

Massi, D., Tarantini, F., Franchi, A., Paglierani, M., Di Serio, C., Pellerito, S., Leoncini, G., Cirino, G., Geppetti, P. and Santucci, M. (2006). Evidence for differential expression of Notch receptors and their ligands in melanocytic nevi and cutaneous malignant melanoma. *Mod Pathol* **19**, 246-54.

Mattick, J. S. (2004). RNA regulation: a new genetics? *Nat Rev Genet* **5**, 316-23.

Maurer-Schultze, B., Siebert, M. and Bassukas, I. D. (1988). An in vivo study on the synchronizing effect of hydroxyurea. *Exp Cell Res* **174**, 230-43.

McGill, M. A. and McGlade, C. J. (2003). Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. *J Biol Chem* **278**, 23196-203.

McKay, J. A., Douglas, J. J., Ross, V. G., Curran, S., Loane, J. F., Ahmed, F. Y., Cassidy, J., McLeod, H. L. and Murray, G. I. (2002). Analysis of key cell-cycle checkpoint proteins in colorectal tumours. *J Pathol* **196**, 386-93.

Meng, R. D., Shelton, C. C., Li, Y. M., Qin, L. X., Notterman, D., Paty, P. B. and Schwartz, G. K. (2009). gamma-Secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity. *Cancer Res* **69**, 573-82.

Messeguer, X., Escudero, R., Farre, D., Nunez, O., Martinez, J. and Alba, M. M. (2002). PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* **18**, 333-4.

Micchelli, C. A. and Perrimon, N. (2006). Evidence that stem cells reside in the adult Drosophila midgut epithelium. *Nature* **439**, 475-9.

Micke, P. and Ostman, A. (2005). Exploring the tumour environment: cancer-associated fibroblasts as targets in cancer therapy. *Expert Opin Ther Targets* **9**, 1217-33.

Midgley, R. and Kerr, D. (1999). Colorectal cancer. *Lancet* **353**, 391-9.

Miele, L., Miao, H. and Nickoloff, B. J. (2006). NOTCH signaling as a novel cancer therapeutic target. *Curr Cancer Drug Targets* **6**, 313-23.

Miele, L. and Osborne, B. (1999). Arbiter of differentiation and death: Notch signaling meets apoptosis. *J Cell Physiol* **181**, 393-409.

Milano, J., McKay, J., Dagenais, C., Foster-Brown, L., Pognan, F., Gadient, R., Jacobs, R. T., Zacco, A., Greenberg, B. and Ciaccio, P. J. (2004). Modulation of notch processing by gamma-

secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci* **82**, 341-58.

Miyamoto, Y., Maitra, A., Ghosh, B., Zechner, U., Argani, P., Iacobuzio-Donahue, C. A., Sriuranpong, V., Iso, T., Meszoely, I. M., Wolfe, M. S. *et al.* (2003). Notch mediates TGF alpha-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **3**, 565-76.

Miyata, T., Maeda, T. and Lee, J. E. (1999). NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. *Genes Dev* **13**, 1647-52.

Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T. and Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Hum Mol Genet* **1**, 229-33.

Mohamed, O. A., Clarke, H. J. and Dufort, D. (2004). Beta-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. *Dev Dyn* **231**, 416-24.

Moore, M., Jackson, V., Sealy, L. and Chalkley, R. (1979). Comparative studies on highly metabolically active histone acetylation. *Biochim Biophys Acta* **561**, 248-60.

Moran, J. L., Johnston, S. H., Rauskolb, C., Bhalerao, J., Bowcock, A. M. and Vogt, T. F. (1999). Genomic structure, mapping, and expression analysis of the mammalian Lunatic, Manic, and Radical fringe genes. *Mamm Genome* **10**, 535-41.

Morgan, T. H. (1917). The theory of the gene. *Am. Nat.* **51**, 513-544.

Mori, M., Mimori, K., Adachi, Y. and Akiyoshi, T. (1996). Cell proliferation kinetics are abnormal in transitional mucosa adjacent to colorectal carcinoma. *Br J Surg* **83**, 870-1.

Mori, M., Shimono, R., Adachi, Y., Matsuda, H., Kuwano, H., Sugimachi, K., Ikeda, M. and Saku, M. (1990). Transitional mucosa in human colorectal lesions. *Dis Colon Rectum* **33**, 498-501.

Morimura, T., Goitsuka, R., Zhang, Y., Saito, I., Reth, M. and Kitamura, D. (2000). Cell cycle arrest and apoptosis induced by Notch1 in B cells. *J Biol Chem* **275**, 36523-31.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**, 1787-90.

Morson, B. (1974). President's address. The polyp-cancer sequence in the large bowel. *Proc R Soc Med* **67**, 451-7.

Moser, A. R., Pitot, H. C. and Dove, W. F. (1990). A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **247**, 322-4.

Mummery-Widmer, J. L., Yamazaki, M., Stoeger, T., Novatchkova, M., Bhalerao, S., Chen, D., Dietzl, G., Dickson, B. J. and Knoblich, J. A. (2009). Genome-wide analysis of Notch signalling in Drosophila by transgenic RNAi. *Nature* **458**, 987-92.

Muncan, V., Sansom, O. J., Tertoolen, L., Phesse, T. J., Begthel, H., Sancho, E., Cole, A. M., Gregorieff, A., de Alboran, I. M., Clevers, H. *et al.* (2006). Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. *Mol Cell Biol* **26**, 8418-26.

Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B. and Polakis, P. (1995). Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. *Proc Natl Acad Sci U S A* **92**, 3046-50.

Mungamuri, S. K., Yang, X., Thor, A. D. and Somasundaram, K. (2006). Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. *Cancer Res* **66**, 4715-24.

Murciano, A., Zamora, J., Lopez-Sanchez, J. and Frade, J. M. (2002). Interkinetic nuclear movement may provide spatial clues to the regulation of neurogenesis. *Mol Cell Neurosci* **21**, 285-300.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev Biol* **174**, 233-47.

Nagase, H., Miyoshi, Y., Horii, A., Aoki, T., Ogawa, M., Utsunomiya, J., Baba, S., Sasazuki, T. and Nakamura, Y. (1992). Correlation between the location of germ-line mutations in the APC gene and the number of colorectal polyps in familial adenomatous polyposis patients. *Cancer Res* **52**, 4055-7.

Nair, P., Somasundaram, K. and Krishna, S. (2003). Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. *J Virol* **77**, 7106-12.

Nakajima, Y., Yamagishi, T., Hokari, S. and Nakamura, H. (2000). Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis: roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). *Anat Rec* **258**, 119-27.

Nakamura, M., Okano, H., Blendy, J. A. and Montell, C. (1994). Musashi, a neural RNA-binding protein required for Drosophila adult external sensory organ development. *Neuron* **13**, 67-81.

Nakamura, T., Tsuchiya, K. and Watanabe, M. (2007). Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. *J Gastroenterol* **42**, 705-10.

Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T. *et al.* (1997). Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. *J Biol Chem* **272**, 22199-206.

Nakashima, K., Takizawa, T., Ochiai, W., Yanagisawa, M., Hisatsune, T., Nakafuku, M., Miyazono, K., Kishimoto, T., Kageyama, R. and Taga, T. (2001). BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. *Proc Natl Acad Sci U S A* **98**, 5868-73.

Nam, Y., Aster, J. C. and Blacklow, S. C. (2002). Notch signaling as a therapeutic target. *Curr Opin Chem Biol* **6**, 501-9.

Nassif, N. T., Lobo, G. P., Wu, X., Henderson, C. J., Morrison, C. D., Eng, C., Jalaludin, B. and Segelov, E. (2004). PTEN mutations are common in sporadic microsatellite stable colorectal cancer. *Oncogene* **23**, 617-28.

Naya, F. J., Huang, H. P., Qiu, Y., Mutoh, H., DeMayo, F. J., Leiter, A. B. and Tsai, M. J. (1997). Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev* **11**, 2323-34.

Naya, F. J., Stellrecht, C. M. and Tsai, M. J. (1995). Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. *Genes Dev* **9**, 1009-19.

Nefedova, Y., Cheng, P., Alsina, M., Dalton, W. S. and Gabrilovich, D. I. (2004). Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. *Blood* **103**, 3503-10.

Negre, N., Ghysen, A. and Martinez, A. M. (2003). Mitotic G2-arrest is required for neural cell fate determination in Drosophila. *Mech Dev* **120**, 253-65.

Ngan, C. Y., Yamamoto, H., Seshimo, I., Tsujino, T., Man-i, M., Ikeda, J. I., Konishi, K., Takemasa, I., Ikeda, M., Sekimoto, M. *et al.* (2007). Quantitative evaluation of vimentin expression in tumour stroma of colorectal cancer. *Br J Cancer* **96**, 986-92.

Nichols, J. T., Miyamoto, A., Olsen, S. L., D'Souza, B., Yao, C. and Weinmaster, G. (2007). DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. *J Cell Biol* **176**, 445-58.

Nickoloff, B. J., Osborne, B. A. and Miele, L. (2003). Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* **22**, 6598-608.

Nickoloff, B. J., Qin, J. Z., Chaturvedi, V., Denning, M. F., Bonish, B. and Miele, L. (2002). Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. *Cell Death Differ* **9**, 842-55.

Nicolas, M., Wolfer, A., Raj, K., Kummer, J. A., Mill, P., van Noort, M., Hui, C. C., Clevers, H., Dotto, G. P. and Radtke, F. (2003). Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* **33**, 416-21.

Nielsen, C. M., Williams, J., van den Brink, G. R., Lauwers, G. Y. and Roberts, D. J. (2004). Hh pathway expression in human gut tissues and in inflammatory gut diseases. *Lab Invest* **84**, 1631-42.

Niessen, K., Fu, Y., Chang, L., Hoodless, P. A., McFadden, D. and Karsan, A. (2008). Slug is a direct Notch target required for initiation of cardiac cushion cellularization. *J Cell Biol* **182**, 315-25.

Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* **3**, 155-66.

Nigam, A. K., Savage, F. J., Boulos, P. B., Stamp, G. W., Liu, D. and Pignatelli, M. (1993). Loss of cell-cell and cell-matrix adhesion molecules in colorectal cancer. *Br J Cancer* **68**, 507-14.

Niimi, H., Pardali, K., Vanlandewijck, M., Heldin, C. H. and Moustakas, A. (2007). Notch signaling is necessary for epithelial growth arrest by TGF-beta. *J Cell Biol* **176**, 695-707.

Nishimura, M., Isaka, F., Ishibashi, M., Tomita, K., Tsuda, H., Nakanishi, S. and Kageyama, R. (1998). Structure, chromosomal locus, and promoter of mouse Hes2 gene, a homologue of Drosophila hairy and Enhancer of split. *Genomics* **49**, 69-75.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S. and Hedge, P. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science* **253**, 665-9.

Noseda, M., Chang, L., McLean, G., Grim, J. E., Clurman, B. E., Smith, L. L. and Karsan, A. (2004a). Notch activation induces endothelial cell cycle arrest and participates in contact inhibition: role of p21Cip1 repression. *Mol Cell Biol* **24**, 8813-22.

Noseda, M., Fu, Y., Niessen, K., Wong, F., Chang, L., McLean, G. and Karsan, A. (2006). Smooth Muscle alpha-actin is a direct target of Notch/CSL. *Circ Res* **98**, 1468-70.

Noseda, M., McLean, G., Niessen, K., Chang, L., Pollet, I., Montpetit, R., Shahidi, R., Dorovini-Zis, K., Li, L., Beckstead, B. *et al.* (2004b). Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. *Circ Res* **94**, 910-7.

Novelli, M. R., Williamson, J. A., Tomlinson, I. P., Elia, G., Hodgson, S. V., Talbot, I. C., Bodmer, W. F. and Wright, N. A. (1996). Polyclonal origin of colonic adenomas in an XO/XY patient with FAP. *Science* **272**, 1187-90.

Nusse, R. The Wnt Homepage, (ed.

O'Brien, C. A., Pollett, A., Gallinger, S. and Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**, 106-10.

Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M. and Lendahl, U. (2001). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. *J Biol Chem* **276**, 35847-53.

Oda, T., Elkahloun, A. G., Pike, B. L., Okajima, K., Krantz, I. D., Genin, A., Piccoli, D. A., Meltzer, P. S., Spinner, N. B., Collins, F. S. *et al.* (1997). Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* **16**, 235-42.

Oellers, N., Dehio, M. and Knust, E. (1994). bHLH proteins encoded by the Enhancer of split complex of Drosophila negatively interfere with transcriptional activation mediated by proneural genes. *Mol Gen Genet* **244**, 465-73.

Ohlstein, B. and Spradling, A. (2006). The adult Drosophila posterior midgut is maintained by pluripotent stem cells. *Nature* **439**, 470-4.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R. (1999). Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *Embo J* **18**, 2196-207.

Oka, C., Nakano, T., Wakeham, A., de la Pompa, J. L., Mori, C., Sakai, T., Okazaki, S., Kawaichi, M., Shiota, K., Mak, T. W. *et al.* (1995). Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development* **121**, 3291-301.

Okabe, M., Imai, T., Kurusu, M., Hiromi, Y. and Okano, H. (2001). Translational repression determines a neuronal potential in Drosophila asymmetric cell division. *Nature* **411**, 94-8.

Okajima, T., Xu, A. and Irvine, K. D. (2003). Modulation of notch-ligand binding by protein O-fucosyltransferase 1 and fringe. *J Biol Chem* **278**, 42340-5.

Okamoto, R., Tsuchiya, K., Nemoto, Y., Akiyama, J., Nakamura, T., Kanai, T. and Watanabe, M. (2009). Requirement of Notch activation during regeneration of the intestinal epithelia. *Am J Physiol Gastrointest Liver Physiol* **296**, G23-35.

Okamoto, R. and Watanabe, M. (2005). Cellular and molecular mechanisms of the epithelial repair in IBD. *Dig Dis Sci* **50 Suppl 1**, S34-8.

Oniscu, A., James, R. M., Morris, R. G., Bader, S., Malcomson, R. D. and Harrison, D. J. (2004). Expression of Sonic hedgehog pathway genes is altered in colonic neoplasia. *J Pathol* **203**, 909-17.

Ono, M., Sakamoto, M., Ino, Y., Moriya, Y., Sugihara, K., Muto, T. and Hirohashi, S. (1996). Cancer cell morphology at the invasive front and expression of cell adhesion-related carbohydrate in the primary lesion of patients with colorectal carcinoma with liver metastasis. *Cancer* **78**, 1179-86.

Oshima, H., Oshima, M., Kobayashi, M., Tsutsumi, M. and Taketo, M. M. (1997). Morphological and molecular processes of polyp formation in Apc(delta716) knockout mice. *Cancer Res* **57**, 1644-9.

Palmeirim, I., Henrique, D., Ish-Horowicz, D. and Pourquie, O. (1997). Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* **91**, 639-48.

Palmer, H. G., Gonzalez-Sancho, J. M., Espada, J., Berciano, M. T., Puig, I., Baulida, J., Quintanilla, M., Cano, A., de Herreros, A. G., Lafarga, M. *et al.* (2001). Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. *J Cell Biol* **154**, 369-87.

Palomero, T., Lim, W. K., Odom, D. T., Sulis, M. L., Real, P. J., Margolin, A., Barnes, K. C., O'Neil, J., Neuberg, D., Weng, A. P. *et al.* (2006). NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci U S A* **103**, 18261-6.

Panin, V. M. and Irvine, K. D. (1998). Modulators of Notch signaling. *Semin Cell Dev Biol* **9**, 609-17.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-12.

Papadopoulos, N., Nicolaides, N. C., Wei, Y. F., Ruben, S. M., Carter, K. C., Rosen, C. A., Haseltine, W. A., Fleischmann, R. D., Fraser, C. M., Adams, M. D. *et al.* (1994). Mutation of a mutL homolog in hereditary colon cancer. *Science* **263**, 1625-9.

Paraskeva, C., Buckle, B. G., Sheer, D. and Wigley, C. B. (1984). The isolation and characterization of colorectal epithelial cell lines at different stages in malignant transformation from familial polyposis coli patients. *Int J Cancer* **34**, 49-56.

Paraskeva, C., Finerty, S., Mountford, R. A. and Powell, S. C. (1989). Specific cytogenetic abnormalities in two new human colorectal adenoma-derived epithelial cell lines. *Cancer Res* **49**, 1282-6.

Park, E. T., Oh, H. K., Gum, J. R., Jr., Crawley, S. C., Kakar, S., Engel, J., Leow, C. C., Gao, W. Q. and Kim, Y. S. (2006). HATH1 expression in mucinous cancers of the colorectum and related lesions. *Clin Cancer Res* **12**, 5403-10.

Parker, B. S., Argani, P., Cook, B. P., Liangfeng, H., Chartrand, S. D., Zhang, M., Saha, S., Bardelli, A., Jiang, Y., St Martin, T. B. *et al.* (2004). Alterations in vascular gene expression in invasive breast carcinoma. *Cancer Res* **64**, 7857-66.

Parks, A. L., Klueg, K. M., Stout, J. R. and Muskavitch, M. A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* **127**, 1373-85.

Parr, C., Watkins, G. and Jiang, W. G. (2004). The possible correlation of Notch-1 and Notch-2 with clinical outcome and tumour clinicopathological parameters in human breast cancer. *Int J Mol Med* **14**, 779-86.

Parsons, D. W., Wang, T. L., Samuels, Y., Bardelli, A., Cummins, J. M., DeLong, L., Silliman, N., Ptak, J., Szabo, S., Willson, J. K. *et al.* (2005). Colorectal cancer: mutations in a signalling pathway. *Nature* **436**, 792.

Patel, N. S., Li, J. L., Generali, D., Poulsom, R., Cranston, D. W. and Harris, A. L. (2005). Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res* **65**, 8690-7.

Pear, W. S., Aster, J. C., Scott, M. L., Hasserjian, R. P., Soffer, B., Sklar, J. and Baltimore, D. (1996). Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med* **183**, 2283-91.

Pece, S., Serresi, M., Santolini, E., Capra, M., Hulleman, E., Galimberti, V., Zurrida, S., Maisonneuve, P., Viale, G. and Di Fiore, P. P. (2004). Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. *J Cell Biol* **167**, 215-21.

Perk, J., Iavarone, A. and Benezra, R. (2005). Id family of helix-loop-helix proteins in cancer. *Nat Rev Cancer* **5**, 603-14.

Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H. and Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* **392**, 190-3.

Persad, S., Troussard, A. A., McPhee, T. R., Mulholland, D. J. and Dedhar, S. (2001). Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. *J Cell Biol* **153**, 1161-74.

Philipp-Staheli, J., Kim, K. H., Payne, S. R., Gurley, K. E., Liggitt, D., Longton, G. and Kemp, C. J. (2002). Pathway-specific tumor suppression. Reduction of p27 accelerates gastrointestinal tumorigenesis in Apc mutant mice, but not in Smad3 mutant mice. *Cancer Cell* **1**, 355-68.

Pignatelli, M. (1993). E-cadherin: a biological marker of tumour differentiation. *J Pathol* **171**, 81-2.

Pines, J. (1993). Cyclins and cyclin-dependent kinases: take your partners. *Trends Biochem Sci* **18**, 195-7.

Pinto, D., Gregorieff, A., Begthel, H. and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* **17**, 1709-13.

Pizao, P. E., Lyaruu, D. M., Peters, G. J., van Ark-Otte, J., Winograd, B., Giaccone, G. and Pinedo, H. M. (1992). Growth, morphology and chemosensitivity studies on postconfluent cells cultured in 'V'-bottomed microtiter plates. *Br J Cancer* **66**, 660-5.

Podolsky, D. K. (2002). Inflammatory bowel disease. *N Engl J Med* **347**, 417-29.

Ponz de Leon, M. and Di Gregorio, C. (2001). Pathology of colorectal cancer. *Dig Liver Dis* **33**, 372-88.

Porter, E. M., Bevins, C. L., Ghosh, D. and Ganz, T. (2002). The multifaceted Paneth cell. *Cell Mol Life Sci* **59**, 156-70.

Potten, C. S., Booth, C., Tudor, G. L., Booth, D., Brady, G., Hurley, P., Ashton, G., Clarke, R., Sakakibara, S. and Okano, H. (2003). Identification of a putative intestinal stem cell and early lineage marker; musashi-1. *Differentiation* **71**, 28-41.

Potten, C. S., Gandara, R., Mahida, Y. R., Loeffler, M. and Wright, N. A. (2009). The stem cells of small intestinal crypts: where are they? *Cell Prolif* **42**, 731-50.

Potten, C. S., Kellett, M., Rew, D. A. and Roberts, S. A. (1992). Proliferation in human gastrointestinal epithelium using bromodeoxyuridine in vivo: data for different sites, proximity to a tumour, and polyposis coli. *Gut* **33**, 524-9.

Potten, C. S., Owen, G. and Booth, D. (2002). Intestinal stem cells protect their genome by selective segregation of template DNA strands. *J Cell Sci* **115**, 2381-8.

Poulson, D. F. (1937). Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster. *Proc Natl Acad Sci U S A* **23**, 133-7.

Pourquie, O. (1999). Notch around the clock. *Curr Opin Genet Dev* **9**, 559-65.

Pourquie, O. (2000). Segmentation of the paraxial mesoderm and vertebrate somitogenesis. *Curr Top Dev Biol* **47**, 81-105.

Powell, S. M., Zilz, N., Beazer-Barclay, Y., Bryan, T. M., Hamilton, S. R., Thibodeau, S. N., Vogelstein, B. and Kinzler, K. W. (1992). APC mutations occur early during colorectal tumorigenesis. *Nature* **359**, 235-7.

Preston, S. L., Wong, W. M., Chan, A. O., Poulsom, R., Jeffery, R., Goodlad, R. A., Mandir, N., Elia, G., Novelli, M., Bodmer, W. F. *et al.* (2003). Bottom-up histogenesis of colorectal adenomas: origin in the monocryptal adenoma and initial expansion by crypt fission. *Cancer Res* **63**, 3819-25.

Pretlow, T. P., Edelmann, W., Kucherlapati, R., Pretlow, T. G. and Augenlicht, L. H. (2003). Spontaneous aberrant crypt foci in Apc1638N mice with a mutant Apc allele. *Am J Pathol* **163**, 1757-63.

Proweller, A., Tu, L., Lepore, J. J., Cheng, L., Lu, M. M., Seykora, J., Millar, S. E., Pear, W. S. and Parmacek, M. S. (2006). Impaired notch signaling promotes de novo squamous cell carcinoma formation. *Cancer Res* **66**, 7438-44.

Pui, J. C., Allman, D., Xu, L., DeRocco, S., Karnell, F. G., Bakkour, S., Lee, J. Y., Kadesch, T., Hardy, R. R., Aster, J. C. *et al.* (1999). Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11**, 299-308.

Purdie, C. A. and Piris, J. (2000). Histopathological grade, mucinous differentiation and DNA ploidy in relation to prognosis in colorectal carcinoma. *Histopathology* **36**, 121-6.

Purow, B. W., Haque, R. M., Noel, M. W., Su, Q., Burdick, M. J., Lee, J., Sundaresan, T., Pastorino, S., Park, J. K., Mikolaenko, I. *et al.* (2005). Expression of Notch-1 and its ligands, Delta-like-1 and Jagged-1, is critical for glioma cell survival and proliferation. *Cancer Res* **65**, 2353-63.

Qiao, L. and Wong, B. C. (2009). Role of Notch signaling in colorectal cancer. *Carcinogenesis* **30**, 1979-86.

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., Fang, D., Hunter, T. and Liu, Y. C. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *J Biol Chem* **275**, 35734-7.

Qualtrough, D., Buda, A., Gaffield, W., Williams, A. C. and Paraskeva, C. (2004). Hedgehog signalling in colorectal tumour cells: induction of apoptosis with cyclopamine treatment. *Int J Cancer* **110**, 831-7.

Qualtrough, D., Hinoi, T., Fearon, E. and Paraskeva, C. (2002). Expression of CDX2 in normal and neoplastic human colon tissue and during differentiation of an in vitro model system. *Gut* **51**, 184-90.

Qualtrough, D., Kaidi, A., Chell, S., Jabbour, H. N., Williams, A. C. and Paraskeva, C. (2007). Prostaglandin F(2alpha) stimulates motility and invasion in colorectal tumor cells. *Int J Cancer* **121**, 734-40.

Radtke, F. and Raj, K. (2003). The role of Notch in tumorigenesis: oncogene or tumour suppressor? *Nat Rev Cancer* **3**, 756-67.

Radtke, F., Wilson, A., Mancini, S. J. and MacDonald, H. R. (2004). Notch regulation of lymphocyte development and function. *Nat Immunol* **5**, 247-53.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-58.

Ramalho-Santos, M., Melton, D. A. and McMahon, A. P. (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* **127**, 2763-72.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R. C. and Melton, D. A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science* **298**, 597-600.

Ramdass, B., Maliekal, T. T., Lakshmi, S., Rehman, M., Rema, P., Nair, P., Mukherjee, G., Reddy, B. K., Krishna, S. and Radhakrishna Pillai, M. (2007). Coexpression of Notch1 and NF-kappaB signaling pathway components in human cervical cancer progression. *Gynecol Oncol* **104**, 352-61.

Rangarajan, A., Syal, R., Selvarajah, S., Chakrabarti, O., Sarin, A. and Krishna, S. (2001a). Activated Notch1 signaling cooperates with papillomavirus oncogenes in transformation and generates resistance to apoptosis on matrix withdrawal through PKB/Akt. *Virology* **286**, 23-30.

Rangarajan, A., Talora, C., Okuyama, R., Nicolas, M., Mammucari, C., Oh, H., Aster, J. C., Krishna, S., Metzger, D., Chambon, P. *et al.* (2001b). Notch signaling is a direct determinant of keratinocyte growth arrest and entry into differentiation. *Embo J* **20**, 3427-36.

Rao, S. S., O'Neil, J., Liberator, C. D., Hardwick, J. S., Dai, X., Zhang, T., Tyminski, E., Yuan, J., Kohl, N. E., Richon, V. M. *et al.* (2009). Inhibition of NOTCH signaling by gamma secretase inhibitor engages the RB pathway and elicits cell cycle exit in T-cell acute lymphoblastic leukemia cells. *Cancer Res* **69**, 3060-8.

Rasmussen, J. P., English, K., Tenlen, J. R. and Priess, J. R. (2008). Notch signaling and morphogenesis of single-cell tubes in the C. elegans digestive tract. *Dev Cell* **14**, 559-69.

Rasul, S., Balasubramanian, R., Filipovic, A., Slade, M. J., Yague, E. and Coombes, R. C. (2009). Inhibition of gamma-secretase induces G2/M arrest and triggers apoptosis in breast cancer cells. *Br J Cancer* **100**, 1879-88.

Real, P. J. and Ferrando, A. A. (2009). NOTCH inhibition and glucocorticoid therapy in T-cell acute lymphoblastic leukemia. *Leukemia* **23**, 1374-7.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-99.

Redmond, L., Oh, S. R., Hicks, C., Weinmaster, G. and Ghosh, A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. *Nat Neurosci* **3**, 30-40.

Reedijk, M., Odorcic, S., Chang, L., Zhang, H., Miller, N., McCready, D. R., Lockwood, G. and Egan, S. E. (2005). High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res* **65**, 8530-7.

Reedijk, M., Odorcic, S., Zhang, H., Chetty, R., Tennert, C., Dickson, B. C., Lockwood, G., Gallinger, S. and Egan, S. E. (2008). Activation of Notch signaling in human colon adenocarcinoma. *Int J Oncol* **33**, 1223-9.

Renan, M. J. (1993). How many mutations are required for tumorigenesis? Implications from human cancer data. *Mol Carcinog* **7**, 139-46.

Renehan, A. G., O'Dwyer, S. T., Haboubi, N. J. and Potten, C. S. (2002). Early cellular events in colorectal carcinogenesis. *Colorectal Dis* **4**, 76-89.

Rescigno, M. (2008). The pathogenic role of intestinal flora in IBD and colon cancer. *Curr Drug Targets* **9**, 395-403.

Reynolds, T. C., Smith, S. D. and Sklar, J. (1987). Analysis of DNA surrounding the breakpoints of chromosomal translocations involving the beta T cell receptor gene in human lymphoblastic neoplasms. *Cell* **50**, 107-17.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C. and De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**, 111-5.

Riccio, O., van Gijn, M. E., Bezdek, A. C., Pellegrinet, L., van Es, J. H., Zimber-Strobl, U., Strobl, L. J., Honjo, T., Clevers, H. and Radtke, F. (2008). Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep* **9**, 377-83.

Rice, P., Longden, I. and Bleasby, A. (2000). EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* **16**, 276-7.

Richon, V. M., Sandhoff, T. W., Rifkind, R. A. and Marks, P. A. (2000). Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. *Proc Natl Acad Sci U S A* **97**, 10014-9.

Rieder, C. L. and Palazzo, R. E. (1992). Colcemid and the mitotic cycle. *J Cell Sci* **102 (Pt 3)**, 387-92.

Rindi, G., Ratineau, C., Ronco, A., Candusso, M. E., Tsai, M. and Leiter, A. B. (1999). Targeted ablation of secretin-producing cells in transgenic mice reveals a common differentiation pathway with multiple enteroendocrine cell lineages in the small intestine. *Development* **126**, 4149-56.

Robey-Cafferty, S. S., Ro, J. Y., Ordonez, N. G. and Cleary, K. R. (1990). Transitional mucosa of colon. A morphological, histochemical, and immunohistochemical study. *Arch Pathol Lab Med* **114**, 72-5.

Robson, H., Anderson, E., James, R. D. and Schofield, P. F. (1996). Transforming growth factor beta 1 expression in human colorectal tumours: an independent prognostic marker in a subgroup of poor prognosis patients. *Br J Cancer* **74**, 753-8.

Rockman, S. P., Currie, S. A., Ciavarella, M., Vincan, E., Dow, C., Thomas, R. J. and Phillips, W. A. (2001). Id2 is a target of the beta-catenin/T cell factor pathway in colon carcinoma. *J Biol Chem* **276**, 45113-9.

Rodilla, V., Villanueva, A., Obrador-Hevia, A., Robert-Moreno, A., Fernandez-Majada, V., Grilli, A., Lopez-Bigas, N., Bellora, N., Alba, M. M., Torres, F. *et al.* (2009). Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. *Proc Natl Acad Sci U S A* **106**, 6315-20.

Rodrigues, N. R., Rowan, A., Smith, M. E., Kerr, I. B., Bodmer, W. F., Gannon, J. V. and Lane, D. P. (1990). p53 mutations in colorectal cancer. *Proc Natl Acad Sci U S A* **87**, 7555-9.

Rodriguez-Gonzalez, J. G., Santillan, M., Fowler, A. C. and Mackey, M. C. (2007). The segmentation clock in mice: interaction between the Wnt and Notch signalling pathways. *J Theor Biol* **248**, 37-47.

Roediger, W. E. (1980). Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut* **21**, 793-8.

Roman, C., Saha, D. and Beauchamp, R. (2001). TGF-beta and colorectal carcinogenesis. *Microsc Res Tech* **52**, 450-7.

Ronchini, C. and Capobianco, A. J. (2000). Notch(ic)-ER chimeras display hormone-dependent transformation, nuclear accumulation, phosphorylation and CBF1 activation. *Oncogene* **19**, 3914-24.

Ronchini, C. and Capobianco, A. J. (2001). Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). *Mol Cell Biol* **21**, 5925-34.

Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O. and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**, 608-12.

Rorth, P. (2007). Collective guidance of collective cell migration. *Trends Cell Biol* **17**, 575-9.

Rosin-Arbesfeld, R., Cliffe, A., Brabletz, T. and Bienz, M. (2003). Nuclear export of the APC tumour suppressor controls beta-catenin function in transcription. *EMBO J* **22**, 1101-13.

Ross, D. A. and Kadesch, T. (2001). The notch intracellular domain can function as a coactivator for LEF-1. *Mol Cell Biol* **21**, 7537-44.

Rousset, M., Laburthe, M., Pinto, M., Chevalier, G., Rouyer-Fessard, C., Dussaulx, E., Trugnan, G., Boige, N., Brun, J. L. and Zweibaum, A. (1985). Enterocytic differentiation and glucose utilization in the human colon tumor cell line Caco-2: modulation by forskolin. *J Cell Physiol* **123**, 377-85.

Rowan, A. J., Lamlum, H., Ilyas, M., Wheeler, J., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W. F. and Tomlinson, I. P. (2000). APC mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits". *Proc Natl Acad Sci U S A* **97**, 3352-7.

Roy, H. K., Smyrk, T. C., Koetsier, J., Victor, T. A. and Wali, R. K. (2005). The transcriptional repressor SNAIL is overexpressed in human colon cancer. *Dig Dis Sci* **50**, 42-6.

Roy, M., Pear, W. S. and Aster, J. C. (2007). The multifaceted role of Notch in cancer. *Curr Opin Genet Dev* **17**, 52-9.

Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S. H., Masiarz, F. R., Munemitsu, S. and Polakis, P. (1993). Association of the APC gene product with beta-catenin. *Science* **262**, 1731-4.

Rudy, D. R. and Zdon, M. J. (2000). Update on colorectal cancer. *Am Fam Physician* **61**, 1759-70, 1773-4.

Ruel, L., Bourouis, M., Heitzler, P., Pantesco, V. and Simpson, P. (1993). Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch. *Nature* **362**, 557-60.

Sade, H., Krishna, S. and Sarin, A. (2004). The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem* **279**, 2937-44.

Sahlgren, C., Gustafsson, M. V., Jin, S., Poellinger, L. and Lendahl, U. (2008). Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci U S A* **105**, 6392-7.

Sakakibara, S., Nakamura, Y., Yoshida, T., Shibata, S., Koike, M., Takano, H., Ueda, S., Uchiyama, Y., Noda, T. and Okano, H. (2002). RNA-binding protein Musashi family: roles for CNS stem cells and a subpopulation of ependymal cells revealed by targeted disruption and antisense ablation. *Proc Natl Acad Sci U S A* **99**, 15194-9.

Sakakibara, S. and Okano, H. (1997). Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. *J Neurosci* **17**, 8300-12.

Sakamoto, K., Yamaguchi, S., Ando, R., Miyawaki, A., Kabasawa, Y., Takagi, M., Li, C. L., Perbal, B. and Katsube, K. (2002). The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. *J Biol Chem* **277**, 29399-405.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A. and Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* **21**, 1-26.

Sanchez-Irizarry, C., Carpenter, A. C., Weng, A. P., Pear, W. S., Aster, J. C. and Blacklow, S. C. (2004). Notch subunit heterodimerization and prevention of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. *Mol Cell Biol* **24**, 9265-73.

Sancho, E., Batlle, E. and Clevers, H. (2004). Signaling pathways in intestinal development and cancer. *Annu Rev Cell Dev Biol* **20**, 695-723.

Sander, G. R. and Powell, B. C. (2004). Expression of notch receptors and ligands in the adult gut. *J Histochem Cytochem* **52**, 509-16.

Sandhu, M. S., White, I. R. and McPherson, K. (2001). Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. *Cancer Epidemiol Biomarkers Prev* **10**, 439-46.

Sangiorgi, E. and Capecchi, M. R. (2008). Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* **40**, 915-20.

Sansom, O. J., Meniel, V., Wilkins, J. A., Cole, A. M., Oien, K. A., Marsh, V., Jamieson, T. J., Guerra, C., Ashton, G. H., Barbacid, M. *et al.* (2006). Loss of Apc allows phenotypic manifestation of the transforming properties of an endogenous K-ras oncogene in vivo. *Proc Natl Acad Sci U S A* **103**, 14122-7.

Sansom, O. J., Reed, K. R., Hayes, A. J., Ireland, H., Brinkmann, H., Newton, I. P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I. S. *et al.* (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev* **18**, 1385-90.

Santagata, S., Demichelis, F., Riva, A., Varambally, S., Hofer, M. D., Kutok, J. L., Kim, R., Tang, J., Montie, J. E., Chinnaiyan, A. M. *et al.* (2004). JAGGED1 expression is associated with prostate cancer metastasis and recurrence. *Cancer Res* **64**, 6854-7.

Sarachu, M. and Colet, M. (2005). wEMBOSS: a web interface for EMBOSS. *Bioinformatics* **21**, 540-1.

Sarmento, L. M., Huang, H., Limon, A., Gordon, W., Fernandes, J., Tavares, M. J., Miele, L., Cardoso, A. A., Classon, M. and Carlesso, N. (2005). Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and p27 Kip1 degradation. *J Exp Med* **202**, 157-68.

Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R. and Nakanishi, S. (1992). Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev* **6**, 2620-34.

Sato, H., Tsuchiya, A. and Abe, R. (1995). Correlation between c-myc protein expression and phases of the cell cycle in human colorectal carcinomas. *Fukushima J Med Sci* **41**, 111-23.

Sato, T., Vries, R. G., Snippert, H. J., van de Wetering, M., Barker, N., Stange, D. E., van Es, J. H., Abo, A., Kujala, P., Peters, P. J. *et al.* (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* **459**, 262-5.

Schafer, K. A. (1998). The cell cycle: a review. *Vet Pathol* **35**, 461-78.

Schafer, M. and Werner, S. (2008). Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol* **9**, 628-38.

Schier, A. F., Neuhauss, S. C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z. *et al.* (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123**, 165-78.

Schonhoff, S. E., Giel-Moloney, M. and Leiter, A. B. (2004). Minireview: Development and differentiation of gut endocrine cells. *Endocrinology* **145**, 2639-44.

Schroder, N. and Gossler, A. (2002). Expression of Notch pathway components in fetal and adult mouse small intestine. *Gene Expr Patterns* **2**, 247-50.

Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-6.

Schroy, P., Rifkin, J., Coffey, R. J., Winawer, S. and Friedman, E. (1990). Role of transforming growth factor beta 1 in induction of colon carcinoma differentiation by hexamethylene bisacetamide. *Cancer Res* **50**, 261-5.

Schuhmacher, C., Becker, I., Oswald, S., Atkinson, M. J., Nekarda, H., Becker, K. F., Mueller, J., Siewert, J. R. and Hofler, H. (1999). Loss of immunohistochemical E-cadherin expression in colon cancer is not due to structural gene alterations. *Virchows Arch* **434**, 489-95.

Scott, R. J., van der Luijt, R., Spycher, M., Mary, J. L., Muller, A., Hoppeler, T., Haner, M., Muller, H., Martinoli, S., Brazzola, P. L. *et al.* (1995). Novel germline APC gene mutation in a large familial adenomatous polyposis kindred displaying variable phenotypes. *Gut* **36**, 731-6.

Scoville, D. H., Sato, T., He, X. C. and Li, L. (2008). Current view: intestinal stem cells and signaling. *Gastroenterology* **134**, 849-64.

Sealy, L. and Chalkley, R. (1978). The effect of sodium butyrate on histone modification. *Cell* **14**, 115-21.

Searfoss, G. H., Jordan, W. H., Calligaro, D. O., Galbreath, E. J., Schirtzinger, L. M., Berridge, B. R., Gao, H., Higgins, M. A., May, P. C. and Ryan, T. P. (2003). Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. *J Biol Chem* **278**, 46107-16.

Sebolt-Leopold, J. S. and Herrera, R. (2004). Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer* **4**, 937-47.

Segditsas, S. and Tomlinson, I. (2006). Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* **25**, 7531-7.

Segre, J. A., Bauer, C. and Fuchs, E. (1999). Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat Genet* **22**, 356-60.

Sestan, N., Artavanis-Tsakonas, S. and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science* **286**, 741-6.

Shah, N. M., Groves, A. K. and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell* **85**, 331-43.

Sharma, V. M., Calvo, J. A., Draheim, K. M., Cunningham, L. A., Hermance, N., Beverly, L., Krishnamoorthy, V., Bhasin, M., Capobianco, A. J. and Kelliher, M. A. (2006). Notch1 contributes to mouse T-cell leukemia by directly inducing the expression of c-myc. *Mol Cell Biol* **26**, 8022-31.

Shawber, C., Boulter, J., Lindsell, C. E. and Weinmaster, G. (1996a). Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev Biol* **180**, 370-6.

Shawber, C., Nofziger, D., Hsieh, J. J., Lindsell, C., Bogler, O., Hayward, D. and Weinmaster, G. (1996b). Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. *Development* **122**, 3765-73.

Shelly, L. L., Fuchs, C. and Miele, L. (1999). Notch-1 inhibits apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar compounds. *J Cell Biochem* **73**, 164-75.

Sheng, M. and Greenberg, M. E. (1990). The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**, 477-85.

Shi, S. and Stanley, P. (2003). Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc Natl Acad Sci U S A* **100**, 5234-9.

Shi, S. R., Imam, S. A., Young, L., Cote, R. J. and Taylor, C. R. (1995). Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J Histochem Cytochem* **43**, 193-201.

Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.

Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K. *et al.* (1997). Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science* **278**, 120-3.

Shields, J. M., Christy, R. J. and Yang, V. W. (1996). Identification and characterization of a gene encoding a gut-enriched Kruppel-like factor expressed during growth arrest. *J Biol Chem* **271**, 20009-17.

Shih, I. M., Wang, T. L., Traverso, G., Romans, K., Hamilton, S. R., Ben-Sasson, S., Kinzler, K. W. and Vogelstein, B. (2001). Top-down morphogenesis of colorectal tumors. *Proc Natl Acad Sci U S A* **98**, 2640-5.

Shimizu, T., Kagawa, T., Inoue, T., Nonaka, A., Takada, S., Aburatani, H. and Taga, T. (2008). Stabilized beta-catenin functions through TCF/LEF proteins and the Notch/RBP-Jkappa complex to promote proliferation and suppress differentiation of neural precursor cells. *Mol Cell Biol* **28**, 7427-41.

Shimojo, H., Ohtsuka, T. and Kageyama, R. (2008). Oscillations in notch signaling regulate maintenance of neural progenitors. *Neuron* **58**, 52-64.

Shioiri, M., Shida, T., Koda, K., Oda, K., Seike, K., Nishimura, M., Takano, S. and Miyazaki, M. (2006). Slug expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients. *Br J Cancer* **94**, 1816-22.

Shou, J., Ross, S., Koeppen, H., de Sauvage, F. J. and Gao, W. Q. (2001). Dynamics of notch expression during murine prostate development and tumorigenesis. *Cancer Res* **61**, 7291-7.

Shpitz, B., Bomstein, Y., Mekori, Y., Cohen, R., Kaufman, Z., Grankin, M. and Bernheim, J. (1997). Proliferating cell nuclear antigen as a marker of cell kinetics in aberrant crypt foci, hyperplastic polyps, adenomas, and adenocarcinomas of the human colon. *Am J Surg* **174**, 425-30.

Shroyer, N. F., Wallis, D., Venken, K. J., Bellen, H. J. and Zoghbi, H. Y. (2005). Gfi1 functions downstream of Math1 to control intestinal secretory cell subtype allocation and differentiation. *Genes Dev* **19**, 2412-7.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. *Proc Natl Acad Sci U S A* **96**, 5522-7.

Shutter, J. R., Scully, S., Fan, W., Richards, W. G., Kitajewski, J., Deblandre, G. A., Kintner, C. R. and Stark, K. L. (2000). Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev* **14**, 1313-8.

Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowski, C., Yu, Q., Ferrando, A. A., Levin, S. D., Geng, Y., von Boehmer, H. *et al.* (2003). Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* **4**, 451-61.

Singh, S. K., Hawkins, C., Clarke, I. D., Squire, J. A., Bayani, J., Hide, T., Henkelman, R. M., Cusimano, M. D. and Dirks, P. B. (2004). Identification of human brain tumour initiating cells. *Nature* **432**, 396-401.

Siu, I. M., Pretlow, T. G., Amini, S. B. and Pretlow, T. P. (1997). Identification of dysplasia in human colonic aberrant crypt foci. *Am J Pathol* **150**, 1805-13.

Sjoblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N. *et al.* (2006). The consensus coding sequences of human breast and colorectal cancers. *Science* **314**, 268-74.

Skovronsky, D. M., Pijak, D. S., Doms, R. W. and Lee, V. M. (2000). A distinct ER/IC gamma-secretase competes with the proteasome for cleavage of APP. *Biochemistry* **39**, 810-7.

Smartt, H. J., Guilmeau, S., Nasser, S. V., Nicholas, C., Bancroft, L., Simpson, S. A., Yeh, N., Yang, W., Mariadason, J. M., Koff, A. *et al.* (2007). p27kip1 Regulates cdk2 activity in the proliferating zone of the mouse intestinal epithelium: potential role in neoplasia. *Gastroenterology* **133**, 232-43.

Smits, R., Kielman, M. F., Breukel, C., Zurcher, C., Neufeld, K., Jagmohan-Changur, S., Hofland, N., van Dijk, J., White, R., Edelmann, W. *et al.* (1999). Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. *Genes Dev* **13**, 1309-21.

Sobin, L. H. (2003). TNM, sixth edition: new developments in general concepts and rules. *Semin Surg Oncol* **21**, 19-22.

Sommer, L., Ma, Q. and Anderson, D. J. (1996). neurogenins, a novel family of atonal-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal progenitor cell heterogeneity in the developing CNS and PNS. *Mol Cell Neurosci* **8**, 221-41.

Song, W., Nadeau, P., Yuan, M., Yang, X., Shen, J. and Yankner, B. A. (1999). Proteolytic release and nuclear translocation of Notch-1 are induced by presenilin-1 and impaired by pathogenic presenilin-1 mutations. *Proc Natl Acad Sci U S A* **96**, 6959-63.

Soriano, J. V., Uyttendaele, H., Kitajewski, J. and Montesano, R. (2000). Expression of an activated Notch4(int-3) oncoprotein disrupts morphogenesis and induces an invasive phenotype in mammary epithelial cells in vitro. *Int J Cancer* **86**, 652-9.

Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F. and Brooks, S. C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res* **50**, 6075-86.

Spana, E. P. and Doe, C. Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron* **17**, 21-6.

Sparks, A. B., Morin, P. J., Vogelstein, B. and Kinzler, K. W. (1998). Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res* **58**, 1130-4.

Sporn, M. B. (1996). The war on cancer. *Lancet* **347**, 1377-81.

Sriuranpong, V., Borges, M. W., Ravi, R. K., Arnold, D. R., Nelkin, B. D., Baylin, S. B. and Ball, D. W. (2001). Notch signaling induces cell cycle arrest in small cell lung cancer cells. *Cancer Res* **61**, 3200-5.

Sriuranpong, V., Borges, M. W., Strock, C. L., Nakakura, E. K., Watkins, D. N., Blaumueller, C. M., Nelkin, B. D. and Ball, D. W. (2002). Notch signaling induces rapid degradation of achaete-scute homolog 1. *Mol Cell Biol* **22**, 3129-39.

Stanger, B. Z., Datar, R., Murtaugh, L. C. and Melton, D. A. (2005). Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci U S A* **102**, 12443-8.

Stappenbeck, T. S. and Gordon, J. I. (2000). Rac1 mutations produce aberrant epithelial differentiation in the developing and adult mouse small intestine. *Development* **127**, 2629-42.

Stappenbeck, T. S., Mills, J. C. and Gordon, J. I. (2003). Molecular features of adult mouse small intestinal epithelial progenitors. *Proc Natl Acad Sci U S A* **100**, 1004-9.

Steinhilb, M. L., Turner, R. S. and Gaut, J. R. (2001). The protease inhibitor, MG132, blocks maturation of the amyloid precursor protein Swedish mutant preventing cleavage by beta-Secretase. *J Biol Chem* **276**, 4476-84.

Steinmetz, K. A. and Potter, J. D. (1991). Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Causes Control* **2**, 325-57.

Stierum, R., Gaspari, M., Dommels, Y., Ouatas, T., Pluk, H., Jespersen, S., Vogels, J., Verhoeckx, K., Groten, J. and van Ommen, B. (2003). Proteome analysis reveals novel proteins associated with proliferation and differentiation of the colorectal cancer cell line Caco-2. *Biochim Biophys Acta* **1650**, 73-91.

Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E. and Artavanis-Tsakonas, S. (1992). Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. *Nat Genet* **2**, 119-27.

Stollman, N. and Raskin, J. B. (2004). Diverticular disease of the colon. *Lancet* **363**, 631-9.

Struhl, G. and Adachi, A. (1998). Nuclear access and action of notch in vivo. *Cell* **93**, 649-60.

Struhl, G. and Greenwald, I. (1999). Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature* **398**, 522-5.

Stylianou, S., Clarke, R. B. and Brennan, K. (2006). Aberrant activation of notch signaling in human breast cancer. *Cancer Res* **66**, 1517-25.

Su, L. K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A. and Dove, W. F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science* **256**, 668-70.

Sukegawa, A., Narita, T., Kameda, T., Saitoh, K., Nohno, T., Iba, H., Yasugi, S. and Fukuda, K. (2000). The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium. *Development* **127**, 1971-80.

Sureban, S. M., May, R., George, R. J., Dieckgraefe, B. K., McLeod, H. L., Ramalingam, S., Bishnupuri, K. S., Natarajan, G., Anant, S. and Houchen, C. W. (2008). Knockdown of RNA binding protein musashi-1 leads to tumor regression in vivo. *Gastroenterology* **134**, 1448-58.

Sutter, T., Doi, S., Carnevale, K. A., Arber, N. and Weinstein, I. B. (1997). Expression of cyclins D1 and E in human colon adenocarcinomas. *J Med* **28**, 285-309.

Suzuki, K., Fukui, H., Kayahara, T., Sawada, M., Seno, H., Hiai, H., Kageyama, R., Okano, H. and Chiba, T. (2005). Hes1-deficient mice show precocious differentiation of Paneth cells in the small intestine. *Biochem Biophys Res Commun* **328**, 348-52.

Swiatek, P. J., Lindsell, C. E., del Amo, F. F., Weinmaster, G. and Gridley, T. (1994). Notch1 is essential for postimplantation development in mice. *Genes Dev* **8**, 707-19.

Taichman, D. B., Loomes, K. M., Schachtner, S. K., Guttentag, S., Vu, C., Williams, P., Oakey, R. J. and Baldwin, H. S. (2002). Notch1 and Jagged1 expression by the developing pulmonary vasculature. *Dev Dyn* **225**, 166-75.

Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M. F. and Taketo, M. M. (1998). Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* **92**, 645-56.

Takayama, T., Katsuki, S., Takahashi, Y., Ohi, M., Nojiri, S., Sakamaki, S., Kato, J., Kogawa, K., Miyake, H. and Niitsu, Y. (1998). Aberrant crypt foci of the colon as precursors of adenoma and cancer. *N Engl J Med* **339**, 1277-84.

Takebayashi, K., Akazawa, C., Nakanishi, S. and Kageyama, R. (1995). Structure and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-5. Identification of the neural precursor cell-specific promoter element. *J Biol Chem* **270**, 1342-9.

Takebayashi, K., Sasai, Y., Sakai, Y., Watanabe, T., Nakanishi, S. and Kageyama, R. (1994). Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. *J Biol Chem* **269**, 5150-6.

Takemaru, K. I. and Moon, R. T. (2000). The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. *J Cell Biol* **149**, 249-54.

Takizawa, T., Ochiai, W., Nakashima, K. and Taga, T. (2003). Enhanced gene activation by Notch and BMP signaling cross-talk. *Nucleic Acids Res* **31**, 5723-31.

Tanaka, R., Takase, Y., Kanachi, M., Enomoto-Katayama, R., Shirai, T. and Nakagoshi, H. (2007). Notch-, Wingless-, and Dpp-mediated signaling pathways are required for functional specification of Drosophila midgut cells. *Dev Biol* **304**, 53-61.

Tanaka, T. (2009). Colorectal carcinogenesis: Review of human and experimental animal studies. *J Carcinog* **8**, 5.

Tarin, D., Thompson, E. W. and Newgreen, D. F. (2005). The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* **65**, 5996-6000; discussion 6000-1.

Taupin, D. and Podolsky, D. K. (2003). Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol* **4**, 721-32.

Taylor, C. W., Kim, Y. S., Childress-Fields, K. E. and Yeoman, L. C. (1992). Sensitivity of nuclear c-myc levels and induction to differentiation-inducing agents in human colon tumor cell lines. *Cancer Lett* **62**, 95-105.

Taylor, E. W. (1965). The Mechanism of Colchicine Inhibition of Mitosis. I. Kinetics of Inhibition and the Binding of H3-Colchicine. *J Cell Biol* **25**, SUPPL:145-60.

Tetsu, O. and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-6.

Tezuka, K., Yasuda, M., Watanabe, N., Morimura, N., Kuroda, K., Miyatani, S. and Hozumi, N. (2002). Stimulation of osteoblastic cell differentiation by Notch. *J Bone Miner Res* **17**, 231-9.

Thayer, M. J., Tapscott, S. J., Davis, R. L., Wright, W. E., Lassar, A. B. and Weintraub, H. (1989). Positive autoregulation of the myogenic determination gene MyoD1. *Cell* **58**, 241-8.

Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., Qi, Y. P., Gysin, S., Fernandez-del Castillo, C., Yajnik, V. *et al.* (2003). Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* **425**, 851-6.

Thiagalingam, S., Lengauer, C., Leach, F. S., Schutte, M., Hahn, S. A., Overhauser, J., Willson, J. K., Markowitz, S., Hamilton, S. R., Kern, S. E. *et al.* (1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nat Genet* **13**, 343-6.

Thiery, J. P. and Sleeman, J. P. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7**, 131-42.

Thompson, J. S., Saxena, S. K. and Sharp, J. G. (2000). Regulation of intestinal regeneration: new insights. *Microsc Res Tech* **51**, 129-37.

Tien, A. C., Rajan, A. and Bellen, H. J. (2009). A Notch updated. *J Cell Biol* **184**, 621-9.

Timmerman, L. A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J. M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J. C. *et al.* (2004). Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. *Genes Dev* **18**, 99-115.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S. L., Nakanishi, S., Guillemot, F. and Kageyama, R. (1996). Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. *Neuron* **16**, 723-34.

Tomita, N., Jiang, W., Hibshoosh, H., Warburton, D., Kahn, S. M. and Weinstein, I. B. (1992). Isolation and characterization of a highly malignant variant of the SW480 human colon cancer cell line. *Cancer Res* **52**, 6840-7.

Ton-That, H., Kaestner, K. H., Shields, J. M., Mahatanankoon, C. S. and Yang, V. W. (1997). Expression of the gut-enriched Kruppel-like factor gene during development and intestinal tumorigenesis. *FEBS Lett* **419**, 239-43.

Trainer, D. L., Kline, T., McCabe, F. L., Faucette, L. F., Feild, J., Chaikin, M., Anzano, M., Rieman, D., Hoffstein, S., Li, D. J. *et al.* (1988). Biological characterization and oncogene expression in human colorectal carcinoma cell lines. *Int J Cancer* **41**, 287-96.

Tsuchiya, K., Nakamura, T., Okamoto, R., Kanai, T. and Watanabe, M. (2007). Reciprocal targeting of Hath1 and beta-catenin by Wnt glycogen synthase kinase 3beta in human colon cancer. *Gastroenterology* **132**, 208-20.

Tun, T., Hamaguchi, Y., Matsunami, N., Furukawa, T., Honjo, T. and Kawaichi, M. (1994). Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. *Nucleic Acids Res* **22**, 965-71.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D. and Kitajewski, J. (1996). Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* **122**, 2251-9.

Valdes-Mora, F., Gomez del Pulgar, T., Bandres, E., Cejas, P., Ramirez de Molina, A., Perez-Palacios, R., Gallego-Ortega, D., Garcia-Cabezas, M. A., Casado, E., Larrauri, J. *et al.* (2009). TWIST1 overexpression is associated with nodal invasion and male sex in primary colorectal cancer. *Ann Surg Oncol* **16**, 78-87.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. *et al.* (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. *Cell* **88**, 789-99.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P. *et al.* (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111**, 241-50.

van den Brink, G. R. (2007). Hedgehog signaling in development and homeostasis of the gastrointestinal tract. *Physiol Rev* **87**, 1343-75.

van den Brink, G. R., Bleuming, S. A., Hardwick, J. C., Schepman, B. L., Offerhaus, G. J., Keller, J. J., Nielsen, C., Gaffield, W., van Deventer, S. J., Roberts, D. J. *et al.* (2004). Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nat Genet* **36**, 277-82.

van den Brink, G. R. and Offerhaus, G. J. (2007). The morphogenetic code and colon cancer development. *Cancer Cell* **11**, 109-17.

Van der Flier, L. G., Sabates-Bellver, J., Oving, I., Haegebarth, A., De Palo, M., Anti, M., Van Gijn, M. E., Suijkerbuijk, S., Van de Wetering, M., Marra, G. *et al.* (2007). The Intestinal Wnt/TCF Signature. *Gastroenterology* **132**, 628-32.

van Es, J. H. and Clevers, H. (2005). Notch and Wnt inhibitors as potential new drugs for intestinal neoplastic disease. *Trends Mol Med* **11**, 496-502.

van Es, J. H., van Gijn, M. E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D. J., Radtke, F. *et al.* (2005). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959-63.

van Noort, M., Meeldijk, J., van der Zee, R., Destree, O. and Clevers, H. (2002). Wnt signaling controls the phosphorylation status of beta-catenin. *J Biol Chem* **277**, 17901-5.

Vandromme, M., Gauthier-Rouviere, C., Lamb, N. and Fernandez, A. (1996). Regulation of transcription factor localization: fine-tuning of gene expression. *Trends Biochem Sci* **21**, 59-64.

Varnum-Finney, B., Purton, L. E., Yu, M., Brashem-Stein, C., Flowers, D., Staats, S., Moore, K. A., Le Roux, I., Mann, R., Gray, G. *et al.* (1998). The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood* **91**, 4084-91.

Veenendaal, L. M., Kranenburg, O., Smakman, N., Klomp, A., Borel Rinkes, I. H. and van Diest, P. J. (2008). Differential Notch and TGFbeta signaling in primary colorectal tumors and their corresponding metastases. *Cell Oncol* **30**, 1-11.

Veeraraghavalu, K., Pett, M., Kumar, R. V., Nair, P., Rangarajan, A., Stanley, M. A. and Krishna, S. (2004). Papillomavirus-mediated neoplastic progression is associated with reciprocal changes in JAGGED1 and manic fringe expression linked to notch activation. *J Virol* **78**, 8687-700.

Veeraraghavalu, K., Subbaiah, V. K., Srivastava, S., Chakrabarti, O., Syal, R. and Krishna, S. (2005). Complementation of human papillomavirus type 16 E6 and E7 by Jagged1-specific Notch1-phosphatidylinositol 3-kinase signaling involves pleiotropic oncogenic functions independent of CBF1;Su(H);Lag-1 activation. *J Virol* **79**, 7889-98.

Velcich, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Viani, S., Kucherlapati, R., Lipkin, M., Yang, K. and Augenlicht, L. (2002). Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* **295**, 1726-9.

Vidal, A. and Koff, A. (2000). Cell-cycle inhibitors: three families united by a common cause. *Gene* **247**, 1-15.

Vincent-Salomon, A. and Thiery, J. P. (2003). Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. *Breast Cancer Res* **5**, 101-6.

Visvader, J. E. and Lindeman, G. J. (2008). Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* **8**, 755-68.

Vleminckx, K., Vakaet, L., Jr., Mareel, M., Fiers, W. and van Roy, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* **66**, 107-19.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. and Bos, J. L. (1988). Genetic alterations during colorectal-tumor development. *N Engl J Med* **319**, 525-32.

Vogelstein, B. and Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nat Med* **10**, 789-99.

Vooijs, M., Ong, C. T., Hadland, B., Huppert, S., Liu, Z., Korving, J., van den Born, M., Stappenbeck, T., Wu, Y., Clevers, H. *et al.* (2007). Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE. *Development* **134**, 535-44.

Vousden, K. H. and Lane, D. P. (2007). p53 in health and disease. *Nat Rev Mol Cell Biol* **8**, 275-83.

Waite, K. A. and Eng, C. (2003). BMP2 exposure results in decreased PTEN protein degradation and increased PTEN levels. *Hum Mol Genet* **12**, 679-84.

Wakamatsu, Y., Maynard, T. M., Jones, S. U. and Weston, J. A. (1999). NUMB localizes in the basal cortex of mitotic avian neuroepithelial cells and modulates neuronal differentiation by binding to NOTCH-1. *Neuron* **23**, 71-81.

Wallis, D., Hamblen, M., Zhou, Y., Venken, K. J., Schumacher, A., Grimes, H. L., Zoghbi, H. Y., Orkin, S. H. and Bellen, H. J. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. *Development* **130**, 221-32.

Wang, L., Chen, L., Zhu, L., Rawle, M., Nie, S., Zhang, J., Ping, Z., Kangrong, C. and Jacob, T. J. (2002). Regulatory volume decrease is actively modulated during the cell cycle. *J Cell Physiol* **193**, 110-9.

Wang, L., Qin, H., Chen, B., Xin, X., Li, J. and Han, H. (2007). Overexpressed active Notch1 induces cell growth arrest of HeLa cervical carcinoma cells. *Int J Gynecol Cancer* **17**, 1283-92.

Wang, Z., Banerjee, S., Li, Y., Rahman, K. M., Zhang, Y. and Sarkar, F. H. (2006). Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. *Cancer Res* **66**, 2778-84.

Wasan, H. S., Park, H. S., Liu, K. C., Mandir, N. K., Winnett, A., Sasieni, P., Bodmer, W. F., Goodlad, R. A. and Wright, N. A. (1998). APC in the regulation of intestinal crypt fission. *J Pathol* **185**, 246-55.

Watabe-Uchida, M., Uchida, N., Imamura, Y., Nagafuchi, A., Fujimoto, K., Uemura, T., Vermeulen, S., van Roy, F., Adamson, E. D. and Takeichi, M. (1998). alpha-Catenin-vinculin interaction functions to organize the apical junctional complex in epithelial cells. *J Cell Biol* **142**, 847-57.

Watt, F. M., Estrach, S. and Ambler, C. A. (2008). Epidermal Notch signalling: differentiation, cancer and adhesion. *Curr Opin Cell Biol* **20**, 171-9.

Weerkamp, F., van Dongen, J. J. and Staal, F. J. (2006). Notch and Wnt signaling in T-lymphocyte development and acute lymphoblastic leukemia. *Leukemia* **20**, 1197-205.

Wehkamp, J., Salzman, N. H., Porter, E., Nuding, S., Weichenthal, M., Petras, R. E., Shen, B., Schaeffeler, E., Schwab, M., Linzmeier, R. *et al.* (2005). Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A* **102**, 18129-34.

Weijzen, S., Rizzo, P., Braid, M., Vaishnav, R., Jonkheer, S. M., Zlobin, A., Osborne, B. A., Gottipati, S., Aster, J. C., Hahn, W. C. *et al.* (2002a). Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. *Nat Med* **8**, 979-86.

Weijzen, S., Velders, M. P., Elmishad, A. G., Bacon, P. E., Panella, J. R., Nickoloff, B. J., Miele, L. and Kast, W. M. (2002b). The Notch ligand Jagged-1 is able to induce maturation of monocyte-derived human dendritic cells. *J Immunol* **169**, 4273-8.

Weinberg, R. A. (1995). The molecular basis of oncogenes and tumor suppressor genes. *Ann N Y Acad Sci* **758**, 331-8.

Weinmaster, G., Roberts, V. J. and Lemke, G. (1992). Notch2: a second mammalian Notch gene. *Development* **116**, 931-41.

Weiser, M. M. (1973a). Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. *J Biol Chem* **248**, 2536-41.

Weiser, M. M. (1973b). Intestinal epithelial cell surface membrane glycoprotein synthesis. II. Glycosyltransferases and endogenous acceptors of the undifferentiated cell surface membrane. *J Biol Chem* **248**, 2542-8.

Weng, A. P., Ferrando, A. A., Lee, W., Morris, J. P. t., Silverman, L. B., Sanchez-Irizarry, C., Blacklow, S. C., Look, A. T. and Aster, J. C. (2004). Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**, 269-71.

Weng, A. P., Millholland, J. M., Yashiro-Ohtani, Y., Arcangeli, M. L., Lau, A., Wai, C., Del Bianco, C., Rodriguez, C. G., Sai, H., Tobias, J. *et al.* (2006). c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev* **20**, 2096-109.

Weng, A. P., Nam, Y., Wolfe, M. S., Pear, W. S., Griffin, J. D., Blacklow, S. C. and Aster, J. C. (2003). Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol Cell Biol* **23**, 655-64.

Wesley, C. S. (1999). Notch and wingless regulate expression of cuticle patterning genes. *Mol Cell Biol* **19**, 5743-58.

Westhoff, B., Colaluca, I. N., D'Ario, G., Donzelli, M., Tosoni, D., Volorio, S., Pelosi, G., Spaggiari, L., Mazzarol, G., Viale, G. *et al.* (2009). Alterations of the Notch pathway in lung cancer. *Proc Natl Acad Sci U S A*.

Wettstein, D. A., Turner, D. L. and Kintner, C. (1997). The Xenopus homolog of Drosophila Suppressor of Hairless mediates Notch signaling during primary neurogenesis. *Development* **124**, 693-702.

Wharton, K. A., Johansen, K. M., Xu, T. and Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-81.

Whitehead, R. H., Young, G. P. and Bhathal, P. S. (1986). Effects of short chain fatty acids on a new human colon carcinoma cell line (LIM1215). *Gut* **27**, 1457-63.

Whiteley, L. O., Hudson, L., Jr. and Pretlow, T. P. (1996). Aberrant crypt foci in the colonic mucosa of rats treated with a genotoxic and nongenotoxic colon carcinogen. *Toxicol Pathol* **24**, 681-9.

Wicki, A., Lehembre, F., Wick, N., Hantusch, B., Kerjaschki, D. and Christofori, G. (2006). Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. *Cancer Cell* **9**, 261-72.

Williams, A. C., Harper, S. J. and Paraskeva, C. (1990). Neoplastic transformation of a human colonic epithelial cell line: in vitro evidence for the adenoma to carcinoma sequence. *Cancer Res* **50**, 4724-30.

Wilson, A. A. and Kotton, D. N. (2008). Another notch in stem cell biology: Drosophila intestinal stem cells and the specification of cell fates. *Bioessays* **30**, 107-9.

Wilson, A. J., Byun, D. S., Popova, N., Murray, L. B., L'Italien, K., Sowa, Y., Arango, D., Velcich, A., Augenlicht, L. H. and Mariadason, J. M. (2006). Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem* **281**, 13548-58.

Wilson, J. W., Deed, R. W., Inoue, T., Balzi, M., Becciolini, A., Faraoni, P., Potten, C. S. and Norton, J. D. (2001). Expression of Id helix-loop-helix proteins in colorectal adenocarcinoma correlates with p53 expression and mitotic index. *Cancer Res* **61**, 8803-10.

Winesett, M. P., Ramsey, G. W. and Barnard, J. A. (1996). Type II TGF(beta) receptor expression in intestinal cell lines and in the intestinal tract. *Carcinogenesis* **17**, 989-95.

Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J. and Harper, J. W. (1999). The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. *Genes Dev* **13**, 270-83.

Wittekind, C., Compton, C. C., Greene, F. L. and Sobin, L. H. (2002). TNM residual tumor classification revisited. *Cancer* **94**, 2511-6.

Woda, B. A., Forde, K. and Lane, N. (1977). A unicryptal colonic adenoma, the smallest colonic neoplasm yet observed in a non-polyposis individual. *Am J Clin Pathol* **68**, 631-2.

Wolpert, L. (1996). One hundred years of positional information. *Trends Genet* **12**, 359-64.

Wong, G. T., Manfra, D., Poulet, F. M., Zhang, Q., Josien, H., Bara, T., Engstrom, L., Pinzon-Ortiz, M., Fine, J. S., Lee, H. J. *et al.* (2004). Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J Biol Chem* **279**, 12876-82.

Wong, J. M., de Souza, R., Kendall, C. W., Emam, A. and Jenkins, D. J. (2006). Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* **40**, 235-43.

Wong, M. H., Huelsken, J., Birchmeier, W. and Gordon, J. I. (2002). Selection of multipotent stem cells during morphogenesis of small intestinal crypts of Lieberkuhn is perturbed by stimulation of Lef-1/beta-catenin signaling. *J Biol Chem* **277**, 15843-50.

Woodhouse, E. C., Chuaqui, R. F. and Liotta, L. A. (1997). General mechanisms of metastasis. *Cancer* **80**, 1529-37.

Wright, T. R. (1970). The genetics of embryogenesis in Drosophila. *Adv Genet* **15**, 261-395.

Wu, G., Lyapina, S., Das, I., Li, J., Gurney, M., Pauley, A., Chui, I., Deshaies, R. J. and Kitajewski, J. (2001). SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. *Mol Cell Biol* **21**, 7403-15.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S. and Griffin, J. D. (2000). MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat Genet* **26**, 484-9.

Xavier, R. J. and Podolsky, D. K. (2007). Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427-34.

Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-71.

Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G. and Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* **8**, 723-30.

Yamada, Y. and Mori, H. (2007). Multistep carcinogenesis of the colon in Apc(Min/+) mouse. *Cancer Sci* **98**, 6-10.

Yang, H. B., Hsu, P. I., Chan, S. H., Lee, J. C., Shin, J. S. and Chow, N. H. (1996). Growth kinetics of colorectal adenoma-carcinoma sequence: an immunohistochemical study of proliferating cell nuclear antigen expression. *Hum Pathol* **27**, 1071-6.

Yang, J., Mani, S. A., Donaher, J. L., Ramaswamy, S., Itzykson, R. A., Come, C., Savagner, P., Gitelman, I., Richardson, A. and Weinberg, R. A. (2004). Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**, 927-39.

Yang, Q., Bermingham, N. A., Finegold, M. J. and Zoghbi, H. Y. (2001a). Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. *Science* **294**, 2155-8.

Yang, W., Bancroft, L., Liang, J., Zhuang, M. and Augenlicht, L. H. (2005). p27kip1 in intestinal tumorigenesis and chemoprevention in the mouse. *Cancer Res* **65**, 9363-8.

Yang, W., Bancroft, L., Nicholas, C., Lozonschi, I. and Augenlicht, L. H. (2003). Targeted inactivation of p27kip1 is sufficient for large and small intestinal tumorigenesis in the mouse, which can be augmented by a Western-style high-risk diet. *Cancer Res* **63**, 4990-6.

Yang, W., Velcich, A., Mariadason, J., Nicholas, C., Corner, G., Houston, M., Edelmann, W., Kucherlapati, R., Holt, P. R. and Augenlicht, L. H. (2001b). p21(WAF1/cip1) is an important determinant of intestinal cell response to sulindac in vitro and in vivo. *Cancer Res* **61**, 6297-302.

Yang, W. C., Mathew, J., Velcich, A., Edelmann, W., Kucherlapati, R., Lipkin, M., Yang, K. and Augenlicht, L. H. (2001c). Targeted inactivation of the p21(WAF1/cip1) gene enhances Apc-initiated tumor formation and the tumor-promoting activity of a Western-style high-risk diet by altering cell maturation in the intestinal mucosal. *Cancer Res* **61**, 565-9.

Yarbro, J. W. (1992). Mechanism of action of hydroxyurea. *Semin Oncol* **19**, 1-10.

Yen, T. H. and Wright, N. A. (2006). The gastrointestinal tract stem cell niche. *Stem Cell Rev* **2**, 203-12.

Yoshiura, S., Ohtsuka, T., Takenaka, Y., Nagahara, H., Yoshikawa, K. and Kageyama, R. (2007). Ultradian oscillations of Stat, Smad, and Hes1 expression in response to serum. *Proc Natl Acad Sci U S A* **104**, 11292-7.

Young, A. and Rea, D. (2000). ABC of colorectal cancer: treatment of advanced disease. *Bmj* **321**, 1278-81.

Zagouras, P., Stifani, S., Blaumueller, C. M., Carcangiu, M. L. and Artavanis-Tsakonas, S. (1995). Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc Natl Acad Sci U S A* **92**, 6414-8.

Zavadil, J., Bitzer, M., Liang, D., Yang, Y. C., Massimi, A., Kneitz, S., Piek, E. and Bottinger, E. P. (2001). Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci U S A* **98**, 6686-91.

Zavadil, J. and Bottinger, E. P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene* **24**, 5764-74.

Zavadil, J., Cermak, L., Soto-Nieves, N. and Bottinger, E. P. (2004). Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *Embo J* **23**, 1155-65.

Zecchini, V., Domaschenz, R., Winton, D. and Jones, P. (2005). Notch signaling regulates the differentiation of post-mitotic intestinal epithelial cells. *Genes Dev* **19**, 1686-91.

Zhang, L., Song, L. and Parker, E. M. (1999). Calpain inhibitor I increases beta-amyloid peptide production by inhibiting the degradation of the substrate of gamma-secretase. Evidence that substrate availability limits beta-amyloid peptide production. *J Biol Chem* **274**, 8966-72.

Zhang, Z., Hartmann, H., Do, V. M., Abramowski, D., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B., van de Wetering, M., Clevers, H., Saftig, P. *et al.* (1998). Destabilization of beta-catenin by mutations in presenilin-1 potentiates neuronal apoptosis. *Nature* **395**, 698-702.

Zhao, J., Kim, K. A. and Abo, A. (2009). Tipping the balance: modulating the Wnt pathway for tissue repair. *Trends Biotechnol* **27**, 131-6.

Zheng, Q., Qin, H., Zhang, H., Li, J., Hou, L., Wang, H., Zhang, X., Zhang, S., Feng, L., Liang, Y. *et al.* (2007). Notch signaling inhibits growth of the human lung adenocarcinoma cell line A549. *Oncol Rep* **17**, 847-52.

Zhong, W., Feder, J. N., Jiang, M. M., Jan, L. Y. and Jan, Y. N. (1996). Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. *Neuron* **17**, 43-53.

Zisman, T. L. and Rubin, D. T. (2008). Colorectal cancer and dysplasia in inflammatory bowel disease. *World J Gastroenterol* **14**, 2662-9.

Zlobec, I., Steele, R., Michel, R. P., Compton, C. C., Lugli, A. and Jass, J. R. (2006). Scoring of p53, VEGF, Bcl-2 and APAF-1 immunohistochemistry and interobserver reliability in colorectal cancer. *Mod Pathol* **19**, 1236-42.

Zlobec, I., Terracciano, L., Jass, J. R. and Lugli, A. (2007). Value of staining intensity in the interpretation of immunohistochemistry for tumor markers in colorectal cancer. *Virchows Arch* **451**, 763-9.

Zweibaum, A. (1993). [Differentiation of human colon cancer cells: a new approach to colon cancer]. *Bull Acad Natl Med* **177**, 63-71; discussion 71-3.

Zweidler-McKay, P. A., He, Y., Xu, L., Rodriguez, C. G., Karnell, F. G., Carpenter, A. C., Aster, J. C., Allman, D. and Pear, W. S. (2005). Notch signaling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies. *Blood* **106**, 3898-906.