The roles of the Apc proteins in homeostasis and tumourigenesis

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A thesis presented for the award of PhD

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DECLARATION

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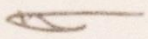
Signed  Date 31/3/13

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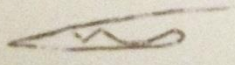
Signed  Date 31/3/13

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Abbreviations and definitions

°C	Degree celsius
µg	Micrograms
µl	Microlitres
µm	Micrometre
aa	Amino acid
ABC	Avidin biotin complex
ADH	Atypical ductal hyperplasia
Ah-Cre	Aryl Hydrocarbon Cre recombinase transgene
APC	Adenomatous poliposis coli
APC2	Adenomatous poliposis coli 2
Apc2$^{-/-}$	Adenomatous poliposis coli 2 transgene
Apc^{min}	Multiple intestinal neoplasia
ASCL2	Achaete Scutelike 2
Axin	Axis inhibitor
Blg-Cre^{+}	Beta-lactoglobulin gene Cre recombinase transgene
Bmi-1	Polycomb ring finger oncogene
bNF	*β*-naphthoflavone
bp	Base pair
BRCA1	Breast cancer type 1 susceptibility
BRCA2	Breast cancer type 2 susceptibility
BrdU	5-Bromo-2-deoxyuridine
BSA	Bovine serum albumin
cDNA	Copy DNA
CK5	Cytokeratin 5
CK8	Cytokeratin 8
cm	Centimetres
CMV	Cytomegalovirus
CPS	Carbamoyl Phosphate synthetase
CRC	Colorectal cancer
Cre	Cre recombinase
C-terminus	Carboxyl-terminus
CV	Central vein
DAB	3,3-diaminobenzidine
DAPI	4',6-diamidino-2-phynylindole
DCIS	Ductal carcinoma *'in situ'*
DEN	Diethylnitrosamine
DEPC	Diethylpyrocarbonate

dH_2O	Distilled H2O
DIG	Digoxigenin
DKO	Double knockout
dlg	Discs large
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EB1	End binding protein 1
EB3	End binding protein 3
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
EGTA	Ethylineglycol tetra-acetic acid
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
g	Grams
GLT1	Glutamate transporter
GS	Glutamine synthetase
GSK-3	Glycogen Synthase Kinase-3
h	Hour
H&E	Haematoxylin and Eosin
HBOC	Hereditary breast and ovarian cancer
HCC	Hepatocellular carcinoma
HIF-1	Hypoxia-inducible factor 1
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
ISC	Intestinal stem cell
IHC	Immunohistochemistry
Kg	Kilogram
K-Ras	Kirsten rat sarcoma viral oncogene
LEC2	Leukocyte cell-derived chemotaxin-2
LEF	Lymphoid enhancer-binding factor
Lgl	Lethal giant larvae
Lgr5	Leucine-rich repeat containing Gprotein coupled receptor 5
LKB1	Liver kinase B1
LN_2	Liquid N2
LOH	Loss of heterozygosity
LoxP	Locus of crossover of bacteriophage P1
LRP	Lipoprotein receptor-related protein
M	Molar
Mad2	Mitotic arrest deficient 2
MCR	Mutational cluster region
mDia	Mammalian diaphanous
mg	Milligrams
MIF	Migratory inhibitory factor
min	Minutes

ml	millilitres
MLH1	MutL homolog 1
mM	millimolar
mm	Millimetres
MMR	Mismatch repair
MOI	Multiplicity of infection
mRNA	Messenger RNA
NGS	Normal goat serum
NRS	Normal rabbit serum
N-terminus	Amino-terminus
OAT	Ornithine aminotransferase
Olfm4	Olfactomedin 4
p	Probability
P/S	Penicillin/ streptomycin
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with TWEEN
PCR	Polymerase chain reaction
PI3k	Phosphatidylinositol-3 Kinase
PJS	Peutz-Jeghers syndrome
PLL	Poly-L-lysine
PMS1	Postmeiotic segregation increased 1
PMS2	Postmeiotic segregation increased 2
PP	Peri-portal
PS	Portal space
PSRC1	Proline/serine-rich coiled-coil 1
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PV	Portal vein
qRT-PCR	Quantitative reverse-transcription PCR
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
Scrib	Scribble
SD	Standard deviation
SDH	Succinate dehydrogenase
siRNA	Small interfering RNA
SSCP	Single-strand conformation polymorphism
TCF	T-cell factor
TF	Transcription factor
UV	Ultraviolet
V	Volts
v/v	Volume per volume
VHL	Von hippel-Lindau
Villin-Cre^{ER}	Villin Cre recombinase estrogen receptor transgene
w/v	Weight per volume
WAP	Whey acidic protein

Wisp1	Wnt-1 inducible signaling protein 1
wk	Weeks
Wnt	Wingless-type murine mammary tumour virus integration site family
WT	Wild type
X	Times
x g	Times gravity
X-gal	5-bromo-4-chloro-3-indolyl-B-D-glactopyranoside
ZO-1	Zonala occudens protein 1

Abstract

The adenomatous polyposis coli (*APC*) gene encodes a multifunctional tumour suppressor protein that is essential for normal development. The most characterised role of APC is its ability to mediate the Wnt signaling pathway, a pathway disrupted in the majority of human cancers. The identification of a second adenomatous polyposis coli gene (*APC2*) which possesses many shared structural characteristics with *APC* and potentially comparable functions raises the possibility that *APC2* also functions both in development and in tumour suppression, and that some redundancy may exist between the two proteins. Analysis of these proteins in the mouse has been hampered due to the lethality of the *Apc* mutation and the lack of a suitable *Apc2* mutation. However, to circumvent the first of these difficulties, Cre-lox technology was employed to conditionally delete *Apc* in adult mouse tissues and so study its function *in vivo*. To circumvent the second difficulty, a novel *Apc2* null allele had become available from the laboratory of Professor Hans Clevers. Remarkably, constitutive deletion of *Apc2* does not lead to embryonic lethality, permitting study of the effects of Apc2 deficiency within adult tissues. In this thesis I aimed to characterise the consequences of Apc2 loss alone, and in the context of tissue specific Apc loss, in a range of tissues. Apc2 deficiency led to subtle changes in Wnt signaling in the intestines and liver however, no detectable differences of this pathway were apparent within the mammary gland. Phenotypically, altered homeostasis was only observed within the intestines. Apc2 deficiency led to an increase in epithelial cell division, an increase in markers of intestinal stemness and increases in intestinal cell migration. However, loss of Apc2 failed to induce tumourigenesis in the intestines or indeed any other tissue. In the context of Apc loss, the effect was dependent upon the tissue. Within the intestines, additional loss of Apc2 altered the immediate phenotype of Apc loss but failed to modify Apc induced tumourigenesis. Within the mammary gland, whilst either Apc protein alone was dispensable, combined loss synergised to disrupt homeostasis and drive tumourigenesis. Contrary to this, in the liver the additional loss of Apc2 attenuated tumourigenesis induced by reduced levels of Apc. Together, these studies highlight the importance of these proteins and their interactions and redundancies in homeostasis and tumourigenesis.

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4 Investigating the effects of Apc2 loss in the context of intestinal *Apc* mutation

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6 Investigating the liver phenotypes associated with reduced levels of Apc proteins

Chapter 1

General introduction

1.1 Cancer

1.1.1 Cancer statistics

It was estimated by the World Health Organisation that in 2008 cancer was the cause of 7.9 million deaths worldwide, accounting for 13% of the recorded deaths (Ferlay et al., 2008). In 2010, it was responsible for 28% of recorded UK deaths (Figure 1.1, Cancer Research UK). People living within developed countries are 1.7 times more likely to develop cancer than

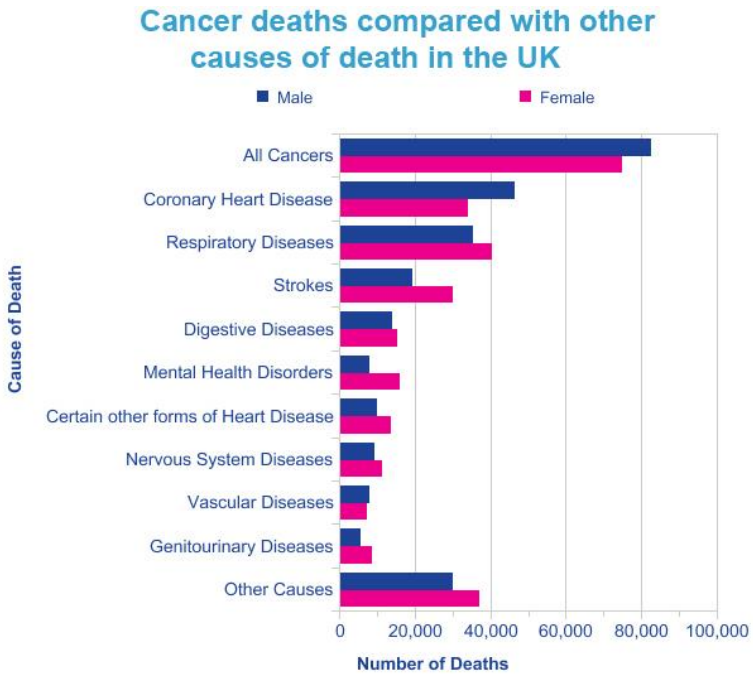

Figure 1.1 In 2010, 28% of all UK deaths were attributed to cancer. Males represented a slightly higher percentage (31%) than females (26%) (Cancer Research UK).

those living in developing countries. Certain cancers such as melanoma of the skin, kidney cancer and multiple myeloma display rates more than three times higher in developed countries. Regardless of geographic location, cancer rates also display sexual dimorphism. Males have both a higher occurrence and a higher rate of cancer related death (Ferlay et al.,

2008). Discrepancies in cancer rates between different populations and genders may be influenced by a range of environmental, racial, cultural and socioeconomic factors. However, the underlying molecular mechanisms which lead to cancer development remain constant.

The recorded incidence of cancer has been on the increase within the UK (Figure 1.2, Cancer Research UK). Whilst many factors have contributed to this increase in a negative way, such as lifestyle or environmental changes, better awareness, screening and diagnosis may positively contribute. For example, advances in screening methods and technology have led to earlier tumour detection and treatment, an important factor in positively influencing

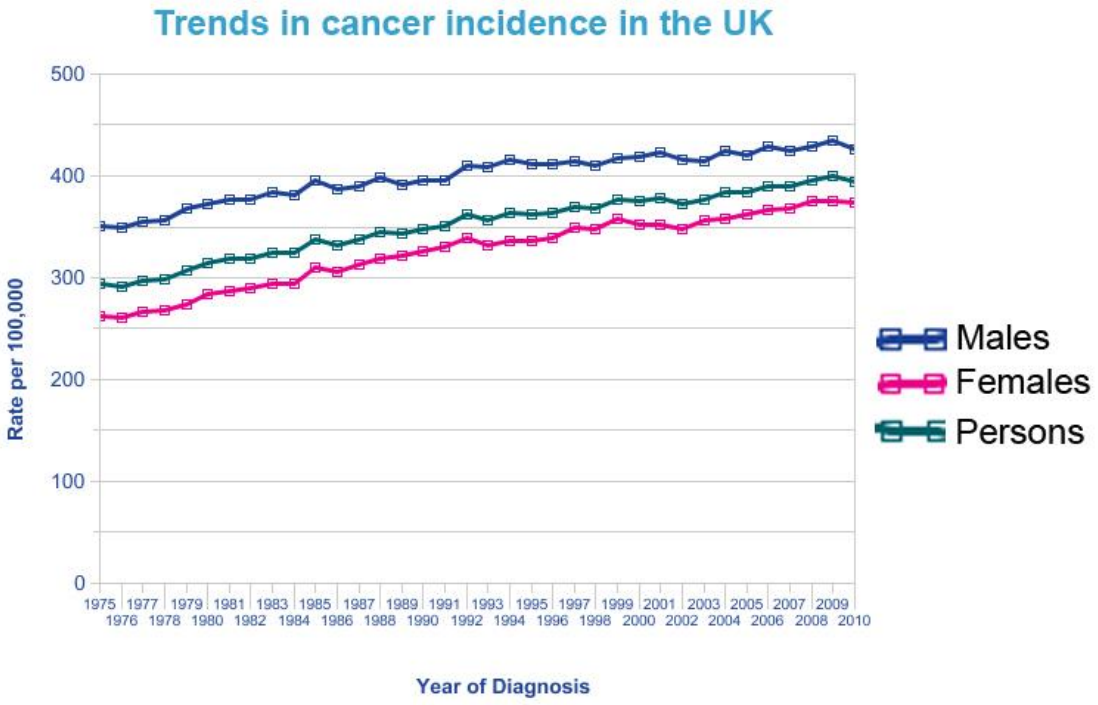

Figure 1.2 Incidence of cancer has increased with time. European age-standardised incidence rates have increased for all cancers in the UK by 22% in males and by 42% in females (Cancer Research UK).

prognosis. Although an increase in cancer incidence has occurred with time, so has the understanding of the disease. Increased understanding has directly led to better treatment strategies and improved 10 year survival rates in a range of cancers (Figure 1.3, Cancer Research UK). However, it is important to stress that although we are getting better at

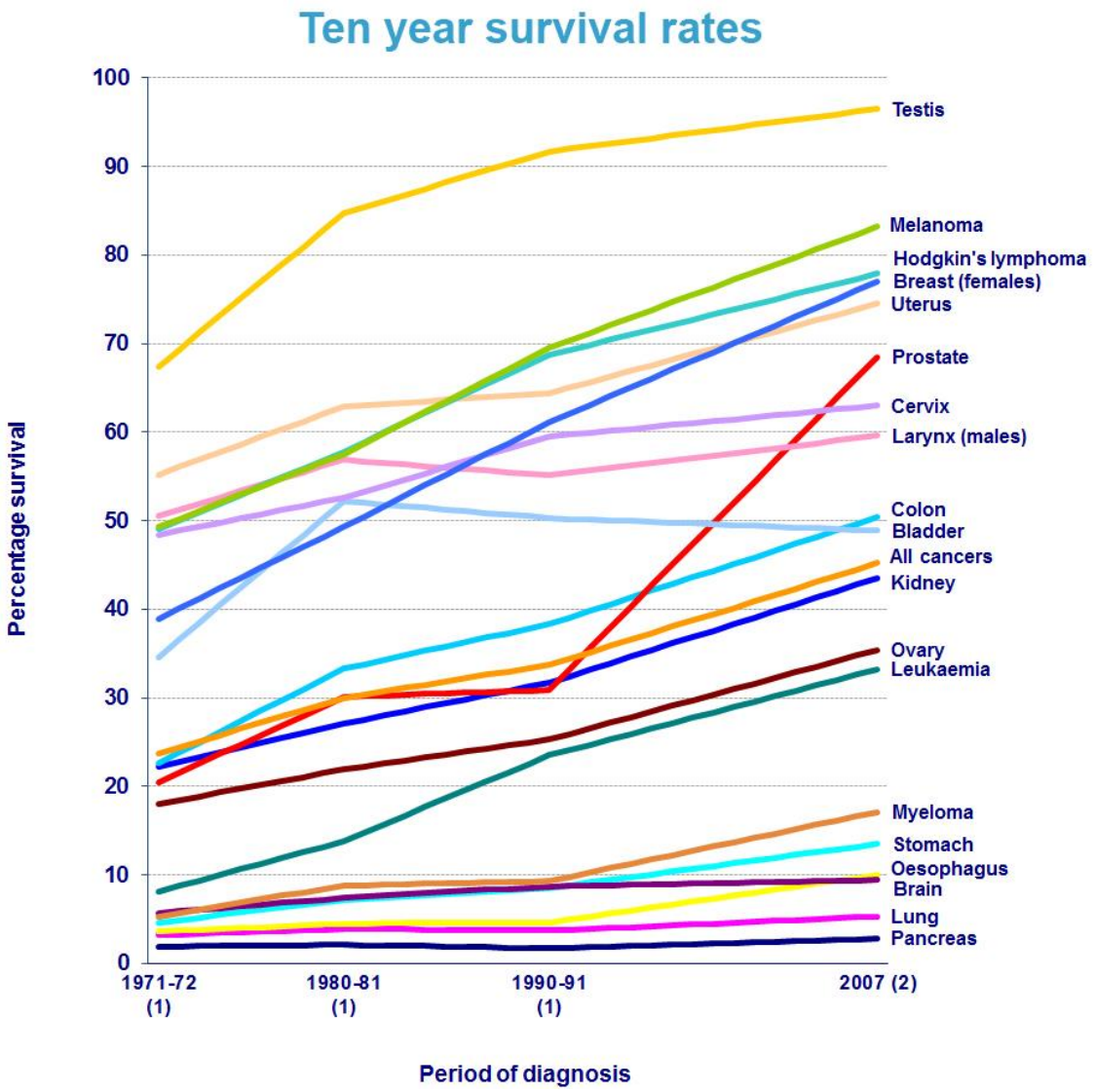

Figure 1.3 Shows the increase in ten-year survival rates from the 1970s to the present for some common cancers. Whilst there has been improvement in survival for most cancers, some display very little change and survival remains low (Cancer Research UK).

treating some cancers such as colon, breast and melanoma, other cancers such as lung and pancreatic cancer have displayed minimal increases in patient survival. Earlier detection and better treatment strategies have helped reduce the sum of all UK cancer related deaths for both genders since the 1990s. (Figure 1.4, Cancer Research UK). Continuing advancement in the understanding of the biological and molecular mechanisms responsible for tumour initiation and progression is key in further increasing the survival of patients and improving overall quality of life.

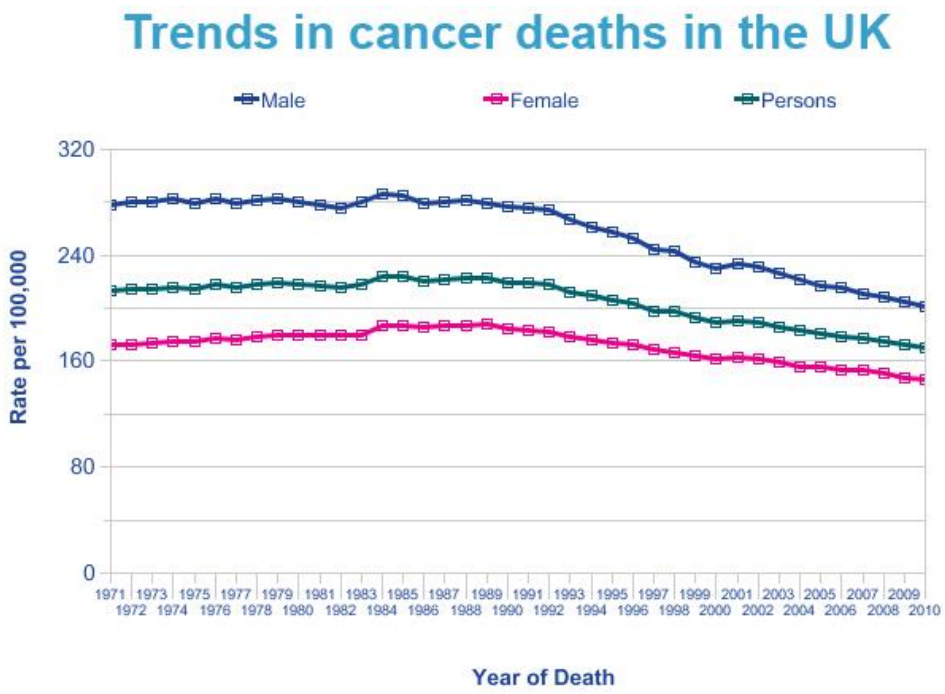

Figure 1.4 In the UK mortality for all cancers combined started to fall in the early 1990s. European age-standardised mortality rates decreased by 26% in males and 20% in females during the 1989-2010 period (Cancer Research UK).

1.1.2 Definition of cancer

Cancer is the collective term for over 100 diseases. It is the endpoint of a process whereby normal human cells have transformed into malignant tumours (Figure 1.5). Cumulative genetic changes initiate and contribute to this transition in a stepwise manner. Whilst different tumours may follow unique pathways of progression, key alterations in regulation of a number of biological processes are required for all cancers (reviewed in Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). Regardless of cellular origin, control of proliferation and/or apoptosis must be bypassed in the early stages, whilst in the later stages cells must acquire invasive and metastatic abilities. In cancer patients with advanced disease, deaths usually occur due to organ or tissue failure. Invading cancer cells possess the ability to outcompete normal physiologically relevant cells, disrupting vital body functions. 90% of all cancer related deaths are attributed to the ability of cancer cells to invade and metastasise (Chaffer & Weinberg, 2011).

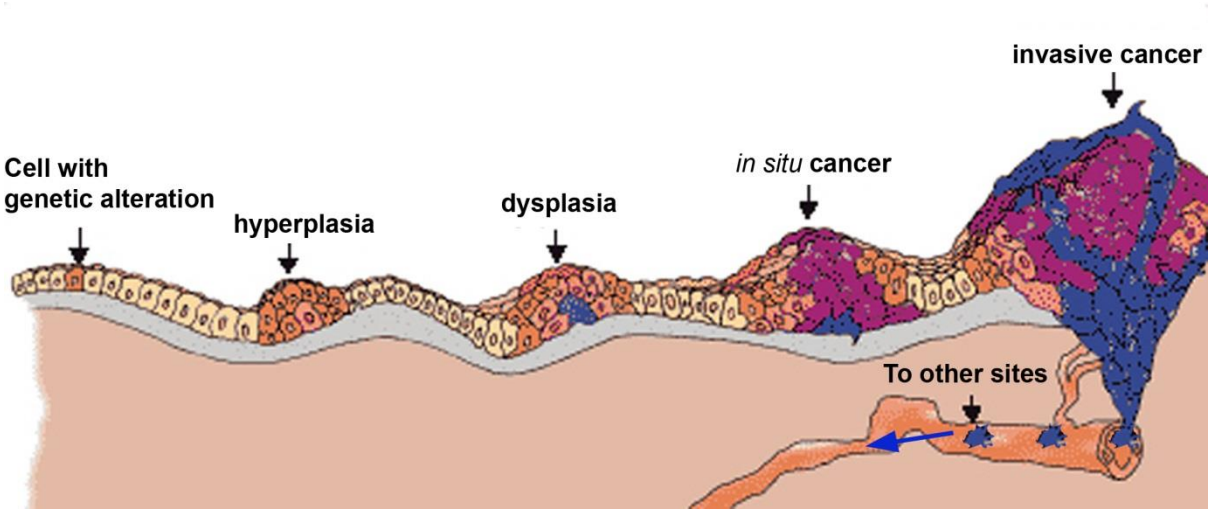

Figure 1.5 Diagram depicting cancer progression. Most cancers are thought to arise due to accumulative mutations in key genes which control a number of regulatory possesses. Hyperplasia is an early step where cells acquire the ability to increase proliferation and/or reduce apoptosis. Subsequent alterations in other genes and regulatory possesses allow these cells to progress into invasive carcinomas through a stepwise progression. In most patients, cancer related death is due to the acquired invasive capability of cancer cells. Cells can 'break off' from the original primary tumour, travel though the lymphatic and/or blood circulatory systems, attach and form secondary tumours in various locations (adapted from www.ndhealthfacts.org/wiki/oncology).

1.1.3 "The hallmarks of cancer"

It was proposed by Hanahan & Weinberg (2000) that six essential alterations in cell physiology could cooperatively dictate malignant growth, coined "the hallmarks of cancer". Following more than a decade of research, four new "hallmarks" have emerged and together these highlight the barriers that cells need to circumvent to develop into cancer (Figure 1.6, discussed in Hanahan & Weinberg, 2011). Some criticisms have been made of these hallmarks, as benign tumours also possess a majority of these features, thus the only true requisite for "cancer" classification are invasive and metastatic capabilities (Lazebnik, 2010). However, regardless of differences in definition, these acquired capabilities are vital components of most cancers and are central to the understanding of the disease. These 'hallmarks' will briefly be discussed.

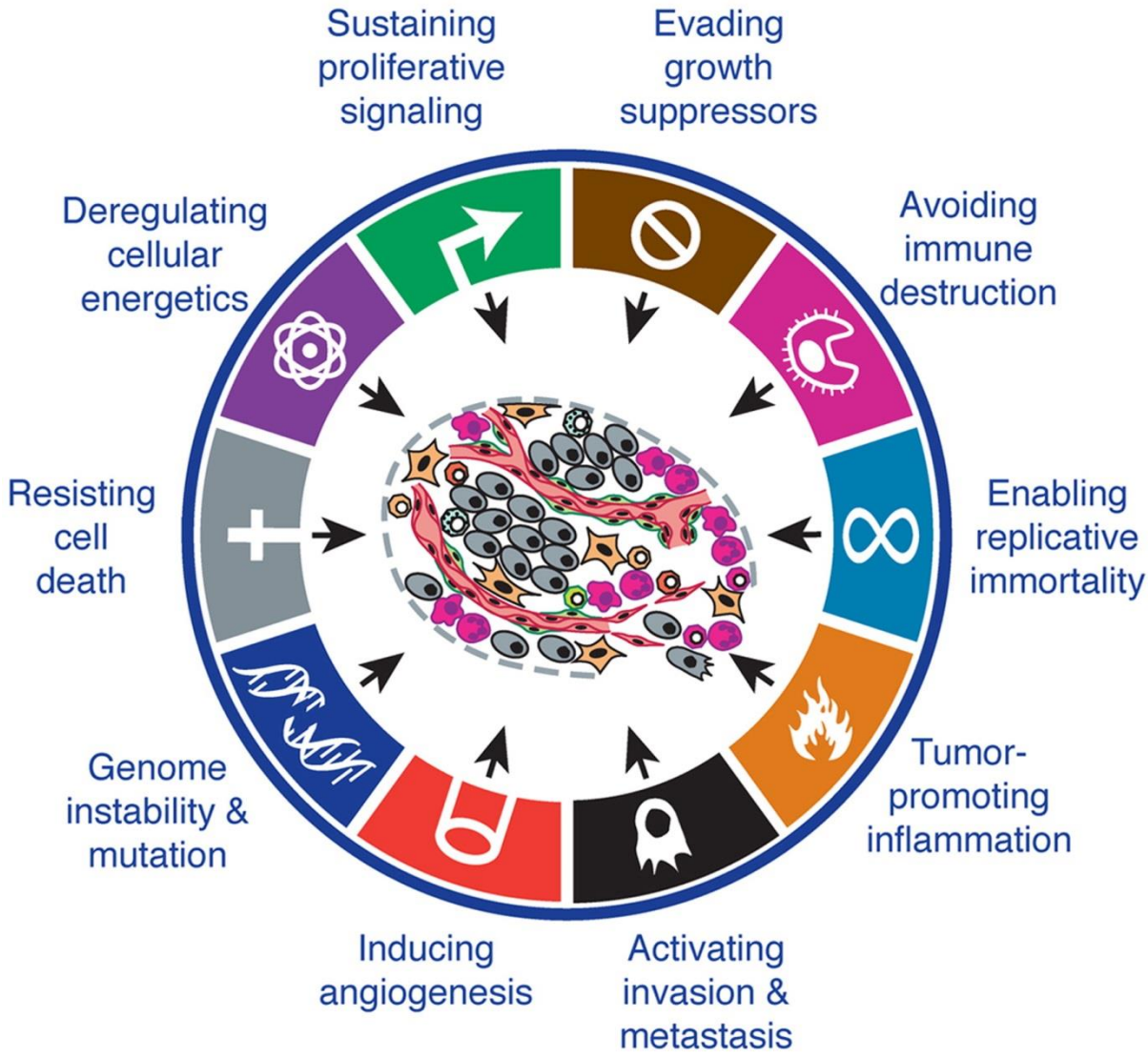

Figure 1.6 "The hallmarks of cancer". Ten traits govern the transformation of cells from normalcy to cancer (Hanahan & Weinberg, 2011).

Sustaining proliferative signaling

The ability to continually proliferate is one of the most fundamental traits of cancer cells. This is achieved through disruption in the regulation of growth signals and/or transduction of these signals.

Enabling replicative immortality

Most normal cell lineages in the body pass through a limited number of successive growth and division cycles. Cancer cell have the ability to circumvent this limitation.

Deregulating cellular energetics

Cancer cells acquire the ability to alter their energy metabolism in order to fuel cell growth and division. This is achieved through reprograming various metabolic pathways.

Inducing angiogenesis

For tumours to progress, neoplastic cells, like normal cells, require nutrients and oxygen and to get rid of waste materials such as carbon dioxide. Increases in tumour mass require increased vasculature. This is achieved through the process of angiogenesis, a process usually quiescent in adult tissues.

Tumour-promoting inflammation

It is known that tumour progression can be influenced by the micro-environment. Inflammation has been shown to help tumour progression. Tumour cells can induce an inflammatory response which subsequently helps neoplastic cells acquire further hallmark capabilities which aid progression.

Evading growth suppressors

In a normal setting, many tumour suppressors operate in various ways to negatively regulate cell proliferation. Tumour cells acquire the ability to circumvent the mechanisms in place that control growth and proliferation.

Resisting cell death

'Programed cell death' serves as a natural barrier to cancer. Neoplastic cell acquire the ability to circumvent control mechanisms which normally impose apoptosis in response to a range of cellular stresses.

Avoiding immune destruction

In a normal setting, the immune system is constantly monitoring and destroying cells that display abnormalities. Neoplastic cells either avoid detection or limit the extent of immunological killing, thus evading eradication.

Activating invasion & metastasis

This is the most advanced stage of cancer, responsible for 90% of related deaths (Chaffer & Weinberg, 2011) and some deem the only true requisite for cancer (Lazebnik, 2010). For invasion and metastasis to occur, cancer cells must acquire the ability to alter cell-cell interactions, cell-extracellular matrix interactions and cellular shape and motility. Cancer cells can now spread within tissues and migrate from the site of origin to new locations in the body forming secondary tumours.

Genome instability & Mutation

Genomic instability is a characteristic of most cancers. Defects in genome maintenance allow and accelerate the necessary genetic changes that are needed for tumourigenesis. It is known that tumourigenesis is a multistep process and each step can be implemented through subsequent accumulation of genetic alterations (Foulds, 1958; Bodmer, 1996; Kinzler & Vogelstein, 1996). How these genetic alterations impact upon each of the necessary cancer hallmarks has been the basis of a broad array of cancer research.

1.1.4 Cancer syndromes

An insight into the genetic alterations that contribute to cancer has come from the study of cancer syndromes. A cancer syndrome is a hereditary genetic disorder that predisposes affected individuals to the development of cancer. It is estimated that 5-10% of all cancers are hereditary (Nagy et al., 2004). Cancer syndromes are caused by the inheritance of genetic mutations in one or more genes, usually tumour suppressor, oncogenes and/or DNA repair genes. Numerous syndromes have been identified and a full list can be found at www.facd.info. Some of the most characterised cancer syndromes will briefly be discussed.

Hereditary breast and ovarian cancer (HBOC)

Patients with HBOC have a 65-85% risk of developing breast cancer (Thompson & Easton, 2002; Brose et al., 2002), ~35% risk of developing ovarian cancer (Easton et al., 1995; Ford et al., 1994; Easton et al., 1993) and a higher risk of developing other cancers in their lifetime. Inherited mutations in *BRCA1* & *BRCA2* genes were identified as responsible for this cancer syndrome (Miki et al., 1994; Wooster et al., 1995). The gene products are responsible for repairing DNA or destroying cells if DNA cannot be repaired (reviewed in Venkitaraman, 2002). When mutated, these functions are lost introducing genomic instability (one of the 'hallmarks' of cancer). Cells can now accumulate further genetic alterations that are required for cancer progression.

Cowden syndrome

Patients with Cowden disease develop hamartomas (Eng et al., 1994) in multiple organs and have an increased risk of breast, thyroid and skin cancer (Lloyd & Dennis, 1963; Carlson et al., 1986; Mallory, 1995). Inherited mutations in the *PTEN* gene was identified as responsible for the majority of cases of this syndrome (Liaw et al., 1997). PTEN is a tumour suppressor gene, its most recognised role is regulating the oncogenic pro-survival PI3K/AKT signaling

pathway. Loss of this function through *PTEN* mutation leads to increased growth and proliferation of cells through increased ribosomal and protein translation (reviewed in Salmena et al., 2008). These attributes would contribute to sustained proliferative signaling, another of the 'hallmarks' of cancer.

Li-Fraumeni syndrome

Patients with Li-Fraumeni syndrome have an 85% risk of developing cancer in their lifetime. These include leukemia, soft tissue & bone sarcomas, breast and brain cancers (Ramzi et al., 2010). Inherited mutations in the *p53* gene were identified as responsible for the majority of cases of this syndrome (Malkin et al., 1990). p53 is a tumour suppressor gene, it regulates the cell cycle and allows for repair and survival or apoptosis following DNA damage or cell cycle abnormalities. Loss of this function through mutation leads to genomic instability and accumulation of genetic alterations needed for tumourigenesis (reviewed in Levine, 1997).

Von hippel-lindau disease

Von hippel-lindau disease is a cancer syndrome that is associated with a germline mutation in the *VHL* tumour suppressor gene. Affected individuals are at an increased risk of developing benign and malignant tumours typically in the central nervous system but also in other locations (Lonser et al., 2003). The VHL protein is a tumour suppressor involved with regulation of genes specific for metabolic and angiogenic changes in response to hypoxia through binding HIF-1 (reviewed in Pugh & Ratcliffe, 1997). Loss of this function of VHL leads to inappropriate levels of HIF-1 and thus transcription of genes allowing necessary metabolic and angiogentic changes essential for tumour progression (Maxwell et al., 1999; Forsythe et al., 1996). Dysregulated cellular energetics and induced angiogenesis are two further 'hallmarks' of cancer.

Hereditary non-polyposis colon cancer (HNPCC)

Patients with HNPCC have a 60% risk of developing colorectal cancer in their lifetime (Hampel et al., 2005) and HNPCC accounts for 2.5-5% of all colorectal cancers (Chapelle,

2004). The majority of inherited mutations which cause HNPCC are within mis-match repair (MMR) genes (mutation in *MLH1* & *MSH2* account for 90% of cases, other mutated genes can include *MSH6*, *PMS1* and *PMS2*) (Lui et al., 1996; Wheeler et al., 2000). MMR genes recognise and help repair abnormalities that arise following DNA replication, recombination and some forms of DNA damage (reviewed in Kolodner, 1996). Disruption of this function through mutation of one or more of the various MMR genes, can lead to tumourigenesis through increased genomic instability (Thibodeau et al., 1996).

The cancer syndromes detailed above clearly show that genetic mutations that predispose to cancer have the potential to induce 'hallmarks' essential for tumourigenesis. These genes are also often found mutated in sporadic tumours. Together, this demonstrates the clear association of genetic abnormalities with tumourigenesis. This is perhaps most elegantly shown in the case of familial adenomatous polyposis, where we are beginning to understand the true molecular basis of this predisposition, as outlined in detail below.

1.2 Adenomatous polyposis coli (APC)

1.2.1 Familial adenomatous polyposis (FAP)

The Adenomatous polyposis coli (*APC*) gene was initially identified due to its association with colorectal cancer. It is highly evolutionally conserved (Aoki & Taketo, 2007) and in humans, located on chromosome 5q21 encoding a 2843aa protein contained in 15 exons (AGCOH). Germline mutation in one copy of the *APC* gene is responsible for the inherited disease familial adenomatous polyposis (FAP) (Kinzler et al., 1991; Groden et al., 1991). This disease is characterised by the development of hundreds to thousands of adenomatous polyps in the colorectum, some of which inevitably progress to carcinoma. Initial development of the polyps usually arises due to somatic mutation of the normal remaining *APC* allele. This demonstrates the importance of APC in preventing the early stages of a progression towards cancer. Once *APC* is lost, subsequent accumulation of mutations in other genes, such as *KRAS* and *P53,* assist the development of polyps in a stepwise progression towards carcinoma (Vogelstein and Kinzler, 1996). *APC* is also mutated in up to 80% of sporadic colorectal cancers (Powell et al., 1992; Nagase & Nakamura, 1993; Ilyas et al., 1996). This demonstrates its importance as a "gatekeeper" of intestinal tumourigenesis.

1.2.2 APC's role in tumour suppression

Along with APC's role in intestinal tumourigenesis, loss of *APC* has been implicated in a range of other cancers (Damjanov et al*.,* 1996; Virmani et al., 2001; Furuuchi et al., 2000; Kashiwaba et al., 1994; Giardiello et al 1996; Hirschman et al 2005; Gruner et al., 1998; Toiyama et al., 2011). Significant effort has been placed into studying the functions of APC and how loss of these functions leads to tumour initiation and progression (reviewed by Aoki and Taketo, 2007; Senda et al., 2007; Hanson et al., 2005; Nathke, 2005; Polakis, 2000; McCartney & Nathke, 2008; Prosperi & Goss, 2011).

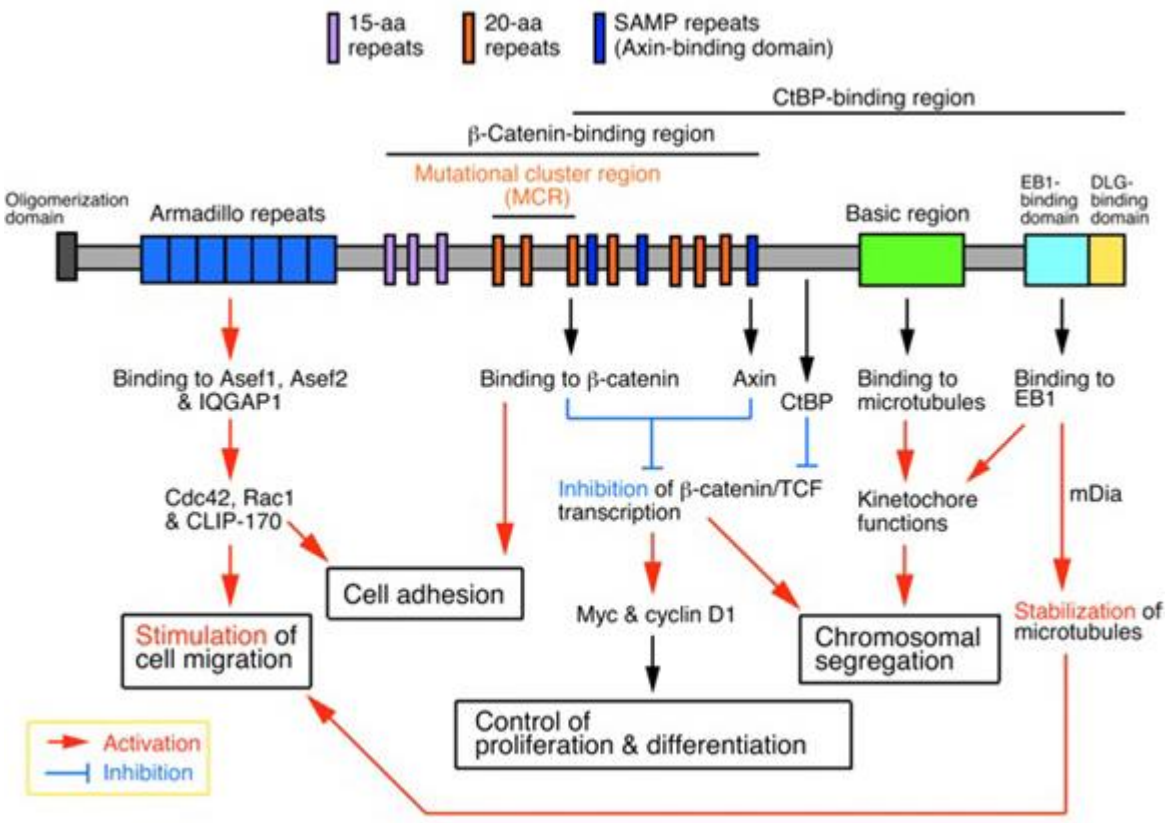

Figure 1.7 Multifunctional roles of the APC protein. APC's most influential role is its ability to mediate Wnt signaling though inhibition of β-catenin/TCF transcription. However, many other interactions are lost following disruption of APC which also contribute to tumourigenesis (Aoki & Taketo, 2007).

APC is a large protein and its role is multifunctional (Figure 1.7). Previous studies suggest its most influential role in tumourigenesis is loss of its ability to regulate Wnt signaling (Hanson et al., 2005; Nathke, 2005; Polakis, 2000). However, other regulatory abilities beyond Wnt control such as those in migration, polarity, adhesion, cell cycle progression, cell death and chromosome instability also contribute to tumorigenesis.

1.2.3 APC and its role in the canonical Wnt signaling pathway

The Wnt signaling pathway is a highly evolutionally conserved signaling pathway, activated by secreted Wnt proteins. It controls many biological events from early embryonic development, right through to adult tissues (for review see Clevers, 2006). The secreted

Wnt ligands are cell signaling glycoproteins which bind to the Frizzled and LRP family member receptors. This is a complex relationship with an abundance of both Wnt ligands, receptors and repressors (Polakis, 2000). However, once the pathway is activated, various factors can be altered at the cellular level such as proliferation, morphology, motility and fate (Polakis, 2000; Oving & Clevers, 2002). Although three separate pathways are believed to be activated upon Wnt ligand/ receptor binding; the Wnt/Ca^{2+}, the planar cell polarity and the canonical pathways (Clevers, 2006), APC's influence is mostly associated with the canonical (Reya & Clevers, 2005). The first connection between APC and the Wnt signaling pathway was made when APC was found to interact with β-catenin, an intracellular signal transducer (Rubinfeld et al., 1993; Su et al., 1993). Now, it is recognised as a vital component of the β-catenin destruction complex assisting in mediation of the Wnt signal (Figure 1.8).

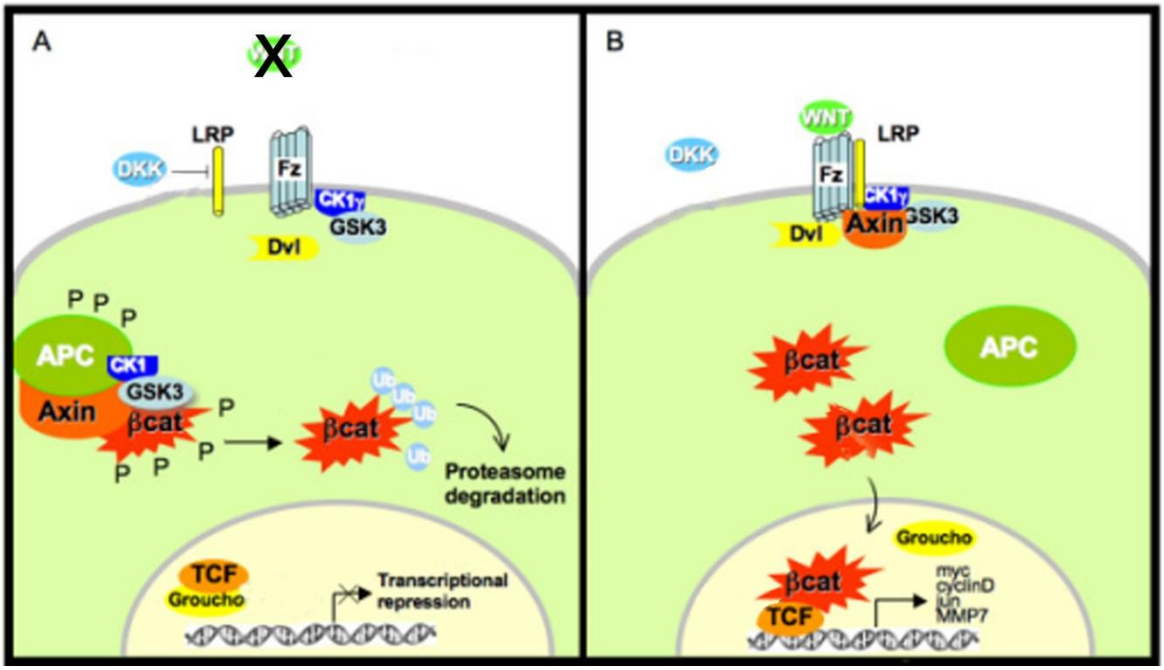

Figure 1.8 The canonical Wnt signaling pathway (this diagram depicts a simplified version). (A) In the absence of Wnt signals, APC forms a multi-protein destruction complex that phosphorylates β-catenin thus labelling it for destruction by the proteasome. In this case, β-catenin is unable to translocate to the nucleus and Wnt gene transcription is repressed. (B) In the presence of a Wnt signal, the destruction complex is disrupted. This prevents β-catenin from being targeted for degradation. β-catenin then translocates to the nucleus where it acts as a co-activator of transcription with the TCF/LEF family of transcription factors. Wnt target genes are subsequently expressed altering cell fate (adapted from Perez-Plasencia, 2008).

In response to a Wnt signal, activation of Wnt gene expression occurs through a multifaceted transduction cascade (Bienz & Clevers, 2000; Fodde, 2002; Polakis, 2000; Reya & Clevers, 2005; Clevers, 2006). Ultimately, β-catenin is freed from destruction and can translocate to the nucleus, upon binding to the N-terminus of LEF/TCF (lymphoid enhancer factor/T cell factor) it activates transcription of a number of Wnt target genes (Molenaar et al., 1996; van de Wetering et al., 1997; Polakis, 2000; Sansom et al., 2004). Wnt target genes are continuing to be identified and a full list can be found on the Wnt homepage (http://www.stanford.edu/~rnusse/wntwindow.html). Wnt activation may promote a range of biological events ranging from cell proliferation and tissue expansion to fate determination and terminal differentiation. Importantly, the cellular action taken upon Wnt activation is determined by distinct transcriptional outputs. This is mostly determined by the developmental identity of the responding cell as oppose to the nature of the signal (Clevers, 2006). However, evidence does suggest that varying levels of β-catenin activation can induce differential profiles of Wnt gene transcription (Kielman et al., 2002).

The Wnt pathway is one of the most commonly dysregulated signaling pathway in human cancers (Reya et al., 2001). Aberrations in this pathway, such as loss of APC, can cause inappropriate nuclear accumulation of β-catenin leading to transcription of a range of β-catenin/TCF gene targets. Some of these include renowned oncogenes: *cMyc, Cyclin D1, AP1, Wisp1* (He et al., 1998; Mann et al., 1999; Testu and McCormick, 1999; Shtutman et al., 1999; Xu et al., 2000). Together, inapt expression of β-catenin/TCF gene targets can cause various unprompted cellular changes such as induced cell division, leading cells down a cascade towards cancer.

1.2.4 The "just right" hypothesis

The APC protein contains three 15aa repeats and seven 20aa repeats (Figure 1.7), both of which are known to bind β-catenin (Rubinfeld et al., 1993; Su et al., 1993). In both hereditary and sporadic colorectal cancers, mutations of the *APC* gene result in truncation

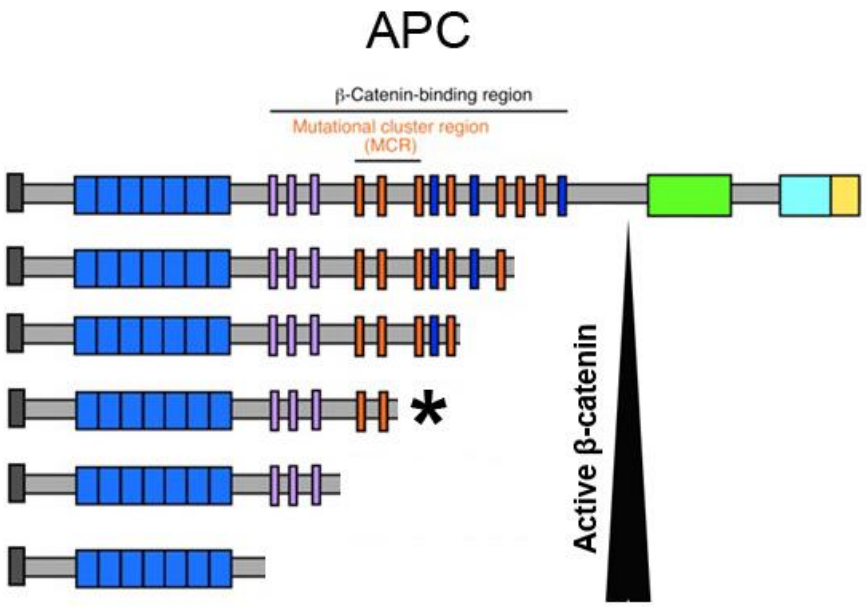

Figure 1.9 APC truncations and Wnt transduction. A range of *APC* mutations have been identified which result in varying degrees of protein truncation. Increased disruption of the β-catenin binding sites inversely correlates with the level of active β-catenin and thus Wnt target gene expression. In human colorectal cancer, mutations usually occur in a specific region (MCR) where some residual β-catenin regulation is retained (marked by asterisk) (adapted from Aoki & Taketo, 2007).

of the APC protein with varying degrees of disruption of these binding sites. Different severities of *APC* mutation can result in different levels of ability to mediate Wnt signaling (Figure 1.9) (Gaspar & Fodde, 2004). A large spectrum of *APC* mutations have been characterised in colorectal cancer and the majority occur within a region known as the mutational cluster region (MCR) (Miyoshi et al., 1992; Ichii et al., 1993). Mutations in this region do not completely abolish APC's ability to regulate β-catenin and analyses of human tumour samples reveal a certain degree of pathway regulation is usually retained (Le et al., 2008; Vermeulen et al., 2010; Sousa et al., 2011). Interestingly, in FAP patients, the position of the germline mutation in *APC* influences the nature of the second mutation in the normal residing allele (Lamlun et al., 1999) and this never results in complete loss of function of the APC protein (Albuquerque et al., 2002). Together, these findings propose a 'just right' level of Wnt activation required for tumour growth (Figure 1.10).

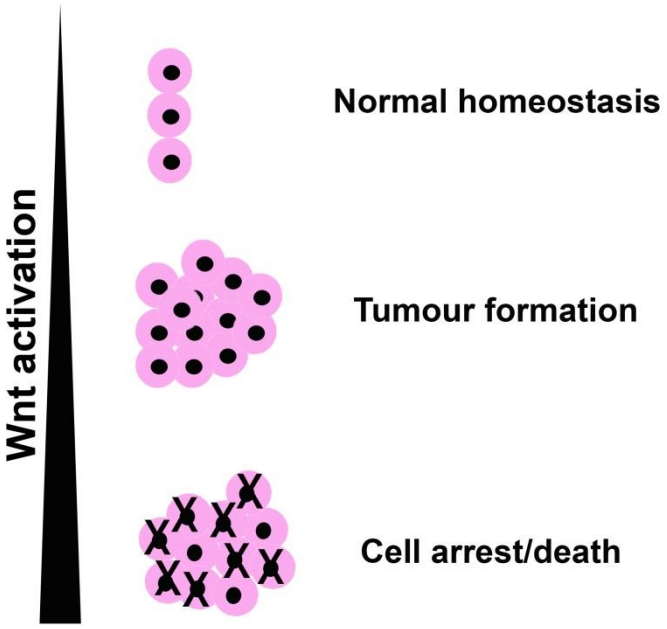

Figure 1.10 The 'just right' hypothesis. Mediocre levels of Wnt activation are optimal for tumour growth whilst levels too high are selected against.

The 'Just right' hypothesis has been demonstrated elegantly in colorectal cancer cell lines. Moderate levels of Wnt activation incur a growth advantage (Lamlum et al., 1999; Albuquerque et al., 2002; Lucero et al., 2010) whilst higher levels induce cell death (Lazarova et al., 2004; Bordonaro et al., 2008). Further, it was shown by Chandra et al. (2012) that down-regulation of truncated APC found in human colorectal cell lines increased β-catenin signaling but inhibited cell expansion '*in vitro*' and tumour outgrowth '*in vivo*' . Human '*In vivo*' data further supports this hypothesis. Mutations in the second half of the last *APC* exon result in typical FAP, whereas mutations earlier in the gene which allow less β-catenin interaction, manifest as attenuated FAP (AFAP) characterised by fewer adenomas as well as delayed adenoma formation (reviewed in Sancho et al., 2004). Together, these studies suggest a fine-tuned balance of Wnt activity is needed and optimal for cell transformation.

Although *APC* mutation and perturbations in the Wnt signaling pathway are well recognised in intestinal tumourigenesis, the importance of APC and 'Just right' levels of Wnt signaling are beginning to be exposed in tumourigenesis in a range of tissues. It is known that patients with FAP can develop a range of tumours outside of the intestinal tract (Gardener,

1951). However, the true consequence of reduced levels of APC in other tissues may be 'masked' by the predisposition to rapid intestinal tumourigenesis. Gaspar et al. (2009) created a mouse model with mild levels of Wnt activation through disrupted *Apc* and these mice were predisposed to mammary tumourigenesis as oppose to intestinal. This study highlights two important actualities: that increased Wnt activation through *Apc* loss can lead to tumourigenesis in other tissues, and that different tissues may have different thresholds levels needed for Wnt activated tumourigenesis. A further study by Buchert et al. (2010) reiterated these findings by analysing the phenotypes of a range of transgenic mice with truncating or hypomorphic mutations in the *Apc* gene. Each mutant displayed a different level of Wnt regulatory ability. Different permissive Wnt signaling thresholds appeared to be required for embryonic development of the head, Hepatocellular carcinoma, liver zonation and adult intestinal polyposis. Together these experiments demonstrate that Wnt signal regulation through Apc is essential in a range of tissues, and that these tissues require different levels of Wnt activation to induce pathophysiological/ tumorigenic phenotypes.

1.2.5 Non-canonical roles of APC

As mentioned previously, the Apc protein is a large protein with many interaction sites. Although its role in controlling Wnt signaling is well known, this may overshadow other interactions vital for homeostasis and tumour suppressor function. It is undeniable that APC's role in tumour suppression is multifunctional (reviewed by Aoki and Taketo, 2007; Senda et al., 2007; Hanson et al., 2005; Nathke, 2005; Polakis, 2000; McCartney & Nathke, 2008; Prosperi & Goss, 2011) and evidence towards the significance of Wnt independent roles is mounting. Interestingly, introduction of exogenous APC into colorectal cancer cells '*in vitro*' induces a G1/S-phase cycle arrest which can only partially be rescued by a non-degradable mutant form of β-catenin (Heinen et al., 2002). Also, some mutant *Apc* alleles which cause profound tumourigenesis do not display vigorous levels of β-catenin

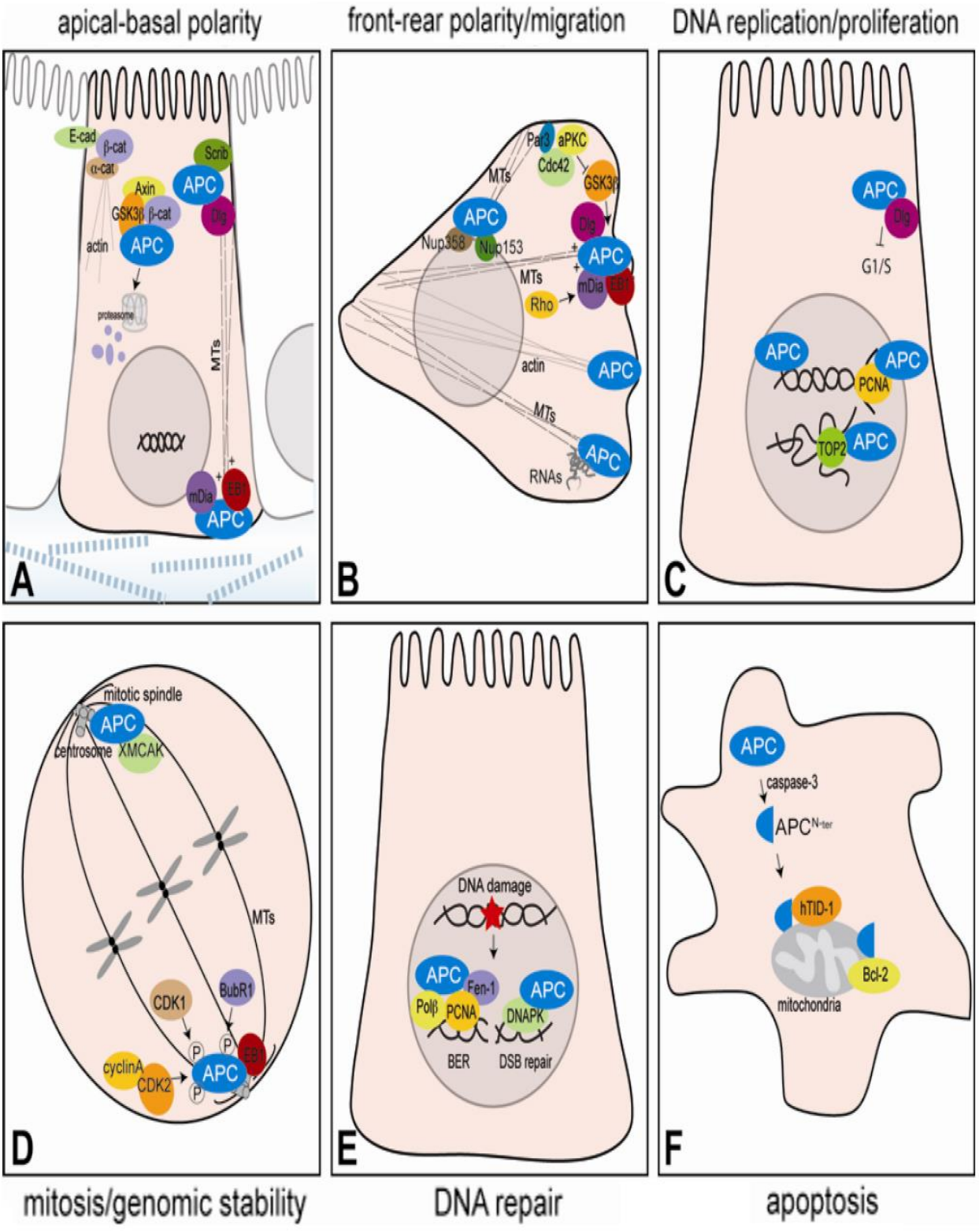

Figure 1.11 Non-canonical functions of APC. This image depicts some of the Wnt independent interactions of the APC Protein. APC has the Ability to interact with the cytoskeleton, junctional and polarity complexes, nuclear proteins and apoptotic factors. Loss of these interactions indubitably contributes to tumourigenesis (Prosperi & Goss, 2011).

stabilization (Pollard et al., 2009; Gaspar et al., 2009). Together, these experiments highlight that some effects of APC loss in tumourigenesis may not solely be explained through Wnt transcriptional control. Wnt independent functions of APC are continuing to be exposed and include interactions with the cytoskeleton, junctional and polarity complexes, nuclear proteins and apoptotic factors. Studies have shown that through these interactions, APC has influences in a variety of cellular functions which include: Polarity, adhesion, migration, genomic stability, cell cycle progression, apoptosis, DNA replication and DNA repair (Figure 1.11). APC's involvement with these functions and how loss contributes to tumourigenesis will each be discussed in turn.

APC's role in Polarity & adhesion

Most epithelium consists of a polarised layer of cells. APC has been shown to localise to the membrane of these cells in a variety of tissues (Reinacher-Schick & Gumbiner, 2001; Rosin-Ardesfeld et al., 2001; Townsley & Bienz, 2000; Prosperi et al., 2009). In a colorectal cancer cell line with mutated *APC* (SW480), introduction of exogenous full length APC induced tighter cell-cells contacts, altered morphology and increased adhesion concomitant with reduced tumorigenic potential (Faux et al., 2004). In mice with heterozygous *Apc* mutation (Apc^{min}), although the most characterised phenotype is an induction of intestinal tumourigenesis (Moser et al., 1993), loss of mammary epithelial integrity and occasional mammary tumorigenesis is manifest. This is purportedly through junctional protein and polarity disruption (Prosperi et al., 2009). These studies suggest a tumour suppressor function of APC in controlling adhesion and polarity.

APC's role in cell-cell contacts and adhesion is unclear but may lie with the fact it localises with both adherens junctions and tight junctions (Reichert et al., 2000; Bienz and Hamada, 2004). These junctions are crucial in establishing and maintaining polarity and adhesion (Ando-Akatsuka et al., 1999). APC may be a direct component of these junctions or may directly regulate them. The exact mechanisms linking junctional disruption and APC loss remain to be elucidated.

APC's role in polarity is somewhat clearer through its ability to interact with both components of polarity complexes and microtubules. Polarity complexes confer the identity of the apical and basolateral membrane domain in polarised epithelial cells (for review see Bryant & Mostev, 2008). APC has been shown to bind both Scribble (Scrib) (Takizawa et al., 2006) and Discs large (Dlg) (Matsumine et al., 1996). Together Scrib and Dlg form a polarity complex with Lethal giant larvae (Lgl). All are required for adherens junction formation, all are required for polarity and all are tumour suppressors (reviewed in Humburt et al., 2008). This fortifies the concept that loss of polarity is important for tumourigenesis. As APC directly binds these proteins, its loss may directly interfere with their polarity function and/or tumour suppressor function, however, the exact relationship remains to be defined.

APC has the ability to interact with microtubules (Nathke et al., 1996; Collin et al., 2008 Dikovskaya et al., 2001). This can occur through both a direct interaction (Munemitsu et al., 1994) and indirect through its binding partner end-binding protein 1 (EB1) (Nakamura et al., 2001). Microtubules are vital in inducing and maintaining polarity in a range of tissues (reviewed in Siegrist & Doe, 2007). Lack of APC correlates with disrupted microtubule stability (Dikovskaya et al., 2001; Kroboth et al., 2006) and studies have shown that APC is essential for the polarity function of microtubules (Dikovskaya et al., 2001; Etienne-Manneville & Hall, 2003; Watanabe et al., 2004).

Polarity and adhesion defects are found in almost all human cancers (Rothenburg et al., 2010; Huang & Muthuswamy, 2010; Schmalhofer et al., 2009). It is proposed that polarity and adhesion can directly enforce tumour suppression through maintaining tissue structurality (Lee & Vasioukhin, 2008). Loss of these features has been shown to contribute to tumourigenesis in both the early and advanced stages of progression (Figure 1.12) (reviewed in Wodarz & Nathke, 2007; Schmalhofer et al., 2009). As APC has the ability to influence these functions through a range of different mechanisms, reduced adhesion and polarity through APC loss highlight a Wnt independent contribution to tumourigenesis.

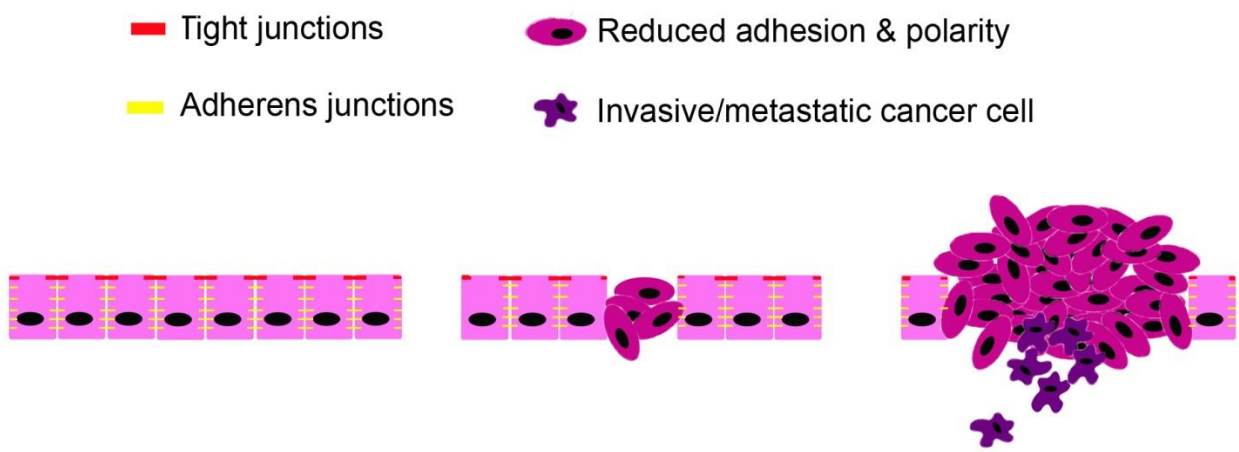

Figure 1.12 Adhesion & polarity in tumourigenesis. Reduced adhesion and polarity marked by loss of tight junctions and adhesion junctions can assist in early and advanced stages of tumour progression. Maintained adhesion & polarity can prevent the formation of tumours by maintaining structure. Loss of this function is needed to permit abnormal growth. In the advanced stages of cancer progression cells acquire the ability to completely detach and invade/metastasise. This is characterised by a complete loss of contact with neighbouring cells and severely altered polarity.

APC's role in migration

Cytoskeletal dynamics dictate cell shape and motility. Given APC possesses the ability to interact with both microtubules (Munemitsu et al., 1994; Nathke et al., 1996; Zumbrunn et al., 2001; Collin et al., 2006) and actin filaments (Dikovskaya et al., 2001; Watanabe et al., 2004; Breitsprecher et al., 2012), the major components of the cytoskeleton, it is unsurprising that its loss affects cellular migration.

Dysregulated levels of APC have been associated with the migratory ability of intestinal epithelial cells *'in vivo'*. Immediate intestinal *Apc* loss in a murine model abrogated epithelial cell migration (Sansom et al., 2004; Andreu et al., 2005). Reduced levels of full length *Apc* expression in the Apc^{min} mouse, along with pre-disposing mice to intestinal tumourigenesis (Moser et al., 1993), attenuated overall intestinal epithelial migration (Oshima et al., 1995; Mahmoud et al., 1997). Conversely, forced overexpression of *APC* in mice resulted in disorganised intestinal epithelial migration (Wong et al., 1996).

Interestingly, concurrent deletion of both *Apc* and *cMyc* from the murine intestinal epithelium rescued epithelial migratory defects (Sansom et al., 2007). As *cMyc* is a direct transcriptional target of β-catenin/TCF and activated upon *Apc* loss, this suggests migratory defects following disrupted Apc may be Wnt dependent. However, numerous studies have proposed that APC's role in migration is Wnt independent (reviewed in Barth et al., 2008; Etienne-Manneville, 2009; Prospery & Goss, 2011). Although cMyc's most characterised role is as part of the Wnt signaling pathway and a global regulator of transcription (Dang et al., 2006), it does have other roles (Cole & Cowling, 2008). For example, it has been shown to directly bind microtubules both '*in vivo'* and *'in vitro'* (Alexandrova et al., 1995; Niklinski et al., 2000) which are known to be a vital structural necessity for migration. Perhaps a novel, Wnt independent mechanism may account for this ability to rescue migration whilst being concomitantly deleted with *Apc*, which may be associated with its ability to interact with microtubules.

APC's translocation from cell-cell contacts to membrane clusters at cell protrusions promoted cell migration and this was found to be independent from its role in β-catenin transcriptional activity (Harris & Nelson, 2010). This, together with numerous other studies in various cell types have linked APC with migration, and suggest that control of this function is both imperative in tumour suppression and independent from Wnt signal mediation (reviewed in Barth et al., 2008; Etienne-Manneville, 2009).

Important insights into why migration is important in tumour suppression have come from studying the intestines. Intestinal epithelial cells are 'born' in a region called the crypt of lieberkuhn (crypt) which is located at the base of the intestinal gland structure. Most of these cells then migrate upwards along a structure called the villus and are 'shed off' into the intestinal lumen (see Chapter 3.1). Loss of migratory ability can aid tumour initiation and progression a number of ways. Foremost, independent of proliferation changes, cells being born that remaining '*in situ'* as oppose to following a designated migratory path will lead to an accumulation of cells (Figure 1.13). This accumulation of cells could be classified as the

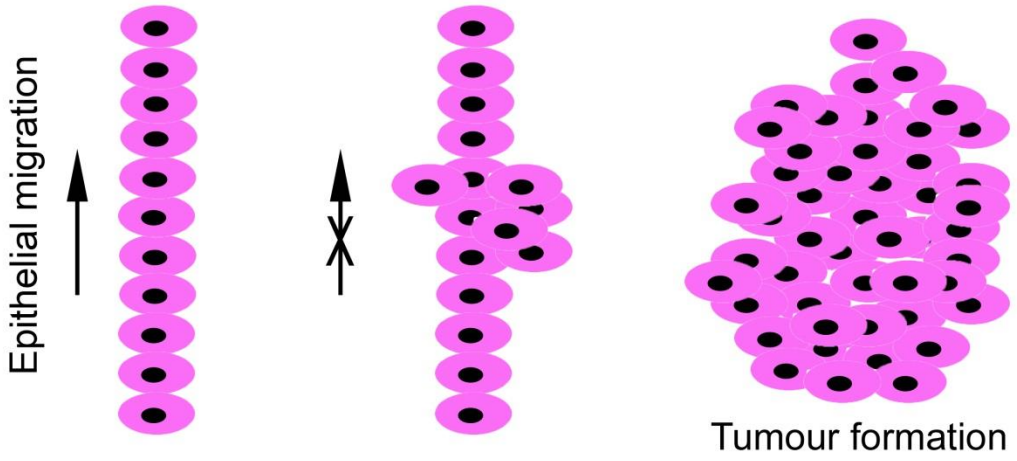

Figure 1.13 Loss of migratory ability can lead to an accumulation of cells that remain '*in situ*' and can represent the earliest stages of tumour formation.

earliest stage of tumour formation. Loss of migratory ability may aid tumourigenesis further by allowing accumulation of genetic alterations. Cells that do not migrate remain '*in situ*' and in contact with ingested material which can be carcinogenic. This prolonged time in contact with ingested material within the gut can lead to an accumulation of genetic changes which assist in tumour progression.

If the non-steroidal anti-inflammatory sulindac is placed in the diet of *Apc*^{min} mice, tumour number is reduced concurrent with restored epithelial migration (Mahmoud et al., 1997). This provides some evidence that cellular migration is associated with tumourigenesis. Also, it was suggested by Oshima et al. (1997) that micro-adenomas, the earliest stage of tumour formation in *Apc*^{min} mice are initiated by lack of directed cell migration. Further, human adenomatous polyps, which are abnormal growths found in the intestinal tract also display and could have been initiated by disorganised migration (Moss et al., 1996). These are a pre-cancerous lesion thus linking migration defects with the early stages of tumourigenesis in humans.

Conversely, in the advanced stages of disease progression, cancer cells must acquire the ability to 'migrate' from their site of origin and metastasise. As APC loss is associated with

migratory disruption, this creates a paradox. Perhaps, tumour cells still retain some migratory ability which is sufficient for their ability to metastasise. Equally, loss of APC may only be sufficient to disrupt migration in particular cell types or settings, once this is altered through tumour progression, migratory ability may be governed by APC independent means. A more plausible explanation may lay with the actualities of the disruption caused by APC loss. It is uncertain whether APC deficient cells lack the cellular capability to migrate or lose the ability to know where to migrate. Cells at any stage of tumourigenesis may still be 'moving' but may just lose their directionality. This may be associated with APC's role in polarity (see above).

To summarise, APC has been implicated in the control of cellular migration and this role is most likely Wnt independent, and disrupted migration through APC Loss may contribute to tumour initiation and progression.

APC's role in genomic stability

Loss of genomic stability is one of the 'hallmarks' of cancer (Hanahan & Weinberg, 2011). The lack of ability to control genomic integrity can allow the necessary genetic alterations needed for tumorigenesis. It is unknown whether genomic instability is a cause or consequence of tumour formation. However, it is known that it accelerates genetic change (Chandhok & Pellman, 2009; Hahn & Weinberg, 2002). Genomic instability can manifest at both the nucleotide and chromosome level. Genetic mutation, chromosome rearrangement and aneuploidy are all characteristics of genomic instability and all are frequently observed in human cancer (Negrini et al., 2010; Salk et al., 2010; Mitelman et al., 1997; Chandhok & Pellman, 2009; Weaver & Cleveland, 2006). How APC loss affects genomic stability at the nucleotide and chromosome level will be addressed.

Genomic stability can be controlled at the nucleotide level through effective DNA repair. DNA is universally being damaged through a range of insults and can lead to mutation. However, mechanisms of repair are constantly in place correcting this damage thus preventing genetic abnormalities which can lead to tumour initiation and progression (reviewed in Hoeijmakers, 2001). Although APC is mostly cytoplasmic and/or membrane

associated, it can also be found within the nucleus (Henderson, 2000; Neufeid & White, 1997) and can directly interact with chromatin (Sierra et al., 2006; Dikovskaya et al., 2012). APC has been implicated in DNA repair through several protein interactions (reviewed in Jaiswal & Narayan, 2008). Further, it was shown by Kouzmenko et al., (2008) that induction of DNA damage stimulated the accumulation of APC at sites of damaged DNA and its knockdown using siRNA reduced the rate of DNA repair. This provides a substantial Wnt independent link whereby APC loss can assist tumourigenesis through disrupting DNA repair.

Genomic instability can also be manifest at a more global level. Chromosome rearrangement and aneuploidy are indicative of genomic instability and are usually implemented through defective mitosis. For these defects to occur, the correct process of chromosome segregation is disrupted along with circumvention of the usual failsafe mechanisms which prevent manifestation of these abnormalities.

Aneuploidy and chromosome rearrangement have been observed in both human and mouse tissues with APC deficiency (Frodde et al., 2001; Su et al., 1995; Miki et al., 1992; Dikovskaya et al., 2007; Caldwell et al., 2007). As mentioned previously, APC has the ability to interact with microtubules both directly and indirectly through EB1 (Nakamura et al., 2001; Nathke et al., 1996; Collin et al., 2006; Munemitsu et al., 1994). Loss of this function may disrupt the microtubules ability to mechanically align and separate paired chromosomes during mitosis. Defects in these processes are commonly found in human cancers displaying genomic instability (reviewed in Pease et al., 2011).

During mitosis, APC is known to localise near the centrosomes and associate with the microtubule-organising centre to maintain chromosome structure (Olmeda et al., 2003) and its loss has been associated with reduced fidelity of chromosome segregation (Fodde et al., 2001; Kaplan et al., 2001). This may be through loss of its role either alone or with EB1 in regulating spindle dynamics and chromosome alignment (Green et al., 2005; Draviam et al., 2006; Green et al., 2003; Dikovskaya et al., 2007) or disruption of its ability to anchor the spindle to the cell cortex during mitosis (Beamish et al., 2009).

Various studies have shown loss of APC leads to the mis-orientation of mitotic spindles which affect proper chromosome segregation (Caldwell et al., 2007; Dikovskaya et al., 2007; Fleming et al., 2009). This may be influenced though loss of direct interaction with microtubules, through a loss of polarity (see above) or a combination of the two. Interestingly, defective regulation of spindle orientation due to APC loss has been reported in intestinal stem cells (Quyn et al., 2010). Spindle orientation can influence the generation of symmetric or asymmetric cell fates (reviewed in Siller & Doe, 2009). The importance of stem cells in cancer and how Wnt Signaling affects these cells is well documented (reviewed in Reya & Clevers, 2005). However, how defective spindle orientation directly contributes to stem cell aberrations in cancer remains to be fully elucidated.

Along with defects in the mechanics of proper chromosome segregation, for genetic abnormalities to persist, failsafe mechanisms which regulate this process must be circumvented. One of these mechanisms is the spindle checkpoint which in normal cells, delays mitotic progression until defects can be corrected (reviewed in Musacchio & Hardwick, 2002). Studies have shown loss or truncation of APC can lead to defects in this checkpoint (Radulescu et al., 2010; Tighe et al., 2004). Further, it was shown by Zhang et al. (2009) that truncated APC interacts with Mad2, an essential mitotic checkpoint protein. This interaction decreases the soluble pool of Mad2 inducing premature anaphase onset inducing mis-segregation of chromosomes.

To summarise, genomic instability is frequently found in human cancer. Loss of APC can lead to genomic instability at both the nucleotide and chromosome level through various mechanisms. This highlights a further Wnt independent mechanism whereby APC loss may influence tumourigenesis.

Other non-canonical roles for APC in tumour suppression

Numerous other roles for APC have been identified and can include a wide array of cellular functions, ranging from control of cell cycle progression and DNA replication to influences in cell death (reviewed in Prosperi & Goss, 2011). New roles of the APC protein are constantly being discovered and how these contribute to tumourigenesis independent of Wnt signalling will continue to be defined.

To summarise, *APC* is a potent tumour suppressor. Although it is a "gatekeeper" of intestinal tumourigenesis, its loss is associated with a wide range of human cancers. The most influential role of APC lies with its ability to mediate the Wnt signaling pathway, a pathway dysregulated in the majority of human cancers. The degree of *APC* mutation and thus the level of Wnt regulatory ability is important in creating a "just right" level of Wnt signaling optimum for tumour growth. Although Wnt regulation is the most characterised function of APC, its role is multi-functional and its loss can influence tumourigenesis in an increasing number of ways.

1.3 Adenomatous polyposis coli 2(APC2)

1.3.1 Discovery of APC2

Embryonic expression of *Apc-like* (*Apc*), which is the *Drosophila* homolog of the *APC* gene, is largely confined to the central nervous system (Hayashi et al., 1997). This observation, along with the fact *Apc* null mutants are viable with defects only observable in the larval photoreceptors (Ahmed et al., 1998), suggested the existence of another *Drosophila* Apc-like protein. In a yeast two-hybrid screen for proteins that bind to the *Drosophila* β-catenin homologue, Apc2 was identified (Yu, et al., 1999). Further, McCartney et al. (1999) confirmed the existence of this second homologue and demonstrated that its gene sequence displayed striking similarities to both *Drosophila Apc* and human *APC.*

APC2 has now been identified in a range of other species including: Zebrafish (Faro et al., 2010), chick (Shintani et al., 2009), mouse (Yamanaka et al., 2002; Shintani et al., 2012) and human (Nakagawa et al., 1998; Van Es et al., 1999; Jarrett et al., 2001). An evolutionary tree of fly, mouse and human APC and APC2 proteins was constructed by Aoki & Takoto (2007) which demonstrates evolutionarily conservation (Figure 1.14). The conservation and protein similarities suggest both APC proteins may have important and redundant roles.

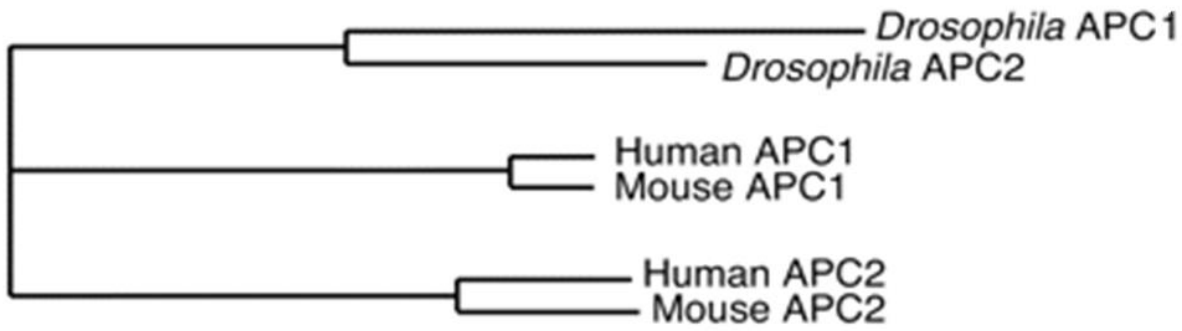

Figure 1.14 Both APC proteins are evolutionarily conserved. However, it is important to note that the two fly *APC* genes are not direct orthologues of the two mammalian genes (Aoki and Taketo, 2007).

In humans, *APC2* is located on chromosome 19p13.3 and encodes a 2274aa protein contained in 14 exons (slightly shorter than the 2843aa coded in 15 exons of *APC*) (Nakagawa et al., 1998; Van Es et al., 1999; Jarrett et al., 2001). It was shown by Van Es et al. (1999) that the highest detected expression of *APC2* mRNA was found in Foetal brain, followed by adult brain. *APC* also displayed this trend although expression levels were lower. Importantly, both *APC*'s were shown to be expressed in the majority of tissues analysed (Figure 1.5). It was also shown by Jarrett et al. (2001) that APC2 was expressed in a wide range of human tissues and cell lines.

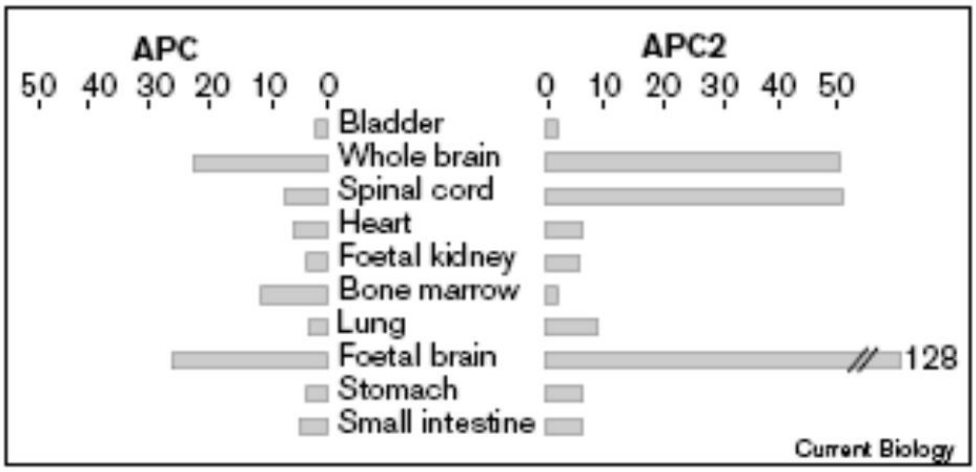

Figure 1.15 Both APC proteins are expressed in a range of tissues. mRNA for both *APC* and *APC2* was detected in a wide range of tissues. High levels of expression for both genes were observed throughout the central nervous system (Van Es et al., 1999).

APC2 displays similarities in protein structure to APC (Figure 1.16). The most similarity is apparent in the N-terminus, the homology then drops steeply towards the C-terminus end. Both proteins are predicted to contain the heptad repeat domains, suggesting both are likely to form homo- or heterodimers. The armadillo domain is also conserved, suggesting both may be involved in cellular adhesion and/or migration. Importantly, although APC2 lacked the three 15aa repeats that can bind β-catenin, both APC proteins contained SAMP domains and 20aa repeats that bind axin and β-catenin respectively. Towards the C-

terminus where the similarity dropped, APC2 is predicted to lack domains that interact with microtubules, mDia, EB1 and Dlg (Nakagawa et al., 1998; Van Es et al., 1999; McCartney et al., 1999). This suggests that APC2 may lack certain functions in comparison to APC relating to microtubules, cytoskeletal organisation, polarity, etc. However, APC2's interactions and binding partners may yet remain to be fully discovered. Indeed, in *Drosophila*, APC2 has been reported to be cytoskeletally-associated (McCartney et al., 1999) and important for spindle orientation (Yamashita et al., 2003). In vertebrates, APC2 has been shown to directly interact with microtubules and F-actin (Shintani et al., 2009; Shintani et al., 2012). It has also been associated with regulation of microtubules through interaction with EB3, a homolog of EB1 (Nakagawa et al., 2000) and DDA3, a downstream target of p53 (Hsieh et al., 2007). Together these studies suggest that APC2 is also a multifunctional protein although these functions remain to fully defined.

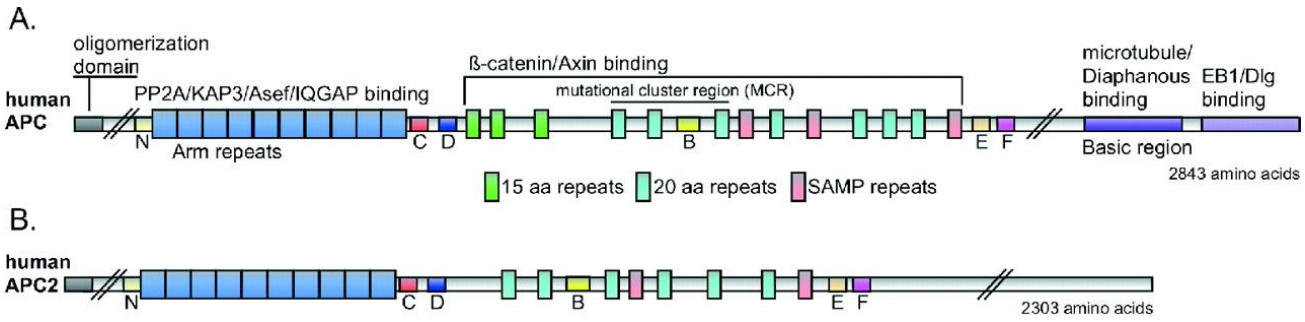

Figure 1.16 APC2 is similar in structure to APC so may have comparable functions. Although APC2 lacks the three 15aa repeats that can bind β-catenin, it contains both SAMP domain and 20aa repeats which bind axin and β-catenin respectively. Homology decreases towards the C-terminus end (McCartney et al., 2006).

1.3.2 APC2's role in the canonical Wnt signaling pathway

Due to the ability of both APC proteins to bind β-catenin and axin, APC2 was also predicted to have a role in negative regulation of the Wnt signaling pathway. Indeed, this was found to be the case (McCartney et al., 1999). In *Drosophila*, Apc2 forms a destruction complex with

homologs of the mammalian GSK-3 and axin (Yu et al., 1999). Apc and Apc2 are partially redundant in Wnt signaling and both may regulate signal transduction throughout all *Drosophila* development, in the majority of tissues (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b). There appears to be an absolute requirement of an Apc protein in order for β -catenin degradation and shuttling out of the nucleus to occur, and the combined activity of Apc and Apc2 within the same cell may allow for tight regulation of these processes (Ahmed et al., 2002). Functional redundancies have also been exposed through complementary Wnt gene transcription rescue, further highlighting the interplay between these proteins in Wnt signaling (Kunttas-Tatli et al., 2011; Roberts et al., 2012).

APC2 has also been shown to deplete cytoplasmic β-catenin via direct interaction in human tissue and cell lines (Nakagawa et al., 1998; Van Es et al., 1999; Roberts et al., 2012). However, its ability to rescue Wnt target gene expression in a Wnt activated colorectal cell line is somewhat reduced in comparison to APC (Nakagawa et al., 1998). This suggests APC2 may have a reduced ability to degrade β-catenin. This may be through a reduced binding affinity, APC2 does lack the 15aa repeats that also bind β-catenin found in APC (Nakagawa et al., 1998; Van Es et al., 1999; McCartney et al., 1999) or through reduced β-catenin availability. Indeed, β-catenin phosphorylation is performed by APC2 in a destruction complex spatially and functionally isolated from the APC associated destruction complex (Maher et al., 2009).

In human colorectal cell lines with reduced APC, transfection with APC2 can rescue β-catenin destruction and reduce Wnt-responsive gene transcription (Nakagawa et al., 1998; Van Es et al., 1999; Roberts et al., 2012). This, together with complimentary rescue in *Drosophila* suggests that functional redundancies in regulating Wnt transduction may be evolutionarily conserved.

1.3.3 Non-canonical roles of APC2

Drosophila

Like APC, APC2 has also been implicated in functions beyond regulation of Wnt signaling. In *Drosophila*, Apc2 has been reported to localise to the apical membrane of polarised epithelial cells (McCartney et al., 1999). Yu et al. (1999) found Apc2 to be concentrated in the apicolateral adhesive zones of these cells, along with the *Drosophila* homologs of β-catenin and E-Cadherin, both of which are integral components of adherens junctions. Loss of function of Apc2 leads to dissociation of these junctions and defects in adhesion (McCartney et al., 1999; Hamada & Bienz, 2002). The mechanisms behind these defects remain to be fully defined.

Studies in *Drosophila* also revealed a role for Apc2 in cell division (McCartney et al., 2001). Apc2 is shown to localise to both spindle poles and the cell cortex where astral microtubules are thought to contact cortical actin. This suggests a role in anchoring the spindle to the cell cortex. Mutants of Apc2 display defects in syncytial development consistent with anchoring defects (McCartney et al., 2001). A further role for Apc2 is apparent in ensuring asymmetric division of *Drosophila* male germline stem cells (Yamashita et al., 2003). Apc2 helps establish polarity to correctly orientate the mitotic spindle. As mentioned previously, spindle orientation can govern between symmetric and asymmetric cell division (reviewed in Siller & Doe, 2009). Loss of this ability through Apc2 loss may be direct or indirect through dissociation of adherens junctions. Adherens junctions are known to provide polarity clues for spindle orientation (Song et al., 2002).

Together these results indicate Apc2 is involved in adhesion, polarity and cell division in *Drosophila* tissue. However, more studies are needed to define these roles and to elucidate if these roles are evolutionarily conserved.

Vertebrates

To date, only two studies have been published analysing loss of function of Apc2 in vertebrate tissue, both studies focused on loss within neural tissue. It is known Apc2 is expressed at its highest levels in the central nervous system (Nakagawa et al., 1998; Yamanaka et al., 2002), with peak levels detected during foetal brain development (Van Es et al., 1999). Shintani et al. (2009) demonstrated that Apc2 is important in the formation of neural projections and that Apc2 associates with microtubules and stabilises them in growing axons. Upon suppression of Apc2 expression, axonal behaviour became abnormal in a dose dependent manner. This implies Apc2 level plays a critical function in the development of neural circuits. Further, Shintani et al. (2012) created a constitutive mouse model disrupting Apc2. Again, these mice displayed neural defects. Apc2 co-localised to both microtubules and F-actin and loss of function resulted in laminary and migratory defects.

Importantly, β-catenin remained unaltered in either study thus implicating a Wnt independent role of Apc2 in neuronal development. However, it is unclear whether Wnt signaling differences are subtle or Apc compensates for Apc2's loss. Defects in both studies were attributed to microtubule and F-actin regulatory defects. Interestingly, Apc2 lacks both a basic domain and an EB1 binding domain. So, it is predicted that Apc2 cannot bind to microtubules. However, Shintani et al. (2012) have identified a direct microtubule binding site within the unique C-terminus of Apc2. Also, APC2 has been shown to interact with EB3 and DDA3 which are microtubule binding proteins highly expressed in the brain (Nakagawa et al., 2000; Hsieh et al., 2007).

Together, studies from *Drosophila,* chick, mice and human suggest that like APC, APC2 has both Wnt dependent and independent functions. Due to the similarities in functions between APC proteins, it is predicted that APC2 may also have a role in tumourigenesis. If this is the case and how APC2 loss contributes to tumourigenesis remains to be elucidated.

APC2 in cancer

APC2 is located at chromosome 19p13.3 a region lost with Peutz-Jeghers syndrome (JPS) and patients with PJS are more susceptible to breast, testis, gastrointestinal, and ovarian cancers (Mehenni et al., 1998; Bignell et al*.,* 1998). Although loss of the *LKB1* gene is responsible for most of the symptoms of PJS, this gene is not mutated in sporadic cancers thus suggesting the existence of another tumour suppressor within this region of chromosome. Loss of 19p13.3 occurs in many sporadic cancers, including those of the breast, and is remarkably common in sporadic ovarian carcinomas (Wang et al., 1999; Jarrett et al., 2001). Also, 100% loss of heterozygosity (LOH) is found at this site in adenoma malignum of the uterine cervix (Lee et al., 1998). APC2 expression was found to be significantly reduced in most gliomas however single-strand conformation polymorphism (SSCP) analysis revealed no mutations suggesting that some epigenetic mechanism is responsible for this decrease in expression (Nakagawa et al., 1999).

Although known mutations in *APC2* are rare, epigenetic silencing and reduction in expression is now recognised in a variety of cancers through hypermethylation (reviewed in Herman & Baylin, 2003; Jones & Baylin, 2007). Epigenetic abnormalities can cooperate with genetic alterations to effect aberrant gene function that results in cancer (Jones & Baylin, 2002). It is known that levels of promoter methylation correlate with levels of protein expression (Suzuki & Bird, 2008) and this is indeed the case with *APC2* (Figure 1.17). It was demonstrated by Chan et al. (2008) that increased methylation status of *APC2* led to a decrease in protein expression and that hypermethylation occurred in 100% of primary colon tumours and 77% of primary breast tumours. In this study hypermethylation also was noticed in 9 out of 11 breast cancer cell lines analysed. Studies by Mokarram et al. (2009) and Kumar et al. (2009) have confirmed hypermethylation of *APC2* in 90-98% of colorectal tumour samples compared to control tissue in a variety of human populations. Hypermethylation of *APC2* was also associated with progression from inflammatory bowel disease (IBD) to IBD associated neoplasia (Dhir et al., 2008). Interestingly, APC also

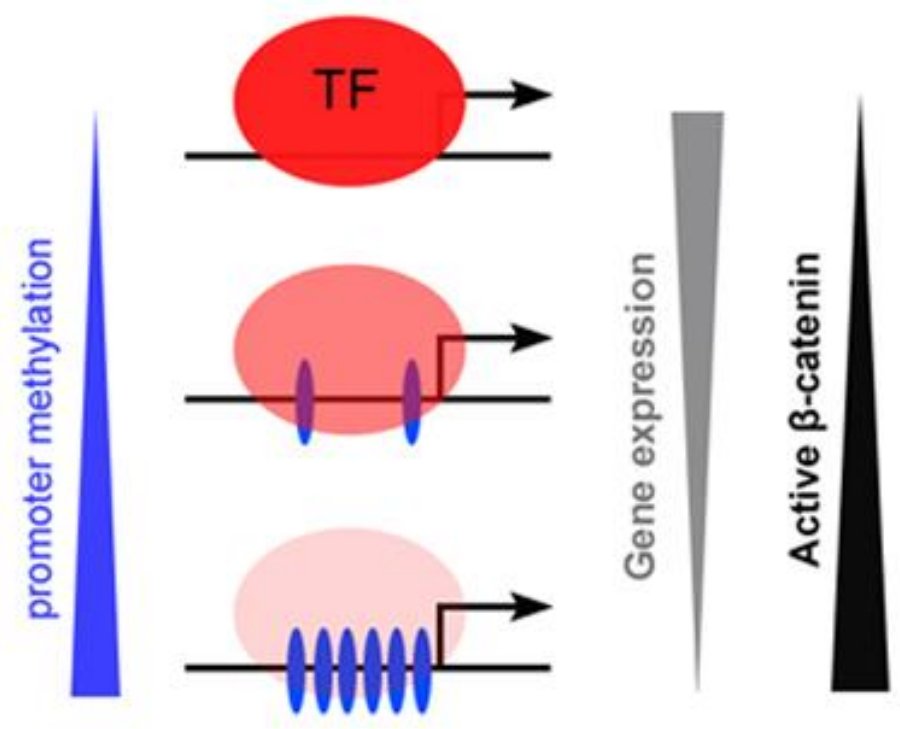

Figure 1.17 *APC2* gene silencing through promoter hypermethylation. Increased methylation status of the CpG islands in the promoter region of *APC2* decreases the affinity for transcription factors (TF) and decreases gene expression. Decreased expression of *APC2* is predicted to increase the amount of active β-catenin and Wnt target gene expression. *APC* has also been shown to display promoter hypermethylation in human tumours.

displays hypermethylation in a wide range of human cancers (Deng et al., 2004; Hsieh et al., 2007; Zare et al., 2009; Jin et al., 2001; Ho et al., 1999; Schlosshauer et al., 2000; Sarrio et al., 2003; Van der Auwera et al., 2008). Given *APC* mutation was the only factor searched for in a range of historic studies, it would be of great interest to re-analyse tumour data to inspect for both mutation and hypermethylation. One could speculate APC loss achieved through a combination of these events may be higher than previously thought in a range of human cancers.

From these studies it is clear that loss of APC2 is associated with human cancer. However it is unknown if APC2 loss has the ability to initiate tumour formation, is associated with tumour progression or is just a passive event. Intriguingly, no studies to date have concurrently analysed both APC proteins in human tumourigenesis.

1.4 Aims

APC's role in human cancer is undeniable. It acts as a "gatekeeper" of intestinal tumourigenesis and is also associated with a wide range of other tumours. APC is multifunctional and its loss induces and promotes tumourigenesis a number of ways. The most influential contribution is loss of its ability to mediate the Wnt signaling pathway, a pathway dysregulated in almost all human cancer. Loss of APC's other functions also contribute to tumourigenesis. Although a vast amount of effort has been placed into studying APC, it has a second family member which possesses similar structural characteristics, functions and is also lost in a range of human cancer. Work in *Drosophila* suggests function redundancies exist between these two homologs and studies in human cancer cell lines demonstrate APC2 can rescue some attributes of APC loss. A lack of published studies highlights a gap in the understanding of the roles of APC2 in mammalian development, homeostasis and/or contribution to tumourigenesis.

We have acquired a mouse model with loss of Apc2 function. The main aims of this thesis are:

- To characterise the consequences of mammalian Apc2 loss. Analysis will focus on a range of tissues including: the intestines, mammary gland and liver.

- To elucidate if Apc2 loss has the potential to induce tumourigenesis in these tissues.

- To analyse Apc2 loss in the context of targeted Apc loss. This will help reveal if any functional redundancies exist within mammalian tissue.

- To delineate any additional contribution of Apc2 to tumourigenesis following altered Apc expression.

Chapter 2

Materials and Methods

*Some protocols in this section were either directly transcribed or adapted from Holisk (2012) or generic protocols within the ARC laboratory.

2.1 Experimental animals

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986 and all other Home Office Guidelines, under valid personal and project licenses.

2.1.1 Transgenic constructs and animals used in the project

A number of genetically modified mice were used in this project. An *Apc2* mutant mouse was kindly gifted from Prof. Clevers laboratory (Van der Meer et al., 2001). This was generated containing a constitutive truncating mutation (termed *Apc2*$^{-/-}$). For targeted *Apc* loss, mice were used containing an *Apc* transgene containing loxP sites positioned in the introns surrounding exon 14 (Shibata et al., 1997). Tissue-specific transgenes included; The *Villin-Cre*^{ER} transgene, which expresses Cre recombinase specifically in the intestinal epithelium following a Tamoxifen induction regime (Colnot et al., 2004), the *Ah-Cre* transgene, which expresses Cre recombinase in the intestinal epithelium and liver cells among other tissues following a *β*-naphthoflavone induction regime (Ireland et al., 2004) and the *Blg-Cre*$^{+}$ transgene, which expressed Cre recombinase specifically in the mammary epithelium, constitutively (Selbert et al., 1998). A Cre *ROSA26* reporter strain was also used to detect recombination events (Soriano et al., 1999)

2.1.2 Animal husbandry

Colony maintenance

Mice were housed in a standard facility that meets all home office standards and had access to the Harlan standard diet (Scientific Diet Services, RM3(E)) and water *ad libitum*.

Breeding

All mice were maintained on a mixed background. Adult animals (6 weeks and older) of known genotype were mainly bred in trios (one male with two females). Pups were left with their mothers until capable of feeding independently. They were weaned on average at four weeks of age.

Identification, ear and tail biopsies

At the time of weaning animals were sexed and separated. Ear clipping was employed for animal identification. The resulting ear pinna was used for DNA extraction and genotyping (described below).

2.1.3 Experimental Procedures

Unless otherwise specified, animals were aged to at least 10 weeks before being subjected to any experimental procedures. Intraperitoneal injections were performed using a 1 ml syringe (BD Plastipak) and 25G needle (BD Microlance 3).

***β*-naphthoflavone induction**

Expression of the *Ah-Cre* recombinase was induced by administration of *β*-naphthoflavone (bNF, Sigma) in corn oil (Sigma). Powdered bNF to a concentration of 10 mg/ml was added to corn oil in an amber bottle in order to protect bNF from light exposure. The solution was heated to 80 ◦C and stirred vigorously to aid complete dissolution of bNF. Aliquots of bNF solution were frozen and stored at −20 ◦C until required. Prior to injection, aliquots were thawed, heated to 80 ◦C and kept at that temperature until immediately before injection to prevent bNF precipitation out of the solution. bNF was injected intraperitoneally at a dose of 80 mg of bNF per kg of body weight. This dose was repeated thrice with 8 hour interval

between injections (4 injections in total). During a single course of injections solution was kept in darkness at 4 ◦C and then any remaining solution was discarded.

Tamoxifen induction

Animals expressing the *Villin-Cre*^{ER} transgene were induced by administration of Tamoxifen (Sigma) in corn oil. Powdered Tamoxifen was added to corn oil in an amber bottle to final concentration of 10 mg/ml. The solution was heated to 80 ◦C with regular shaking to aid Tamoxifen dissolution. Once completely dissolved, the Tamoxifen solution was aliquoted and stored at −20 ◦C. Prior to injection the solution was thawed, heated to 80 ◦C and kept at this temperature until immediately before injection. Tamoxifen was injected intraperitoneally at a dose of 80 mg of Tamoxifen per kg of body weight. This dose was repeated twice with 4 hour interval between injections (3 injections in total). During a single course of injections solution was kept in darkness at 4 ◦C and then any remaining solution was discarded.

2.1.4 PCR Genotyping

Mice were genotyped by PCR using DNA extracted from ear or tail biopsies at weaning age (four weeks) and confirmed at death. Unless provided by earlier publications, all primers used were designed using Primer3 software at http://fokker.wi.mit.edu/primer3/input.htm, checked for specificity using BLAST engine against Ensembl database (http://www.ensembl.-org/Multi/blastview) and synthesised by Sigma Genosys.

DNA purification (Puregene method)

Ear biopsy was placed in a 1.5 ml eppendorf tube and stored at −20 ◦C until analysis. For DNA isolation 250 μl of Cell Lysis Solution (Gentra) and 5 μl of 20 mg/ml Proteinase K (Roche) were added to the tissue and incubated overnight at 37 ◦C with agitation. The next day the contents of the tubes were cooled to room temperature, mixed with 100 μl of Protein Precipitation Solution (Gentra) and centrifuged at 13000 rpm in a microcentrifuge for 10 min. The supernatant was recovered and mixed with 250

μl of isopropanol in a fresh 1.5 ml eppendorf tube and centrifuged at 13000 rpm for 15 min. The supernatant was carefully discarded, and the tubes were left to air-dry for 1 hour. The DNA was then dissolved in 250 μl of PCR-grade water (Sigma) and 2.5 μl of the resulting solution was used in PCR reactions.

Generic protocol for PCR genotyping

PCR reactions were carried out in thin-wall 96-well plates or in thin-wall 0.2 ml strip tubes (Greiner Bio-One) and run using either PTC-100 Peltier (MJ Research), Techne Flexigene (Krackeler Scientific) or GS1 (G-Storm) thermal cycler. Pipetting of the reagents and DNA samples was carried out using filtered pipette tips to avoid aerosol contamination. 2.5 μl of crude genomic DNA extract was loaded into wells using multi-channel pipette. The master-mix containing the remaining components of the reaction (distilled water, GOTaq 5X PCR buffer (Promega), 25 mM Magnesium Chloride (Promega), 25 mM dNTPs (Bioline), Primers (Sigma-Genosys) and GOTaq (Promega)) was prepared according to Table 2.1. 47.5 μl of the appropriate master-mix were added to each well. 96-well plates were sealed with aluminiumfoil tape, while strip-tubes were closed with appropriate caps (Greiner Bio-One). The tubes were gently tapped to dislodge air bubbles and ensure the mixture was at the bottom of the well. Reactions were run using cycle conditions outlined in Table 2.1. The primer sequences used for genotyping of the particular transgenes and the size of respective products are provided in Table 2.2.

Visualisation of PCR products

After completion of PCR reactions the products were visualised by agarose gel electrophoresis. The reactions using colourless 5X PCR buffer (Promega) were mixed with 5μl of DNA loading dye (50% Glycerol (Sigma), 50% distilled water, 0.1% (w/v) Bromophenol Blue (Sigma)). All samples and an appropriate marker (e.g. 100 bp ladder (Promega)) were loaded onto 2% agarose gel (4 g agarose (Eurogentech), 200 ml 1X Tris-Borate-EDTA (TBE) buffer (Sigma), 10 μl of 10 mg/ml ethidium bromide (Sigma) or 10μl Safeview (NBS Biologicals)). The products of Ah-Responder PCR were analysed using 4% agarose gel. Gels were run in 1X TBE buffer at 120 V for 30 minutes. Products were visualised in UV light using GelDoc apparatus (BioRad).

Table 2.1: Genotyping PCR reaction conditions

PCR Reaction Components:	
DNA extract (See section 2.1.4)	2.5 μl
Master Mix:	
PCR-grade H_2o (Sigma)	31.7 μl
GOTaq PCR Buffer (5X, Promega)	10 μl
Magnesium Cloride (25 mM, Promega)	5 μl
dNTPs (25 mM, Bioline)	0.4 μl
Forward Primer (100 mM, Sigma Genosys)	0.1 μl
Reverse Primer (100 mM, Sigma Genosys)	0.1 μl
Taq Polymerase(GOTaq)	0.2 μl
Total Reaction Volume:	50 μl

Table 2.1(cont.)

Cycling conditions (Time; Temperature)	
Initial denaturation	2.5 min; 94 ◦C
Cycle number	30
Step 1 (Denaturation)	30 sec; 95 ◦C
Step 2 (Annealing)	30 sec; 55 ◦C
Step 3 (Elongation)	1 min; 72 ◦C
Final Extension	5 min; 72 ◦C
Hold	15◦C ∞

Table 2.2: Primer sequences used for PCR genotyping

ApcLoxP

Gene Forward Primer Sequence (5-3)	GTTCTGTATCATGGAAAGATAGGTGGTC
Reverse Primer Sequence (5-3)	CACTCAAAACGCTTTTGAGGGTTGATTC
Product Size	WT: 226bp ApcLoxP: 314

Apc2$^-$

Gene Forward Primer Sequence (5-3)	ATTGTATCATGGAAAGATAGGTCGTC
Reverse Primer Sequence (5-3)	CTCACTCAAAACGCTTTTGAGGGTTGA
Product Size	WT: 450bp *Apc2$^-$:* 400bp

LacZ

Gene Forward Primer Sequence (5-3)	CTGGCGTTACCCAACTTAAT
Reverse Primer Sequence (5-3)	ATAACTGCCGTCACTCCAAC
Product Size	WT: 0bp *LacZ*: 500

Villin-Cre^{ER}

Gene Forward Primer Sequence (5-3)	CAA GCC TGG CTC GAC GGC C
Reverse Primer Sequence (5-3)	CGC GAA CAT CTT CAG GTT CT
Product Size	WT: 0bp *Villin-Cre^{ER+}*: 1000bp

Ah-Cre

Gene Forward Primer Sequence (5-3)	ATT GCC CCT GTT TCA CTA TC
Reverse Primer Sequence (5-3)	CCT GAC TAG CAT GGC GAT AC
Product Size	WT: 0bp *Ah-Cre*$^{+}$: 1000bp

Blg-Cre

Gene Forward Primer Sequence (5-3)	TGACCGTACACCAAAATTTG
Reverse Primer Sequence (5-3)	ATTGCCCCTGTTTCACTATC
Product Size	WT: 0bp *Blg-Cre*$^{+}$: 1000bp

2.2 Tissue harvesting and processing

2.2.1 Tissue harvesting

Mammary Glands

Following a schedule 1 approved method of culling (cervical dislocation), animals were dissected using a micro-dissection kit. The fur was sprayed with 70% ethanol and an incision made along the mid-line of the abdomen, through just the skin leaving the peritoneal wall intact. Incisions through the skin were then made transversely along the neck and groin regions. The skin was 'peeled' back from the peritoneal wall and mammary glands dissected, placed into small cassettes and 'stretched out' for fixing.

Other tissues

An incision was made along the mid-line of the abdomen, through both, skin and peritoneal wall. Liver, kidney, spleen, pancreas, lung, stomach, appendix and any abnormal tissue was collected and fixed. The small intestine between stomach and caecum and large intestine between the appendix and anus was isolated and flushed with ice cold 1X PBS (Invitrogen) using a 60 ml syringe (BD Plastipak). Intestines were then opened longitudinally on blotting paper and either fixed overnight in methacarn (methanol, chloroform and acetic acid; 4:2:1) for tumour scoring or processed as follows. The first 10cm were opened up and a 2-4mm longitudinal section dissected for RNA (see section 2.2.4). The reminding tissue from the first 10cm's is then 'swiss-rolled'. The following 8 cm were cut into four 2 cm pieces and bundled together with surgical microtape. The large intestine between the caecum and the rectum was also 'swiss-rolled' if not fixed for tumour scoring.

2.2.2 Tissue fixation

Once collected, tissue samples were immersed in ice cold formalin (4% neutral buffered formaldehyde in saline, Sigma) and incubated for 24 hours at 4 ◦C. Mammary glands were fixed for 2 hours in 4% paraformaldehyde. After initial fixation, samples were either processed straight away as described below or transferred into ice cold 70% ethanol and stored at 4 ◦C until processing. Fixation method used for whole mount and *β*-galactosidase staining is described in section 2.2.5.

2.2.3 Tissue processing for lightmicroscopy

Tissue dehydration

Tissue samples were arranged in histocasettes and processed using an automatic processor (Leica TP1050). Samples were dehydrated in increasing concentrations of ethanol (70% for 1 hour, 95% for 1 hour, 100% 2 x 1 hour 30 min, 100% for 2 hours), Xylene 2 x 1 hour and paraffin 1 x 1 hour and 2 x 2 hours. After dehydration, samples were embedded in paraffin wax.

Tissue sectioning

A microtome (Leica RM2135) was used to cut 5 μm thick sections from paraffin blocks. Sections were then floated onto slides coated with poly-L-lysine (PLL) and baked at 58 ◦C for 24 hours.

2.2.4 Tissue preservation for RNA

Tissues intended for RNA extraction were removed and frozen as quickly as possible to avoid RNA and protein degradation, respectively. Liver samples were frozen using liquid nitrogen or dry ice and stored at -80. Intestinal tissue intended for RNA extraction was processed using Weiser solution (section 2.2.6).

2.2.5 Staining of tissue whole mounts

Carmine alum staining of mammary whole mount tissue

Whole mammary glands were washed 3 times with 1XPBS following fixation, then left in carmine alum solution (1g carmine, 2.5g aluminum in 500ml distilled water) on rocker overnight. Glands were then washed again 3 times with 1XPBS, dehydrated in increasing concentrations of ethanol and placed in xylene for 2 hours to clear fat. Glands were then mounted on slide using glycerol. Stained wholemounts were illuminated with Leica CLS50X light source and visually analysed under Olympus SZX12 low-magnification stereo microscope. Where needed, pictures were taken with Olympus C4040ZOOM 4.1 Megapixel digital camera.

LacZ staining of whole mount tissue

Mammary or liver whole mounts were fixed then stained using X-Gal staining solution: 1 mM $MgCl_2$ (Sigma), 3 mM potassium ferricyanide (Sigma),3 mM potassium ferrocyanide (Sigma) in 1X PBS. The solution was stored in a tin foilwrapped bottle at −20 ◦C and stock X-Gal solution (5% in DMF, Promega) 0.02% was added immediately prior to staining. The tissue was incubated with the staining solution overnight or until the sufficient level of staining was achieved at 37 ◦C. Once stained, tissues were washed with 1X PBS and fixed with formalin to avoid further staining. Stained whole mounts were illuminated with Leica CLS50X light source and visually analysed under Olympus SZX12 low-magnification stereo microscope. Where needed, pictures were taken with Olympus C4040ZOOM 4.1 Megapixel digital camera.

2.2.6 Isolation of epithelium enriched cell populations

Isolation of the intestinal epithelial cells using Weiser solution

The protocol was adapted from (Flint et al., 1991). The first 10 cm of the small intestine from the stomach was flushed with ice cold 1X PBS (Invitrogen), opened longitudinally and a 2-4mm longitudinal section placed in 50 ml falcon tube (Greiner Bio-One) containing 15 ml of freshly prepared ice cold modified Weiser chelating solution (5.56 mM disodium hydrogen orthophosphate (Sigma), 8 mM potassium dihydrogen orthophosphate (Sigma), 96 mM sodium chloride (Sigma), 1.5 mM potassium chloride (Sigma), 27 mM tri-Sodium

citrate (Sigma), 0.5mM dithiothreitol (DTT, Sigma), 1.5% sucrose (Sigma), 1% D-sorbitol (Sigma), 6.07 mM EDTA (Sigma), 4 mM EGTA (Sigma), pH 7.3). The intestine was washed in three changes of ice cold Weiser solution by gentle shaking. After the washes, the tube containing 15 ml of Weiser solution was attached to the vortex mixer and shaken at the lowest setting for 15 min. The tube was then shaken vigorously by hand and the solution was transferred into a fresh tube. Fresh 15 ml of Weiser solution was added to the tube containing the intestine and the shaking steps were repeated. Two 15 ml fractions were joined together and the intestine was discarded. 30 ml of Weiser solution containing the epithelial cells were centrifuged at 1500-2000 rpm for 5 min. The resulting pellet was resuspended in 20 ml of ice cold 1X PBS and centrifuged. The wash in 1X PBS was repeated twice, the pellet was resuspended in 3 ml of 1X PBS and aliquoted into three 1.5 ml eppendorf tubes. The tubes were centrifuged using a mini-centrifuge for 1 minute. The supernatant was removed, the pellet was snap-frozen in LN_2 and stored at −80 ◦C until needed.

2.2.7 Isolation, culture and infection of mammary epithelium

Isolation of mammary epithelium

The fourth mammary fat pads were harvested (mice were culled in batches of three per genotype) for mammary epithelum. This was done in a sterile hood, the mice were culled, immersed in 70% ethanol, and then mammary glands accessed as before. Lymph nodes were removed then the remaining gland was placed in L15/10% FCS + P/S (Leibowitz L15 medium (L15) with L-Glutamine (Invitrogen, Paisley, UK) plus 10% v/v Heat Inactivated Foetal Calf Serum (FCS) (Invitrogen), Penicillin at 100 IU ml-1 and Streptomycin at 100 μg ml-1 (Invitrogen)) on ice until all glands were harvested. Fat pads were quickly dipped 3 times in 70% ethanol and then transferred to fresh L15 /10% FCS+P/S. Fat pads (from 3 mice) were finely minced three times on the McIlwain Tissue Chopper (tissues were stirred and then collected back in the middle of the cutting disc with a sterile Pasteur pipette between each chop). The minced tissue was transferred to a 50 ml Falcon tube (Tube A) containing 10mls collagenase /trypsin digestion mix (30mg of collagenase/15mg tripsine) (4^{o}C) using cell scrapers. The tube was then placed horizontally on a shaker platform and incubated for one hour at 37^{o}C (75rev/min). The digested mixture was then transferred into a 15ml Falcon tube and centrifuged at 1500rpm for 5 minutes. The fat layer and supernatant was poured off into a fresh

15 ml Falcon tube (Tube B) (leaving the tube A laid on its side). The pellet of tube A was then immediately transferred to a fresh 15 ml Falcon in 5 ml L15/10% FCS. Tube B was spun 1500rpm for 5 minutes. The fat layer and supernatant was then discarded and the pellet was taken up in in 5 ml L15/10% FCS and then pooled with the one from Tube A and centrifuged at 250 x *g* for 5 minutes. The new pellet was resuspended in 5mls Sigma Red Blood Cell Lysis Buffer and incubated for 5 minutes at RT. The sample was then centrifuged at 1500rpm for 5 minutes and resuspended in a fresh in 10 ml L15/10%FCS without P/S and pelleted at 1500rpm for 5 minutes.

To remove most of the mammary fibroblasts from the sample, it was resuspended in 15 ml of DMEM/10% FCS (Dulbecco's Modified Eagle's Medium (DMEM) with L-Glutamine (Invitrogen) with 10% v/v FCS, Penicillin at 100 IU ml-1 and Streptomycin at 100 ug ml-1) and transferred to a T-80 tissue culture flask. The flask was then incubated for one hour at 37^{o}C 5%CO2 5%O2. The majority of fibroblasts attach to the tissue culture plastic in this time whilst most of the organoids do not. After the incubation, the flask was shaken in a horizontal plane with moderate vigour to ensure the organoids are in suspension and they were pipetted off. The flask was rinsed with 10 ml L15/10% FCS+P/S, shaken as before and then this wash was pipetted off. The organoid suspension and the wash were then spun at 1500rpm for 5 minutes. The supernatants were then poured off and the pellets resuspended in 1 ml L15/10% FCS (sample composed of mammary epithelial organoids, tissue lymphocytes and some remaining fibroblasts).

Primary mammary epithelial culture

The epithelial organoids were plated out to generate bulk cell by resuspending them in CT:I:EGF medium (1:1 v/v DMEM / Ham's F12 mix (Invitrogen), 10% v/v FCS, Penicillin at 100 IU ml-1, Streptomycin at 100 ug ml-1, 4 mM L-glutamine (Invitrogen), 5 ug/ml bovine pancreatic insulin (I) (Sigma, cell culture tested solution), 10 ng/ml cholera toxin (CT) (Sigma) and 10 ng/ml epidermal growth factor (EGF) (Sigma, murine submaxillary, cell culture tested)) and transferred to flasks. Cultures were maintained at 37oC 5% CO2 5% O2. Over a period of several days, the epithelial cells mobilise and spread out from the organoids to form a monolayer.

Adenoviral infection

All viral work was carried out in accordance with GMO safety regulations and approval. To delete *Apc* from cultured mammary epithelium AD5-CMV-CRE (Vector Labs) was added to the culture medium (see above) at a Multiplicity of infection (MOI) of 10, 50 & 100. The AD5-CMV-CRE was left in the culture for 12 hours then the cultures were washed and maintained in CT:I:EGF medium without AD5-CMV-CRE. An MOI of 50 provided efficient recombination in comparison to cell death. Cultures were imaged 36 hours following infection.

Visualisation

Cultures with and without infection were imaged using an Olympus inverted IX71 microscope.

2.3 Histological analysis

2.3.1 Haematoxylin and Eosin (H&E staining of tissue sections)

Slides containing tissue sections were dewaxed and rehydrated as described in section 2.4. Slides were stained for 5 min in Mayer's Haemalum (R. A. Lamb), washed in running tap water for further 5 min followed by 5 min staining in 1% aqueous Eosin (R.A. Lamb) solution. Eosin was washed off by two 15 second washes in water. Stained sections were dehydrated and mounted as described in section 2.4.

2.3.2 Quantitative histological analysis of H&E stained sections

Histological quantification of the tissue sections was carried out using an Olympus BX41 light microscope. Where appropriate, microphotographs were taken with a Colorview III camera (5 megapixel, Soft Imaging Systems) aided with AnalySIS software (v3.2, Build 831, Soft Imaging Systems) or Moticam 5000 camera (5 megpixel, Motic Instruments) aided with Motic Images Advanced software (v.3.2, Motic China Group). Quantitative and statistical analysis of histological parameters was carried out as described in section 2.6.

Scoring of crypt length

Crypt length was scored by counting the number of cells from the base of the crypt to the crypt-villus junction. Scoring was carried out for 50 half-crypts per section and average of 50 crypts were scored per mouse.

Scoring of villus length

To score villus length, the total number of cells from the crypt bottom to the villus tip was counted on 50 half crypt-villus axes per slide. The average villus length was calculated as average total crypt-villus length minus average crypt length for each mouse.

Scoring of Mitotic index

Mitotic figures were scored on H&E stained sections based on their morphological appearance. Cells were included if chromosome alignment/segregation was microscopically visable (metaphase, anaphase and telophase). Number of mitotic figures was counted in at least 1000 cells. Mitotic index was calculated as average number of mitotic figures per total cells counted.

Scoring of Apoptotic index

Apoptotic cells were scored based on their characteristic appearance on H&E stained sections. Cells were characterised as apoptotic by the microscopic presence of apoptotic bodies, nuclear fragments and/or membrane detachment from neighbouring cells. The number of apoptotic bodies was counted in at least 1000 cells. Apoptotic index was calculated as average number of apoptotic cells per total cells counted.

2.3.3 Use of specific stains for quantification of histological traits

Staining with Alcian Blue

Alcian Blue staining was used to identify mucin-containing goblet cells. Tissue sections were dewaxed and rehydrated as described in section 2.4. Slides were immersed in Alcian Blue staining solution (1% (w/v) Alcian Blue (Sigma), in 3% (v/v) acetic acid (Fisher

Scientific)) for 30 seconds followed by washing in running water for 5 minutes. Slides were washed in running water for 5 min and then dehydrated and mounted as described in section 2.4. Stained sections were examined and the number and position of goblet cells was scored in 50 half crypt-villus axes per mouse. The average number of goblet cells per half crypt-villus axis was calculated and position of these cells was calculated and analysed for each mouse as described in section 2.6.

Lysozyme staining

Immunohistochemistry with an anti-lysozyme antibody (see section 2.4) was used to identify Paneth cells. Both Paneth cell number and position was scored. Paneth cell quantity was scored as the number of lysozyme positive cells in 50 half-crypts per section. The average number of Paneth cells per half-crypt was calculated for each mouse and analysed as described in section 2.6. Paneth cell position was counted from the bottom of the crypt, the bottom-most cell being #1. Paneth cell positions from all the mice of the same genotype were pooled together and analysed as described in section 2.6.

β-catenin staining

Immunohistochemistry with an anti-β-catenin antibody (see section 2.4) was used to assess its protein location. Within the intestines cells containing nuclear localised β-catenin were scored. The average number of displayed cells nuclear localised β-catenin per crypt was calculated from 50 crypts per mouse and analysed as described in section 2.6.

Ki67 staining

Immunohistochemistry with an anti-Ki67 antibody (see section 2.4) was used to identify proliferating cells. The number of proliferating cells was scored as a percent of total cells counted. At least 1000 cells were analysed per tissue per mouse. Data was then analysed as described in section 2.6.

Cleaved Caspase-3 staining

Immunostaining with cleaved caspase-3 antibody (see section 2.4) was used to confirm the morphology-based apoptotic index. The number of Caspase-3 positive cells was scored

as a percent of total cells counted. At least 1000 cells were counted per tissue per mouse. Data was then analysed as described in section 2.6.

BrdU staining

Tissue samples harvested from mice injected with BrdU at 2 or 24 hours prior to dissection were stained with a BrdU antibody as described in section 2.4. The number and position of BrdU positive cells at 2 and 24 hours post labelling were used to assess proliferation activity and cell migration respectively. The number of BrdU positive cells was scored in 50 half-crypts per slide. The average number of BrdU positive cells per half-crypt was calculated for each mouse and analysed as described in section 2.6. The position of BrdU labelled cells was counted from the bottom of the crypt, the bottom-most cell being #1. Cell positions from all the mice of the same genotype were pooled together and analysed as described in section 2.6.

2.4 Immunohistochemistry (IHC)

All standard IHC was carried out according to the generic protocol outlined below. Specific conditions and modifications for particular antibodies are provided in Table 2.3. Unless otherwise specified, all incubation steps were carried out at room temperature (approximately 22 ◦C) in humidified chamber to avoid drying out of the sections or if left overnight at 4◦C. To contain the applied solutions to the tissue, slides were gently dried around the tissue section with tissue paper and the area surrounding the tissue was circled with a water-resistant ImmEdge pen (Vector Labs) typically before endogenous peroxidase block or serum block stage.

2.4.1 Generic immunohistochemistry protocol

Dewaxing and rehydration of tissue sections

Tissue sections were dewaxed by two 5 min washes in xylene (Fisher Scientific) followed by rehydration washes in decreasing concentrations of ethanol (Fisher Scientific): two 2

min washes in 100%, one 2 min wash in 95% and one 2 min wash in 70% ethanol. Slides were then transferred into dH_2O and washed for 5 min.

Antigen retrieval

Antigen retrieval was achieved by heating slides in a microwave in 1X citrate buffer (LabVision) for 2 x 7 min (850 W). Slides were then left to cool in the solution for 30-60 min. Slides were then washed in dH_2O for 5 mins followed by 2 X 5 min washes in washing buffer (1X TBS (Sigma) in dH_2O with 0.1% (v/v) TWEEN-20 (Sigma)).

Endogenous peroxidase activity block

Activity of endogenous peroxidases was blocked by incubating tissue sections with hydrogen peroxide. Either 30% hydrogen peroxide (Sigma) diluted in dH_2O to appropriate concentration or a commercial peroxidase blocking solution (Envision+ Kit, DAKO) were used. The appropriate hydrogen peroxide concentrations and incubation times for specific antibodies are provided in Table 2.3. When using commercial peroxidase block, the tissue was circled with water-resistant pen, placed in a slide chamber and enough solution was applied to cover the tissue. When using hydrogen peroxide from the stock solution, slides were placed in a Coplin jar containing enough solution to cover the tissue. After incubation with the peroxidase blocking solution, slides were briefly rinsed in dH_2O and washed 3 x 5 min in washing buffer.

Non-specific antibody binding block

To diminish non-specific antibody binding, tissue sections were treated either with normal serum (DAKO) or BSA (Sigma) diluted to an appropriate concentration in washing buffer. The specific blocking agent, concentrations and incubation times for particular antibodies are outlined in Table 2.3. Typically, the serum used for blocking was derived from the species, in which the secondary antibody was raised (i.e. if a secondary antibody was raised in rabbit, normal rabbit serum was used to block non-specific binding). If not already done, the tissue containing area was circled with a water-resistant pen, slides

were overlaid with 200 μl of blocking solution and incubated for the time specified.

Primary antibody treatment

Following incubation, blocking solution was removed and 200 μl of primary antibody diluted in blocking solution was applied without washing of sides. The dilutions used with specific antibodies are outlined in Table 2.3. All slides were left in the cold room (4◦C) overnight for incubation, following this the slides were washed 3 x 5 min in washing buffer.

Secondary antibody treatment

The slides were incubated with an appropriate secondary antibody; either an appropriate Horseradish peroxidase (HRP) conjugated secondary antibody (Envision+ Kit, DAKO) used undiluted or if signal amplification was required, a suitable biotinylated secondary antibody (DAKO) was diluted 1:200 in blocking and applied following signal amplification (see table for details 2.3). After incubation, slides were washed 3 x 5 min in washing buffer.

Signal amplification

In instances, when anti-mouse or anti-rabbit Envision+ kits (DAKO) could not be used, a signal amplification step was introduced into the protocol. Avidin-Biotin Complex reagent (Vectastain ABC kit, Vector labs) was prepared according to manufacturer's instructions 30 min prior to use and stored at room temperature. Tissue sections were covered with 200 μl of ABC reagent and incubated for 30 min at room temperature, followed by 3 x 5 min washes in washing buffer.

Signal visualisation with 3.3'-diaminobenzidine (DAB)

Antibody binding was visualised by colourimetric detection with 3,3'-diaminobenzidine (DAB). Tissue sections were covered with 200 μl of DAB solution (2 drops of DAB chromogen mixed with 1 ml of DAB substrate (Envision+ Kit, DAKO)). Slides were incubated for 5–10 min or until a sufficient level of staining was attained followed by a 5 min wash in dH_2O.

Counterstaining, dehydration and mounting

Tissue sections were counterstained in Mayers Haemalum (R. A. Lamb) for 30 sec and washed in running tap water for 5 min. Slides were then dehydrated by consecutive washes in increasing concentrations of ethanol (1 x 3 min 70%, 1 x 3 min in 95%, 2 x 3 min in 100%) and 2 x 5 min washes in xylene. Coverslips were mounted over dehydrated sections using DPX mounting medium (R. A. Lamb).

Table 2.3: Antibody specific conditions for IHC

Lysosome IHC

Primary Antibody	***Peroxidase block***	***Blocking solution***
Neomarkers (RB-372-A), 1:100	1.5% H_2O_2, 15min	10% NGS, 30 min
Signal amplification	***Secondary Antibody***	
No	Envision+ HRP conjugated anti-rabbit, 30min	

BrdU IHC

Primary Antibody	***Peroxidase block***	***Blocking solution***
BD biosciences (347580), 1:150	DAKO, 5min	1% BSA, 30 min
Signal amplification	***Secondary Antibody***	
No	Envision+ HRP conjugated anti-mouse, 30min	

β-catenin IHC

Primary Antibody	***Peroxidase block***	***Blocking solution***
BD transduction labs (610154) 1:300	DAKO, 5min	10% NRS, 30 min
Signal amplification	***Secondary Antibody***	
No	Envision+ HRP conjugated anti-mouse, 30min	

Ki-67 IHC

Primary Antibody	***Peroxidase block***	***Blocking solution***
Vector labs (VPK452) 1:20	0.5% H_2O_2, 30min	20% NRS, 30 min
Signal amplification	***Secondary Antibody***	
ABC kit (Vector labs)	Biotinylated anti-mouse (DAKO), 30min	

Table 2.3 (cont.)

Cleaved caspase-3 IHC

Primary Antibody	*Peroxidase block*	*Blocking solution*
Cell signaling (9661), 1:200	3% H_2O_2, 10min	5% NGS, 30 min

Signal amplification	*Secondary Antibody*
ABC kit (Vector labs)	Biotinylated anti-mouse (DAKO), 30min

cMyc IHC

Primary Antibody	*Peroxidase block*	*Blocking solution*
Santa Cruz (sc-788), 1:200	1.5% H_2O_2, 15min	5% NGS, 30 min

Signal amplification	*Secondary Antibody*
No	Envision+ HRP conjugated anti-rabbit, 30min

CD44 IHC

Primary Antibody	*Peroxidase block*	*Blocking solution*
BD Pharmigen (550638), 1:50	1.5% H_2O_2, 15min	10% NRS, 30 min

Signal amplification	*Secondary Antibody*
ABC kit (Vector labs)	Biotinylated anti-rat (DAKO), 30min

*NRS: normal rabbit serum, NGS: normal goat serum, BSA: bovine serum albumin.

2.4.2 Fluorescent immunohistochemistry protocol

For fluorescent immunohistochemistry, slides were processed as above (section 2.4.1) up until the endogenous peroxidase activity block stage. This step was excluded as antibodies were visualised using fluorescently conjugated secondary antibodies. The serum block used was dependent upon the antibodies used (detailed in Table 2.4) but constant with an incubation time of 30 min. Some slides were double labelled using two primary antibodies and two corresponding fluorescently labelled secondary antibodies. Care was taken when selecting antibodies to avoid cross-reactivity. The protocol remains similar to the generic IHC protocol until addition of the secondary antibodies. All secondary antibodies were used at a concentration of 1:200 diluted in blocking serum however, exposure to daylight of the secondary antibody solution and stained sections was kept to a minimum to avoid photo-

bleaching. The slides were incubated with secondary antibodies for 1-2 hours. Following the final Wash with wash buffer after incubation with the secondary antibodies, slides were wahed in 1 X PBS (Invitrogen) and mounted without dehydration using Vectashield hardset mounting medium with DAPI (DAPI labels nuclear material blue) (Vector Labs). Slides were kept in the dark at 4◦C and imaged within 2 weeks. Images were taken using an Olympus BX61 with correct filters and lamp for detecting fluorescent probes.

Table 2.4: Antibody specific conditions for fluorescent IHC

CK5 & CK8

Primary antibodies	***Secondary antibodies***
CK5, Abcam (ad24647), 1:200	anti-rabbit Alexafluor 594, Invitrogen (A11037)
CK8, Abcam (ab14053), 1:250	anti-chicken Alexafluor 488, Invitrogen (A11039)

Blocking serum
5% NGS

Apc

Primary antibodies	***Secondary antibodies***
APC, Santa Cruz (ad24647), 1:50	anti-rabbit Alexafluor 488, Invitrogen (A11008)
	anti-rabbit Alexafluor 594, Invitrogen (A11037)

Blocking serum
5% BSA

Apc2

Primary antibodies	***Secondary antibodies***
APC2, Zymed (384000), 1:100	anti-rabbit Alexafluor 594, Invitrogen (A11037)
	anti-rabbit Alexafluor 488, Invitrogen (A11008)

Blocking serum
10% NGS

β-galactosidase

Primary antibodies	***Secondary antibodies***
B-galactosidase, Millipore (Ab1211), 1:10,000	anti-rabbit Alexafluor 594, Invitrogen (A11037)

Blocking serum
5% NGS

Table 2.4 (cont.)

β-catenin

Primary antibodies	*Secondary antibodies*
β-catenin, BD transduction Labs (610154), 1:200	anti-mouse Alexafluor 594, Invitrogen (A11005)

Blocking serum
10% NGS

Zo-1 & E-cadherin

Primary antibodies	*Secondary antibodies*
Zo-1, Zymed (402300), 1:400	anti-rabbit Alexafluor 594, Invitrogen (A11037)
E-cadherin, BD transduction Labs (610182), 1:200	anti-mouse Alexafluor 488, Invitrogen (A11001)

Blocking serum
20% NGS

Vimentin & E-cadherin

Primary antibodies	*Secondary antibodies*
Vimentin, Santa Cruz (sc7557), 1:300	anti-rabbit Alexafluor 488, Invitrogen (A11008)
E-cadherin, BD transduction Labs (610182), 1:200	anti-mouse Alexafluor 594, Invitrogen (A11005)

Blocking serum
20% NGS

α-tubulin

Primary antibodies	*Secondary antibodies*
α-tubulin, Invitrogen (A11126), 1:400	anti-mouse Alexafluor 488, Invitrogen (A11001)

Blocking serum
2.5% BSA

β-catenin & Lec2

Primary antibodies	*Secondary antibodies*
β-catenin, BD transduction Labs (610154), 1:200	anti-mouse Alexafluor 594, Invitrogen (A11005)
Lect 2, (Gift from Satoshi Yamagoe), 1:500	anti-rabbit Alexafluor 488, Invitrogen (A11008)

Blocking serum
5% NGS

Table 2.4 (cont.)

GS & CPS

Primary antibodies	***Secondary antibodies***
GS, BD Transduction Labs (ALX803055), 1:400	anti-mouse Alexafluor 594, Invitrogen (A11005)
CPS, Santa Cruz (sc30060), 1:600	anti-rabbit Alexafluor 488, Invitrogen (A11008)

Blocking serum
10% NGS

*NGS: normal goat serum, BSA: bovine serum albumin.

2.5 Gene Expression Analysis

2.5.1 RNA extraction from tissue samples

Homogenisation of tissue samples

Tissue samples were removed from -80 storage and placed in screw-cap tubes containing 1 ml Trizol reagent (Invitrogen) and 1.4 mm ceramic beads (Lysing matrix D tubes, MP Biomedical), Samples were then homogenised using Precellys24 homogeniser (Bertin Technologies) at 6500 RPM for 2 x 45 second cycles. Tubes were centrifuged at 11000 x g for 10 min at 4 ◦C to pellet the beads and tissue debris.

RNA extraction

Supernatant was transferred into fresh 1.5 ml eppendorf tubes containing 200 μl of chloroform (Fisher Scientific). Tubes were then shaken vigorously by hand for 30 seconds and allowed to stand at room temperature for 3 min. Tubes were centrifuged at 11000 x g for 15 min at 4 ◦C. The top, aqueous, phase (approximately 400 μl) containing RNA was removed and transferred into fresh 1.5 ml eppendorf tubes containing 400 μl of isopropanol. Tubes were gently inverted until well mixed.

RNA purification and DNAse treatment

RNA purification was performed using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. All centrifugation steps were performed at 11000 x g in a refrigerated bench top centrifuge cooled to 4 ◦C. The mix from the previous step was

transferred into purification columns placed in a collection tubes. Columns were centrifuged for 30 seconds and the flow-through was discarded. Columns were washed by adding 500 μl of RPE wash buffer (RNeasy kit, Qiagen) and centrifuging for 30 seconds. The flow-through was discarded and the wash was repeated, followed by 2 min centrifugation. The flow-through was discarded and columns were centrifuged once again for 1 min to remove the remnants of the buffer from columns. To elute RNA, columns were placed into fresh 1.5 ml eppendorf tubes, 100 μl of RNAse-free dH_2O was pipetted onto columns and allowed to stand for 3 min at 4 $^\circ$C. Eluate containing RNA was collected by centrifuging columns for 1 min and immediately placed on ice. RNA was then DNAse treated using the DNA-free Kit (Ambion) following manufacturer's instructions.

The concentration and purity of RNA was assessed using NanoDrop 1000 (Thermo Scientific). RNA samples were stored at −80 $^\circ$C until needed.

Determination of RNA quality

The quality of the extracted RNA was analysed using denaturing agarose gel electrophoresis as described in Qiagen Bench Guide (Qiagen). RNA samples containing 2 μg of RNA were mixed with the appropriate amount of RNA 5X loading buffer (0.25% Bromophenol blue (Sigma), 4 mM EDTA (Sigma), 0.9 M formaldehyde (Sigma), 20% glycerol (Sigma), 30.1 % formamide (Sigma), 4X FA gel buffer (see below)), incubated at 65 $^\circ$C for 3–5 min and quenched on ice. Samples were then loaded onto a denaturing gel (1% agarose (Eurogentech), in 1X FA gel buffer (20 mM 3-(N-Morpholino)propanesulfonic acid (MOPS, Sigma), 5 mM sodium acetate (Sigma), 1 mM EDTA (Sigma), 0.22 M formaldehyde (Sigma), 0.1 μg/ml Ethidium bromide (Sigma) in dH_2O)). The gel was run in FA gel running buffer (20 mM 3-(N-Morpholino)propanesulfonic acid (MOPS, Sigma), 5 mM sodium acetate (Sigma), 1 mM EDTA (Sigma), 0.25 M formaldehyde (Sigma) in dH_2O) at 50 V for 1 hour. RNA was then visualised under UV light on a GelDoc (BioRad). Assessment of RNA quality was based on the sharpness of 18S and 28S ribosomal RNA bands.

2.5.2 Preparation of cDNA for quantitative analysis

cDNA synthesis was performed as described by Untergasser A. (http://www.untergasser.de/lab/protocols/cdna synthesis superscript ii v1 0. Htm) using Superscript II RNAse H reverse transcriptase kit (Invitrogen). 1 μg of RNA was mixed with 5 μl of 100 ng/μl random hexamers (Promega), and made up to 20 μl with DNAse free dH_2O (Sigma). The mix was then incubated at 70 $^\circ$C for 10 min, 25 $^\circ$C for 10 min and held at 4 $^\circ$C long enough to add 20 μl of enzyme mix (8 μl 5X First Strand Buffer, 4 μl 0.1 M DTT, 0.8 μl 25 mM dNTPs (Bioline), 1 μl Superscript II enzyme, 6.2 μl RNAse-free dH_2O) per reaction. The reaction mix was then incubated at 25 $^\circ$C for 10 min, 37 $^\circ$C for 45 min, 42 $^\circ$C for 45 min, 70 $^\circ$C for 15 and stored at 4 $^\circ$C. 160 μl of DNAse free dH_2O was added to 40 μl of reaction mix and 1 μl of the resulting cDNA was used per reaction in quantitative RT-PCR. Negative control cDNA samples were synthesised in the same manner but without addition of Superscript II enzyme to the enzyme mix.

2.5.3 Quantitative real-time PCR analysis

Primer design for qRT-PCR

Primers for qRT-PCR analysis (unless previously published) were designed using Primer3 software at http://fokker.wi.mit.edu/primer3/input.htm, checked for mispriming using BLAST engine against Ensembl database (http://www.ensembl.org/Multi/blastview) and synthesised by Sigma Genosys. A number of conditions were considered when designing primers. Primers were designed to reside across exon boundaries to avoid amplification of genomic DNA. Primers were to yield a PCR product of 100–200 bp in size and to have an annealing temperature close to 60 $^\circ$C. The primer sequences used are specified in Table 2.5.

Table 2.5: Primer sequences used for qRT-PCR

Gene	Forward primer	Reverse Primer
β-actin	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA
β-catenin	AGTCCTTTATGAATGGGAGCAA	TCTGAGCCCTAGTCATTGCATA
cMyc	CTAGTGCTGCATGAGGAGACAC	GTAGTTGTGCTGGTGAGTGGAG
Cyclin D1	ACGATTTCATCGAACACTTCCT	GGTCACACTTGATGACTCTGGA
Axin 2	GCAGCTCAGCAAAAAGGGAAAT	TACATGGGGAGCACTGTCTCGT
CD44	ATCGCGGTCAATAGTAGGAGAA	AAATGCACCATTTCCTGAGACT
MIF	GGTATTGCGTGGTACACTGCT	TGTAGGTTTCCAGCATCACATC
WIF1	AACAAGTGCCAGTGTCGAGAGG	GCCTTTTTAAGTGAAGGCGTGTG
Ephrin B2	AAACCCTGATGGACTCTACGAC	TTGTTCTGGCTTGACTCAAAGA
Ephrin B3	TAACGCTGTGGAGGTCTCTGTA	CCTTGCTTTGCTTTGTAACTCC
Ascl2	CAGGAGCTGCTTGACTTTTCCA	GGGCTAGAAGCAGGTAGGTCCA
Lgr5	TGCTCGGACCTGGGGCTCTC	GCCCGTGAACGCTCCCTTGG
Bmi 1	AAGCTTGTCTATTGAGTTCTTTGA	TCTCAAGTGCATCACAGTCATT
Groucho	GGAGAGAGCTCCTGAAGTTTCC	TTCTCTTTGTTCCTCTTCAATGG
Dickkopf 2	TCACTATTCCAACCATGACCTG	CTTCTTGCGTTGTTTGGTACAG

qRT-PCR reaction set-up

All reactions were run on StepOnePlus real-time PCR system supplemented with StepOne software v2.1 (Applied Biosystems). All reactions were run in a minimum of three technical and three biological replicates. Samples devoid of cDNA served as negative controls to test for contamination with genomic DNA. A housekeeping gene (typically *β*-actin) was used in each run as a reference gene. Reactions were run using DyNAmo HS SYBR Green qPCR kit (Finnzymes) according to the manufacturer's instructions. qRT-PCR reactions for the intestinal stem cell markers (Lgr5 and Ascl2) were confirmed using TaqMan assay (Applied Biosystems) following the manufacturer's instructions and using custom designed TaqMan probes (Applied Biosystems).

Forward and reverse primers (100 mM) were mixed in equal quantities and diluted to 10 mM concentration. 0.4 μl of the resulting 10 mM primer mix was then used per reaction. For all reactions 1 μl of cDNA was loaded into a thin wall 96-well PCR plate (Applied Biosystems). 19 μl of Master Mix containing appropriate primers or TaqMan probes were added to cDNA samples and plates were sealed with optically clear sealing film (Applied Biosystems). All SYBR Green reactions were run using the same cycling conditions: 95 $^{\circ}$C for 10 min followed by 40 cycles (95 $^{\circ}$C for 15 seconds, 60 $^{\circ}$C for 30 seconds, 72 $^{\circ}$C for 30

seconds), To ensure that a single product was amplified by the primer set, a melting curve was run after cycling from 60 ◦C to 95◦C with a 0.5◦C increment. TaqMan reactions were run using the following conditions 50 ◦C for 2 min, 95 ◦C for 10 min followed by 40 cycles (95 ◦C for 15 seconds, 60 ◦C for 1 min).

Analysis of qRT-PCR data

Data from the samples with reproducible cycle time (C_t) values and a single peak melting curve (when applicable) were analysed using the $2_{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). ΔC_t values were calculated using C_t value of the reference gene from the same sample.Average ΔC_t values were calculated for all control samples and $\Delta\Delta C_t$ value for each individual sample was calculated in reference to the average 'control' ΔC_t. Average $\Delta\Delta C_t$ values were calculated for samples from each biological group and the Mann-Whitney U test was used to test for significant differences between means. The average $\Delta\Delta C_t$ from each biological group was transformed into fold change using formula $2\Delta\Delta Ct$.

2.5.4 In situ hybridisation

In situ hybridisation was used to examine the expression of the intestinal stem cell markers *Ascl2* and *Olfm4*. This technique utilised digoxigenin (DIG) labelled anti-sense RNA probes (ribo probes) complementary to Ascl2 and Olfm4 mRNA sequence. Probe binding was detected using an anti-digoxigenin alkaline phosphatase-conjugated antibody by development of purple staining. The probe for *Ascl2* was provided by Dr. Simon Tunster (Cardiff university). The probe for *Olfm4*, cloned in a pBluescript II SK+ vector, was provided by Hans Clever's group (Hubrecht Institute, Netherlands). All glassware used in the protocol was heat-treated by baking at 200 ◦C overnight and RNAse-free (Sigma) or DEPC treated dH_2O was used to prepare all solutions.

Probe synthesis

The probes were synthesised by linearisation of plasmid DNA, RNA polymerase reaction from the T7 promoter and DIG labelling. 30 μg of plasmid DNA was linearised

by restriction digest with NotI restriction enzyme (NEB) according to manufacturer's instructions. The restriction digest was carried out overnight at the appropriate temperature, and 5 μl of reaction was run on an agarose gel alongside undigested vector to confirm complete linearisation. Linearised DNA was then purified using DNA purification columns (Qiagen). DNA concentration of the purified plasmid was determined using NanoDrop 1000 (Thermo Scientific), and adjusted to 1 μg/μl using 10 mM Tris (pH 8.0) as required. The linearised plasmid DNA was used as a template for the transcription of DIG-labelled riboprobes using T7 RNA polymerase (Roche). The components of the riboprobe-labelling reaction are outlined in Table 2.5.

Table 2.6: Reaction constituents for DIG labelling

Component	***Volume***
RNase Free H2O	12 μl
Transcription Buffer (10X) (Roche)	2 μl
DIG RNA Labelling mix (Roche)	2 μl
RNasin (Promega)	1 μl
T7 RNA Polymerase (Roche)	2 μl
DNA (1 μg/μl)	1 μl

The labelling reaction was carried out for 2 hours at 37 ◦C, followed by DNA template digestion by incubation with 20 units of DNase I (Ambion) for 15 minutes at 37 ◦C. Riboprobes were purified using the RNeasy Mini kit (Qiagen) according to manufacturer's instructions. Probes were eluted with 100 μl of RNAse-free H_2O and divided into 10 μl aliquots, which were transferred to storage at -80 ◦C.

Probe hybridisation

Slides were dewaxed in 2 x 10 min changes of xylene, and rehydrated by 1 min washes in ethanol of decreasing concentration: 2 x 100%, 95%, 85%, 75%, 50% and 30%. Slides were incubated in 1X saline for 5 min followed by 5 min wash in 1X PBS. Endogenous alkaline phosphatase activity was blocked by a 30 min incubation in 6% hydrogen peroxide (in 1X PBS) at room temperature. Slides were washed 2 x 5 min in 1X PBS, fixed in ice cold 4% PFA (Sigma) (in 1X PBS) for 20 minutes followed by 2 x 5 min washes in 1X PBS. Slides were incubated in Proteinase K (Roche) (20 μg/ml Proteinase K, 50 mM Tris, 5

mM EDTA in dH_2O) for 5 min at room temperature, washed for 5 min in 1X PBS, postfixed in 4% PFA for 5 minutes followed by 2 min wash in DEPC treated H_2O. To reduce non-specific probe binding, slides were incubated with acetic anhydride (0.01 M acetic anhydride (Sigma), in 0.1 M triethanolamine hydrochloride (Sigma)), for 10 min at room temperature. Slides were then consecutively washed in 1X PBS and 1X saline for 5 min at room temperature. Slides were rehydrated through 1 min washes in 30%, 50%, 70%, 85%, 95% and 2 x 100% EtOH solutions (70% ethanol wash was carried out for 5 min to prevent salt precipitation). Slides were air dried for 30 minutes and transferred into sealable boxes lined with tissue paper saturated in moisture buffer (5X saline sodium citrate (SSC) buffer (Sigma), 50% (v/v) formamide (Sigma) in dH_2O). The probe was heated at 80 ◦C for 3 minutes, and quenched on ice, and hybridisation buffer (5X SSC, 50% formamide, 1% SDS, 0.05 mg/ml heparin (Sigma), 0.05 mg/ml calf liver tRNA (Merck) in dH_2O) was thawed at 80 ◦C. Adequate amounts of probe and hybridisation buffer were mixed (1 μl probe per 100 μl hybridisation buffer per slide), and applied to slides. A piece of parafilm was placed on top of the slide to prevent probe mixture evaporation. The box was sealed with electrical tape and incubated at 65 ◦C overnight in a water bath.

Post-hybridisation treatment

Slides were removed from the box and incubated in pre-warmed 5X SSC buffer at 65 ◦C for 30 minutes. Slides were then washed 2 x 30 min in solution I (50% formamide, 5X SSC, 1% (v/v) SDS (Sigma)) pre-warmed to 65 ◦C followed by 3 x 10 min washes in solution II (0.5 M NaCl (Sigma), 0.01 Tris pH 7.5 (Sigma), 0.1% TWEEN-20 (Sigma)) at room temperature. Unhybridised probe was digested by a 45 min incubation with RNase A (Roche) (0.02 mg/ml in solution II) at 37 ◦C, followed by a 10 min wash in solution II at room temperature. Slides were washed 2 x 30 min in solution III (50% formamide, 5X SSC) preheated to 65 ◦C. Slides were washed 2 x 10 min in PBS/T (0.1% TWEEN-20 in 1X PBS) and pre-blocked in 10% heat-inactivated sheep serum (DAKO) in PBS/T for 3 hours, covered with a piece of parafilm to prevent drying up of the solution. Anti-DIG alkaline phosphatase conjugated antibody (Roche) was pre-absorbed in 1% heat inactivated sheep serum in PBS/T containing 6 mg/ml small intestine tissue powder for 3 hours at 4 ◦C and diluted to a final concentration of 1:2000 in 1% sheep serum in PBS/T.

Slides were washed in PBS/T for 5 min, overlaid with 100 μl of antibody solution, covered with a piece of parafilm, and incubated overnight at 4 $^{\circ}$C.

Signal detection

Slides were washed in PBS/T for 5 min, followed by 2 x 5 min and subsequent 3 x 30 min washes in PBS/T. Slides were preconditioned in 3 x 5 min washes of NTMT (0.1 M sodium chloride, 0.1 M Tris pH 9.5, 0.05 M magnesium chloride (Sigma), 0.1% TWEEN-20) containing 2 mM Levamisole (Sigma). Slides were completely covered with BM Purple substrate (Roche), sealed in a dark box and incubated at room temperature until the desired level of signal was developed. Slides were then washed in PBS/T for 10 min, followed by a 30 min wash in dH_2O, and counterstained by immersion in Eosin Y Solution (Sigma) for up to 10 seconds. Excess eosin was washed off under running tap water and slides were air dried for 30 min. Slides were washed briefly in xylene and mounted in DPX mounting media (R. A. Lamb).

2.6 Quantitative and statistical analysis of data

2.6.1 Quantitative analysis of histological parameters

Quantitative analysis of tissue sections was carried out using an Olympus BX41 light microscope. Sample IDs were covered on all slides before scoring to avoid possible bias. Areas to be quantified were carefully selected to ensure that they belong to approximately the same region of the tissue and are free of sectioning artefacts. Quantifiable traits, such as crypt and villus length (within the intestine), mitosis and apoptosis levels, number of differentiated cells of particular types, etc. were scored on appropriately stained tissue sections. A minimum of 50 half-crypt or half-crypt-villus axes were stained per mouse in intestinal studies or at least 1000 cells in other tissues. At least 3 different animals per genotype were examined to allow for statistical analysis. Scores from mice of the same genotype were used to calculate average ‘group’ value as well as standard deviation.

Average ‘group’ values were plotted as histograms with error bars representing standard deviation within each group. MS Excel 2010 was primarily used for graphical representation of the data. Individual ‘mouse’ values were also used to test for significant difference between means as described below. For all tests, the use of one- or two-tailed test was decided on a case-by-case basis, depending on the appropriate null hypothesis.

2.6.2 Comparison of means

Quantitative data was first tested for normal distribution using normal plot of residuals and Anderson-Darling test in Minitab statistical analysis software (version 15.1.30.0). Normally distributed data (Anderson-Darling test $p \geq 0.05$) was then tested for significant difference between means using One-Way ANOVA using SPSS software (version 17.0.2). If data from more than two groups were compared, One-Way ANOVA test was used to establish the difference across all the groups and Post-Hoc Tukey’s test was subsequently employed to perform pair-wise comparisons between groups. These tests were carried out using SPSS software. A significant difference between means was accepted, when p value was equal or less than 0.05. Non-normally distributed data (Anderson-Darling test $p \leq 0.05$) was tested for significant difference between means using Mann-Whitney U test. The test was performed in SPSS software. A significant difference between means was accepted, when p value was equal or less than 0.05.

2.6.3 Kolmogorov-Smirnov Z test

Kolmogorov-Smirnov Z test was used to test for the significant difference in distribution between two data sets. To assess the distribution of specific cells (i.e. BrdU positive, Goblet or Paneth cells) the positions of positive cells were recorded using a following method. Each half-crypt was represented as a column of numbers in a spreadsheet with positive cells recorded as 1 and negative cells recorded as 0. Counting was started from the crypt-base so that the row in the column corresponded to cell position on the crypt-villus axis. This was repeated for 50 half-crypts per mouse. The sum of all 1 and 0 in any given row (at any given

position) in every mouse was calculated and sums of all the mice of the same genotype were pooled together. These intermediate data sets were used to build graphs of cumulative frequency of positive cells manually using MS excel. To test for the difference in distribution between the biological groups a K-S statistic was calculated and significance tested using formulas: see http://www.physics.csbsju.edu/stats/KS-test.html. Statistically significant difference between distributions was accepted, when p value was less or equal 0.05.

2.6.4 Kaplan-Meier survival analysis

Survival analysis and generation of survival curves was performed using the Kaplan-Meier method with the aid of SPSS software . Statistical significance between survival time of different experimental cohorts was assessed using the Log-Rank method. Differences in survival probability were accepted as significant, when p value was less or equal 0.05.

Chapter 3

Investigating the effects of Apc2 loss in the small intestinal epithelium

3.1 Introduction

APC has been extensively studied in both development and tumorigenesis. Initially identified as the defective gene responsible for inherited FAP, it is characterized by numerous adenomas polyps in the intestines of which some inevitably progress to carcinoma (Kinzler et al., 1991; Groden et al., 1991; Nishisho et al., 1991). Patients carry one mutated copy of *APC* and a 'second hit' (mutation of the normal residing *APC* allele) initiates polyp formation. Tumour progression through to carcinoma occurs due to subsequent mutations in other oncogenic genes. Loss of function mutations of *APC* were also found in the majority of sporadic colorectal cancers (Powell et al., 1992; Nagase & Nakamura, 1993). Mutations in *APC* are recognised as an early event in colorectal tumourigenesis (Powell et al., 1992) and although its role in tumour suppression is multifunctional (reviewed by Aoki & Taketo, 2007; Senda et al., 2007), previous studies suggest the most influential role lies with its ability to mediate Wnt signaling (Hanson et al., 2005; Nathke, 2005; Polakis, 2000).

Interestingly, APC2 possesses many shared structural characteristics with APC and may therefore have comparable functions. It had been shown APC2 can deplete cytoplasmic β – catenin via direct interaction and can complement APC loss in human colon carcinoma cells (Nakagawa et al., 1998; Van Es et al., 1999). This is performed by APC2 in a destruction complex spatially and functionally isolated from the APC associated destruction complex (Maher et al., 2009). There is now accumulating evidence that APC2 may also act as a tumour suppressor in humans. *APC2* is located within a chromosome region lost with Peutz-Jeghers syndrome (PJS). Patients with PJS are more susceptible to certain cancers including testis, ovarian, breast and colon (Mehenni et al., 1998; Bignell et al*.,* 1998). However, mutations in the PJS gene, *LKB1,* are not present in most sporadic cases of these cancers, suggesting the existence of other tumour suppressor loci in this region of chromosome. It was also shown that *APC2* displays allelic imbalance in most breast and ovarian tumours

(Jarrett et al., 2001) and exhibits significant LOH in certain sporadic cancers (Sobottka et al., 2000; Yang et al., 2004; Wang et al., 1999).

Although known mutations in *APC2* are rare, epigenetic silencing and reduction in expression is now recognised in a variety of cancers through hypermethylation (reviewed in Herman & Baylin, 2003; Jones & Baylin, 2007), including; neurological cancer (Nakagawa et al., 1999), breast cancer (Chan et al., 2008) and indeed colorectal cancer (Schuebel et al., 2007; Hsieh et al., 2007; Chan et al., 2008). Epigenetic abnormalities can cooperate with genetic alterations to effect aberrant gene function that results in cancer (Jones & Baylin, 2002). Hypermethylation of *APC2* is associated with progression from inflammatory bowel disease (IBD) to IBD associated neoplasia (Montgomery et al., 2008). It was demonstrated by Chan et al. (2008) that increased methylation status of *APC2* led to a decrease in protein expression and that hypermethylation occurred in 100% of primary colon tumours compared to control tissue. Studies by (Mokarram et al., 2009) and (Kumar et al., 2009) have confirmed hypermethylation of *APC2* in 90-98% of colorectal tumour samples compared to control in a variety of human populations.

In mice, constitutive Apc loss causes embryonic lethality (Moser et al., 1995) whilst heterozygosity leads to intestinal tumour formation (Moser et al., 1990). To circumvent embryonic lethality, studies have utilised cre-lox technology to obtain homozygous Apc loss within the intestine (Sansom et al., 2004; Andreu et al., 2005). Immediate loss of Apc results in changes in proliferation, migration and differentiation, primarily thought to be through aberrant Wnt signaling. The importance of normal Wnt signaling in small intestinal epithelial maintenance has been known for some time (Korinek et al., 1998). Perturbed Wnt has implications for intestinal stem cells and indeed cancer initiation/progression (for review see Reya & Clevers, 2005). Studies have shown intestinal stem cell populations depend on active Wnt signaling for their self-renewal (Fevr et al., 2007). Inversely, over-activated Wnt signaling achieved through Apc loss leads to an increase in stem cell expression markers (Jubb et al., 2006; Merlos-Suarez et al., 2011). The intestinal epithelium composes a cellular environment which is critically reliant on Wnt signalling for its homeostasis. Furthermore, it is a commonly used model to study colorectal cancer (reviewed in Heyer et al., 1999) and would therefore represent an excellent tissue to study the consequences of Apc2 loss.

In this chapter I wished to characterise Apc2 loss with particular emphasis on the small intestine. Parameters analysed were those normally associated with Apc loss with an aim to elucidate comparable functions.

The main functions of the small intestine include digestion, absorption and secretion of gastrointestinal hormones. The physiology of the small intestines enables it to maximize efficiency in conducting these functions (Figure 3.0). The epithelium of the small intestines consists of four main differentiated cell types (enterocytes, goblet, paneth and enteroendocrine) of which the majority are replenished every 4 to 5 days (excluding paneth cells). Cells are born and divide in a region known as the crypts of lieberkühn (crypts), whilst paneth cells differentiate and migrate to the bottom of these crypts, all other differentiated cell types migrate towards the lumen of the small intestine along finger like projections termed villi. Once cells reach the tip of the villus they are 'shed off' into the lumen.

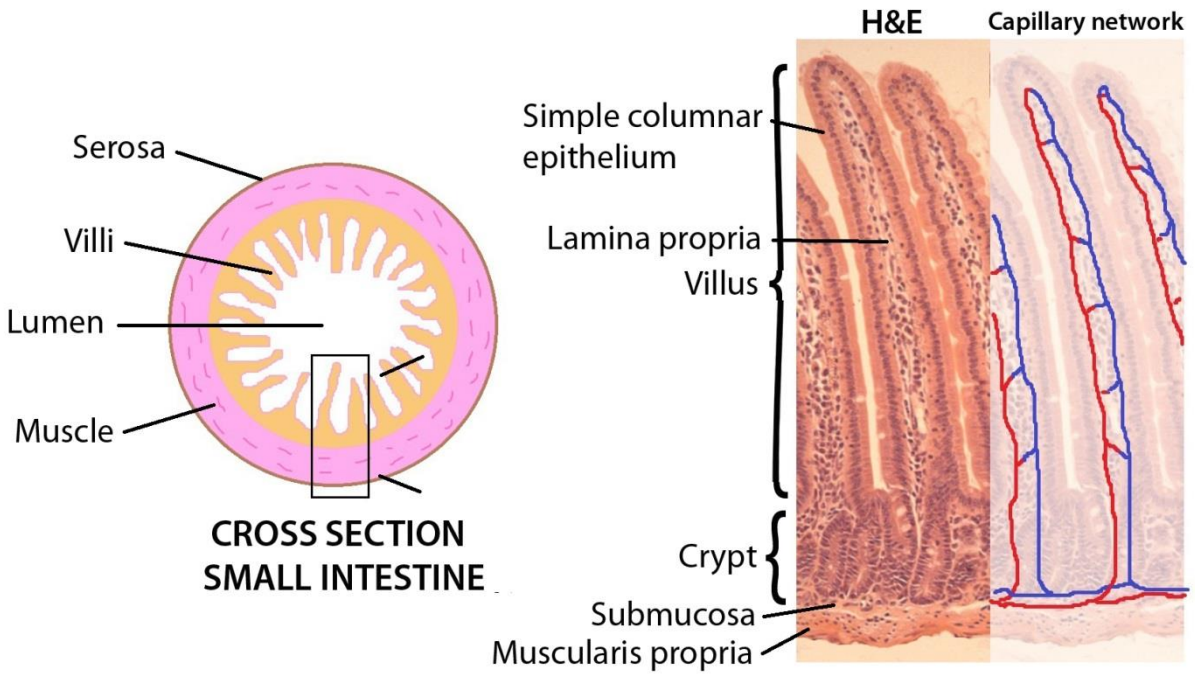

Figure 3.0 Diagram depicts a cross section through the small intestine and a corresponding H&E stained section of small intestinal epithelia. The useful surface of the small intestine is vastly increased due to the formation of finger-like projections termed villi. Water, electrolytes and nutrients are absorbed from the digested contents within the lumen through the intestinal epithelium and into a network of capillaries that run along the villi.

3.2 Results

3.2.1 *APC2* is down-regulated in human colorectal cancer

As previously mentioned, increasing evidence implicates APC2 in tumour suppression. In order to assess the potential importance of APC2 in the pathogenesis of human colorectal cancer (CRC), we analysed the *APC2* gene expression profile in matched normal and tumour samples from 18 patients (courtesy of Dr F Song, Liverpool University). A significant reduction of *APC2* expression levels in tumour samples was shown (see Figure 3.1, Median fold change of -2.754, Wilcoxon Signed Rank Test: Significant with P=0.001). This significance is indicative of a mechanistic link associating APC2 expression levels and tumorigenesis.

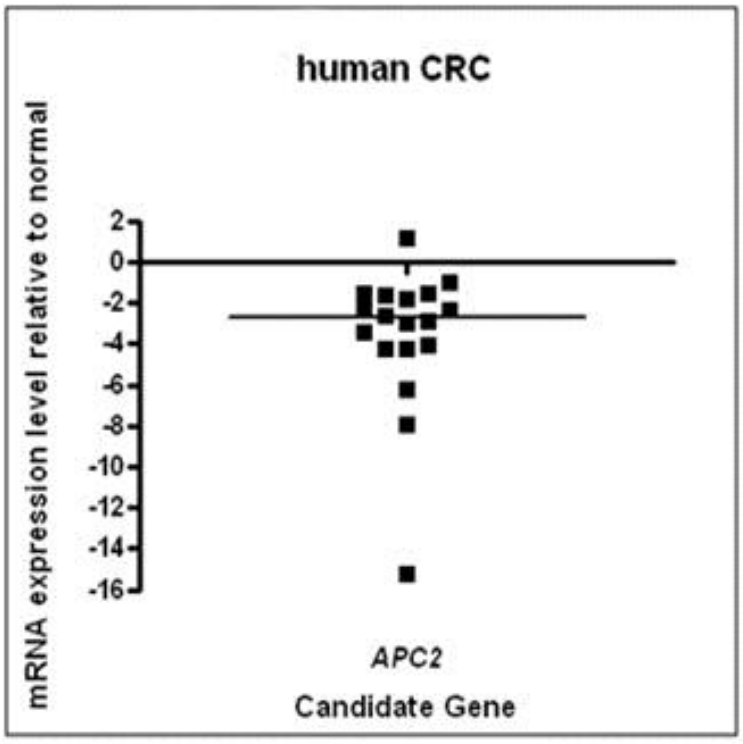

Figure 3.1 *APC2* is down regulated in human colorectal cancer. Quantitative RT-PCR was used to compare *APC2* expression levels between colorectal tumour samples and corresponding macroscopically normal surrounding tissue from 18 patients. There was a significant reduction of *APC2* expression in tumour samples (Median fold change of -2.754, Wilcoxon Signed Rank Test, P=0.001).

3.2.2 Constitutive *Apc2* mutation

Due to a suggested link between APC2 and tumorigenesis and the fact that homology exists between APC2 and APC (Apc is indispensable for development and tumour suppression), an *Apc2* mutant mouse was generated and kindly gifted to us from Prof. Hans Clevers' laboratory (termed *Apc2*$^{-}$). Briefly, Van der Meer et al., (2001) generated this mouse using genetic engineering to disrupt *Apc2* by introducing a stop codon into the open reading frame, resulting in a shortened gene product (Figure 3.2A). PCR was used to confirm and identify mutated animals (Figure 3.2B). Both Apc and Apc2 share the ability to bind β-catenin and Axin. However, in *Apc2*$^{+/-}$ mice (which appear phenotypically indifferent from *Wt* {*Apc2*$^{+/+}$}), one copy of Apc2 is constitutively truncated lacking β-catenin and Axin binding sites (Figure 3.2C).

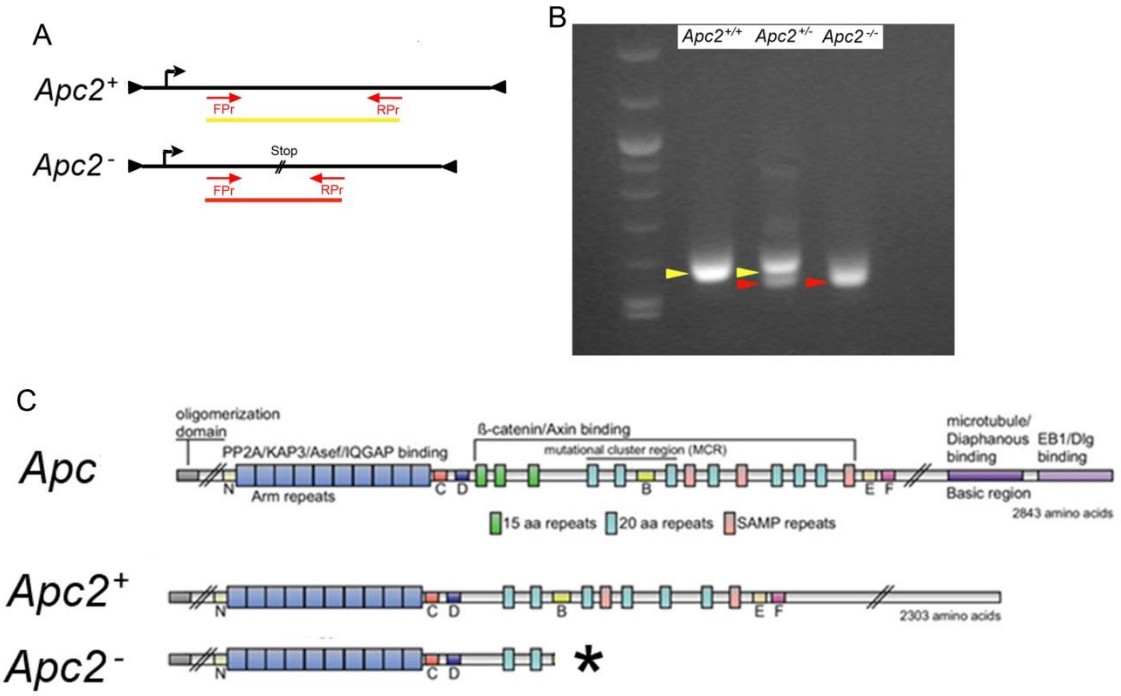

Figure 3.2 Global Apc2 loss via a constitutive truncating mutation. (**A**) Schematic representation of targeted *Apc2* mutation and primer positions for detection. The *Apc2* locus was genetically modified by excising a fragment out of the open reading frame, introducing a STOP Codon (Van der Meer et al., 2001). Specifically designed primers allow for PCR mediated detection of *Apc2* mutation (FPr= forward primer, RPr=reverse primer, yellow and red indicate expected PCR product length). (**B**) PCR reaction designed to detect mutant allele (yellow arrowhead=*Apc*$^{+}$ PCR fragment, red arrowhead= *Apc*$^{-}$ PCR fragment). (**C**) Schematic representation of translated Apc, Apc2^{+} and Apc2^{-} protein. Both Apc proteins contain β-catenin and Axin binding sites. Apc2^{-} protein lose these interaction sites and mimic truncation usually found in cancer related APC loss (adapted from Webb et al., 2009).

In contrast to the embryonic lethality associated with homozygous constitutive *Apc* mutation (Moser et al., 1995; Oshima et al., 1995), *Apc2*$^{-/-}$ mice were viable. However, a substantial proportion of *Apc2*$^{-/-}$ mice, whilst maintained on a mostly *C57BL/6 genetic background*, developed hydrocephalus shortly after birth. Hydrocephalus in *Apc2*$^{-/-}$ mice usually presents at around 2-5 weeks of age where mice with symptoms become ill and were culled. *Apc2*$^{-/-}$ mice that survive this time period did not develop hydrocephalus and can live healthily beyond 100 days of age. Macro- and microscopic examination of the gross morphology of the intestines from mice with hydrocephalus did not identify any differences when compared to age matched *Apc2*$^{+/+}$ controls. However, in depth analysis of intestines from hydrocephalic mice was avoided to ensure any differences observed in *Apc2*$^{-/-}$ intestine were true consequences of Apc2 loss within the intestines, rather than features resulting from hydrocephalic associated brain issues such as altered eating habits or hormone regulation. In order to elucidate if any brain defects resided in hydrocephalus-free surviving *Apc2*$^{-/-}$ mice, a small battery of behavioural tests was employed. Locomotor activity, rotarod and cylinder test revealed no behavioural differences between *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ mice older than 100 days of age (data not shown). This data, along with the observation that surviving *Apc2*$^{-/-}$ mice appeared healthy, suggests no residing brain defects persist. APC2 has been reported to be expressed in a wide range of tissues, including the intestines (Van Es et al., 1999). In order to test the significance of Apc2 within the intestines, cohorts of *Apc2*$^{+/+}$, *Apc2*$^{+/-}$ and *Apc2*$^{-/-}$ mice were established and sacrificed at approximately 3 months of age. In depth intestinal characterisation was then performed focusing on the small intestines.

3.2.3 Loss of Apc2 does not lead to morphological changes in intestinal crypt-villus architecture or alter terminal differentiated cell composition/position

No intestinal lesions were noticed in any genotype at 3 months of age both macro- and/or microscopically. Histological analysis of H&E stained intestinal sections revealed no major alterations in intestinal crypt or villus morphology between experimental and control tissues (Figure 3.3A). Crypt size (Figure 3.3B) or villus length (Figure 3.3C) was measured by cell number and displayed no significant differences between experimental and control tissues (both *Apc2*$^{+/-}$ & *Apc2*$^{-/-}$ vs *Apc2*$^{+/+}$ comparisons $n \geq 4$, Mann-Whitney *U*-test $p \geq 0.54$).

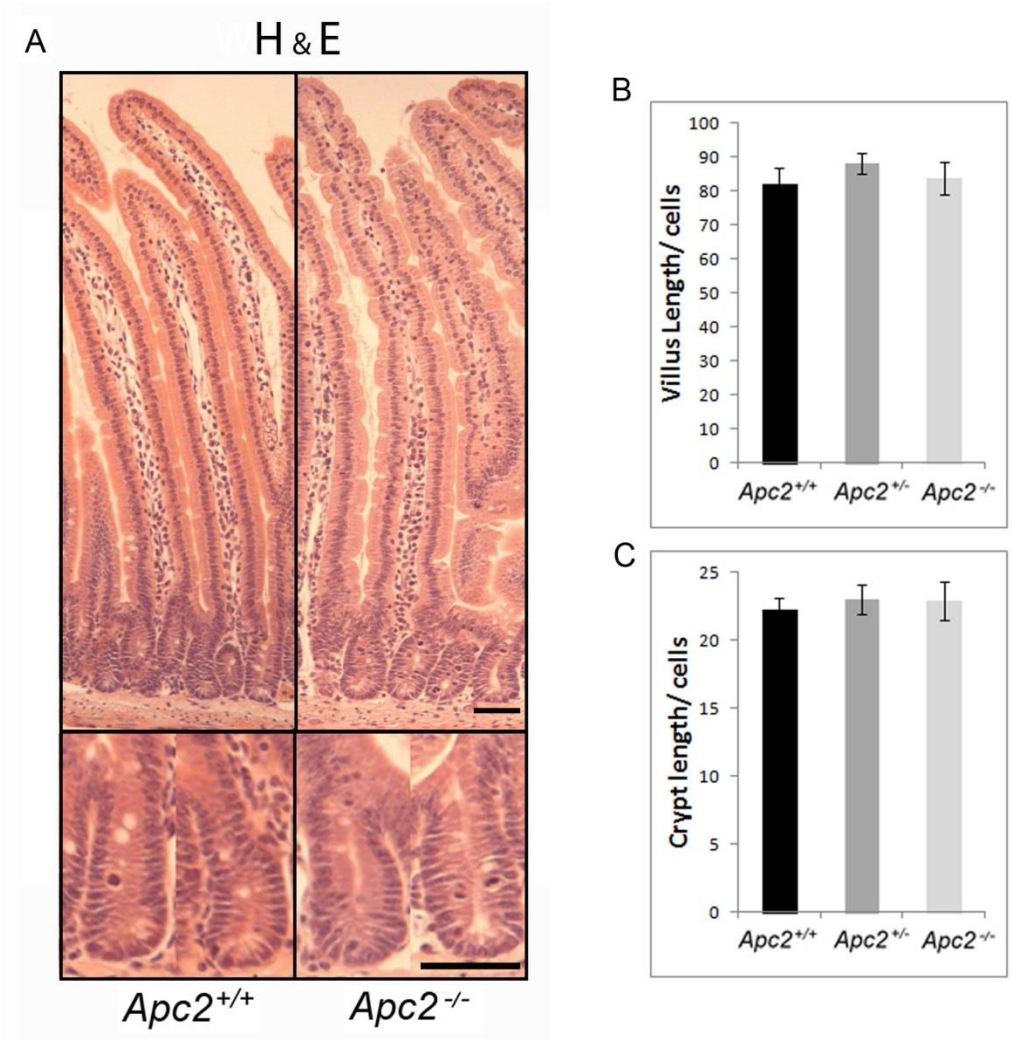

Figure 3.3 Apc2 loss does not lead to alterations in intestinal crypt-villus morphology (**A**) H&E stained intestinal tissue from control and *Apc2*$^{-/-}$ mice culled at 120 days of age revealed no gross microscopic alterations to crypt-villus architecture (Scale bar represents 50μm). Quantitative analysis of villus length (**B**) and crypt size (**C**) between cohorts of *Apc2*$^{+/+}$ (black bar), *Apc2*$^{+/-}$(dark gray bar) and *Apc2*$^{-/-}$ (light gray bar) mice revealed no significant difference between any genotype (error bars represent standard deviation, p>0.3, Mann-Whitney *U*-test, n≥5).

Wnt signaling has been closely coupled with intestinal differentiation and Apc deficient intestines display perturbed differentiation (Sansom et al., 2004; Andreu et al., 2005).To assess differentiated cell types in Apc2 deficient intestines, sections from *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ intestines were co-labelled with cytokeratin 5 and 8 antibodies, using florescent IHC (Figure 3.4A). Cytokeratin 5 labels the secretory lineages (composed of goblet, enteroendocrine

and paneth cells) whilst cytokeratin 8 labels the absorptive lineage (consisting solely of enterocytes). Together this double stain can label the majority of terminally differentiated intestinal epithelia and the absence of stain can expose the non-terminally differentiated cell population. Indeed this stain can expose differentiation changes in Apc deficient intestinal models (unpublished data). No differences were observed between *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ mice, indicating that both genotypes contain comparable undifferentiated, absorptive and secretory lineages. Given that Apc deficient intestines display perturbed differentiation with alterations in paneth and goblet cell populations (Sansom et al., 2004; Andreu et al., 2005), in depth analysis of these cell types was carried out in *Apc2*$^{-/-}$ intestines. Intestinal sections from *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ were stained with alcian blue (goblet cell specific stain) and lysozyme (paneth specific stain) (Figure 3.4B). There was no difference between *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ in total number of paneth cells (2.93±0.18 vs 3.05±0.21 paneth cells per half crypt respectively, p=0.42, Mann-Whitney *U*-test, n=5) or goblet cells (9.21±1.98 vs 9.51±1.39 goblet cells per half crypt respectively, p=0.56, Mann-Whitney *U*-test, n=5). Positional analysis was performed and represented as the percentage chance of a cell being positively labelled (X axis) vs position of that cell (Y axis, 0 represents base of crypt) (Figure 3.4C). No differences were observed in distributions of paneth or goblet cells between *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ intestines. Kolmogorov-Smirnov Z test proved no statistical difference in distribution between *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ in either paneth (p=0.42, n=4) or goblet (p=0.51, n=4) cells.

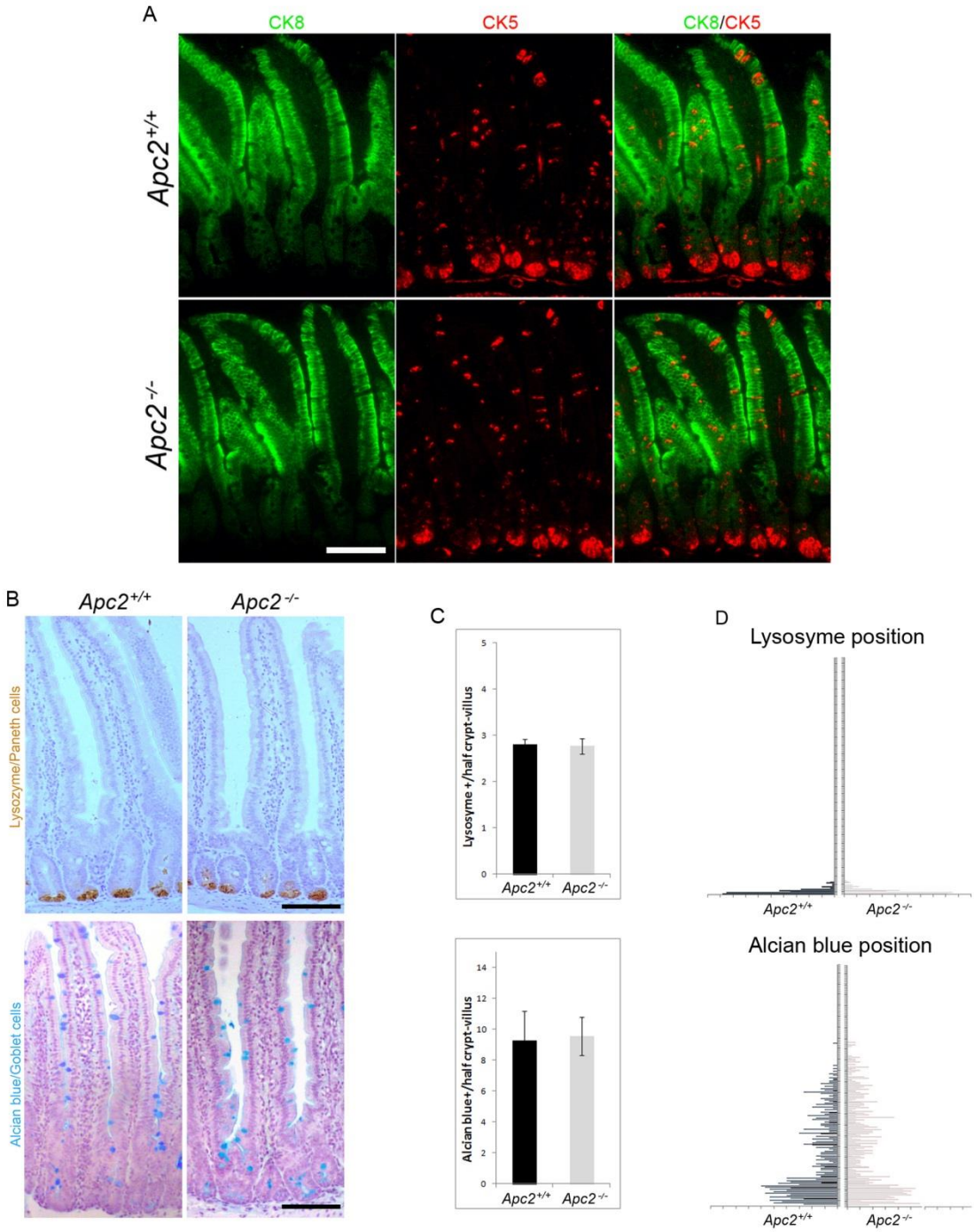

Figure 3.4 Loss of Apc2 does not have an effect on terminally differentiated cell types. (**A**) Sections of aged matched *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ intestines were stained for differentiated cells using double label fluorescent IHC for cytokeratin 8 (CK8) and cytokeratin 5 (CK5) (Scale bar represents 100μm). No differences were noticed between *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ intestines in absorptive (CK8) or secretory (CK5) lineages. (**B**) Aged matched *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ intestines were stained for lysozyme (paneth cells) and alcian blue (goblet cells). Scale bar represents 100μm. There was no difference in either total number (**C**) (error bars represent standard deviation, p>0.4, Mann-Whitney *U*-test, n=5) or position (**D**) (cell position {Y axis} vs percent chance positively labelled {X axis}, for graph description see methods, p>0.25, Kolmogorov-Smirnov Z test, n≥4).

3.2.4 Apc2 deficiency does not affect apoptosis however it does cause an increase in the number of mitotic figures

Apc2 has the proven ability to mediate Wnt signaling (Nakagawa et al., 1998; Van Es et al., 1999). Furthermore, modification of intestinal Wnt signaling as a consequence of Apc deletion is known to result in substantial proliferative and apoptotic changes (Sansom et al., 2004). Thus, intestinal proliferation and apoptosis was analysed in mice deficient for Apc2. H&E stained intestinal sections from all genotypes were used to quantify mitotic figures (Figure 3.5A) and apoptotic bodies (Figure 3.5B) (for description see methods **2.3.2**). Mitotic and apoptotic index were defined as a percent of total crypt cells. There was a statistically significant increase in mitotic figures in Apc2 deficient intestines (*Apc2*$^{+/+}$ 0.80±0.15 vs *Apc2*$^{-/-}$ 1.61±0.18 percent of cells displaying mitotic figures, p≤0.05, Mann-Whitney *U*-test, n=5) whilst apoptosis remained unaltered between genotypes (*Apc2*$^{+/+}$ 0.26±0.07 vs *Apc2*$^{+/-}$ 0.31±0.11 vs *Apc2*$^{-/-}$ 0.28±0.15 percent of cells displaying apoptotic bodies, for all comparisons p>0.2, Mann-Whitney *U*-test, n≥4). To confirm increases in mitotic index, mice were injected 2 hours before death with bromodeoxyuridine (BrdU). Cells entering S-phase incorporate BrdU which can be detected on intestinal sections using an anti-BrdU antibody (Figure 3.5C). Quantitative analysis of BrdU positive cells per half crypt-villus (Figure 3.5D) demonstrated that *Apc2*$^{-/-}$ crypts displayed an increase in proliferation compared to *Apc2*$^{+/+}$ and this increase was in a gene dose dependent manner (*Apc2*$^{+/+}$ 5.29±0.29 vs *Apc2*$^{-/+}$ 6.90±0.66 vs *Apc2*$^{-/-}$ 8.91±1.41 positive BrdU cells per half crypt-villus, for both *Apc2*$^{+/+}$ vs *Apc2*$^{-/+}$ and *Apc2*$^{+/+}$ vs *Apc2*$^{-/-}$ p≤0.05, Mann-Whitney *U*-test, n≥4). Positional analysis of BrdU positive cells indicated dividing cells were still confined to the crypt region consistent with the location of the usual proliferative compartment. However, there was an increase in the likelihood of a cell being positively labelled across all crypt positions again in a gene dose dependent manner (Figure 3.5E).

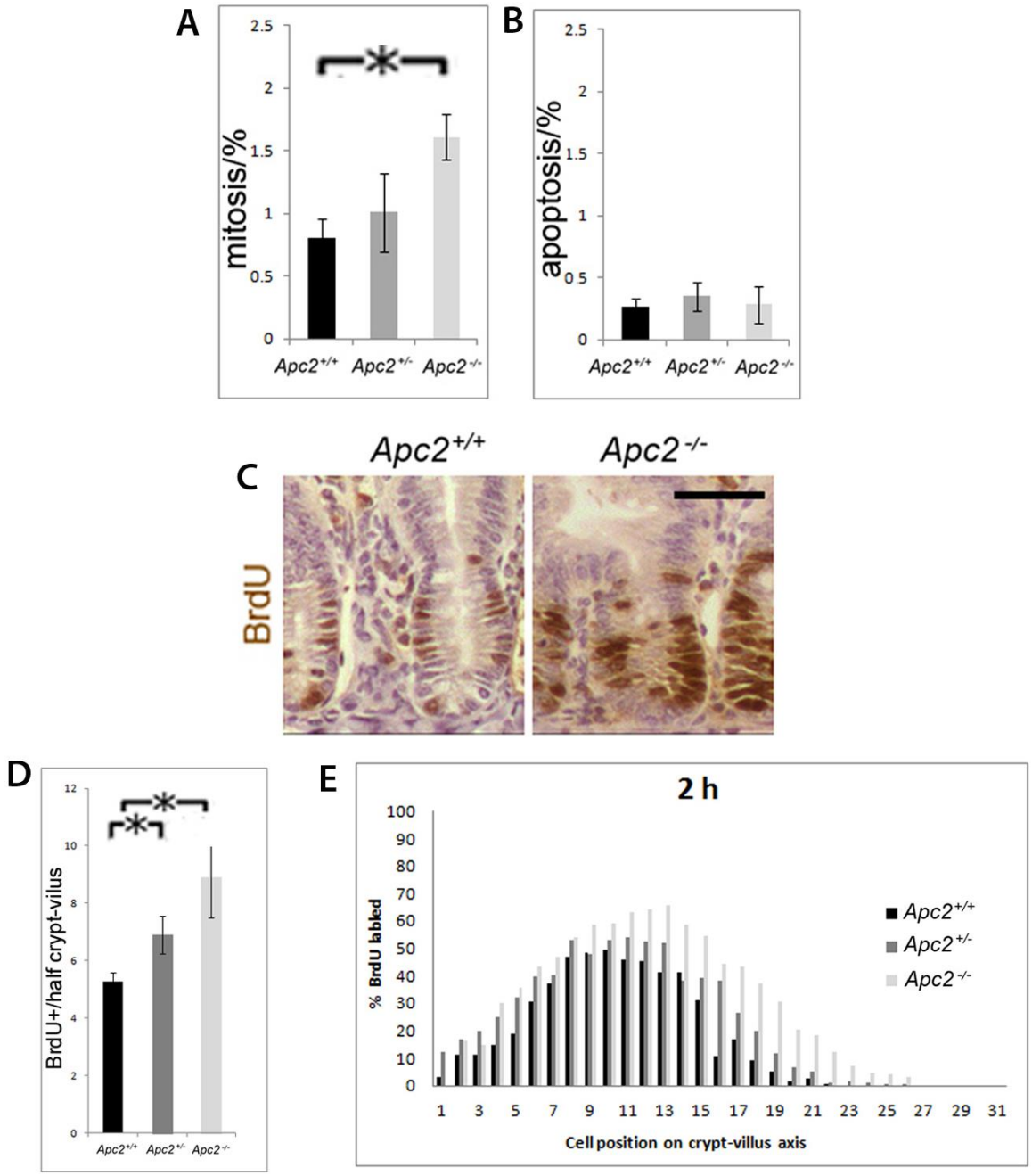

Figure 3.5 Loss of Apc2 caused an increase in proliferative index. Sections of aged matched *Apc2*$^{+/+}$, *Apc2*$^{+/-}$ and *Apc2*$^{-/-}$ intestines were stained with H&E and scored for epithelial incidence of mitotic figures and apoptotic fragments (see methods). (**A**) Quantitative analysis between *Apc2*$^{+/+}$, *Apc2*$^{+/-}$ and *Apc2*$^{-/-}$ intestinal sections showed a statistical increase in mitotic index between *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n=5). (**B**) Apoptosis was unaltered following Apc2 loss (error bars represent standard deviation, p>0.2, Mann-Whitney *U*-test, n≥4). (**C**) Aged matched cohorts from each genotype were injected 2h before death with BrdU then sections stained using an anti-BrdU antibody (scale bar represents 50μm). (**D**) Quantitative analysis between *Apc2*$^{+/+}$, *Apc2*$^{+/-}$ and *Apc2*$^{-/-}$ sections revealed a statistical increase in BrdU positive cells (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n≥4). (**E**) Positional analysis suggests an increase in mitosis across all cell positions along the crypt axis in a dose dependent manner.

3.2.5 Loss of Apc2 leads to an increase in intestinal epithelial migration along the crypt-villus axis

In order to analyse the effects of Apc2 loss on epithelial cell migration a BrdU pulse chase experiment was carried out. Cohorts of *Apc2*$^{+/+}$, *Apc2*$^{+/-}$ and *Apc2*$^{-/-}$ mice were injected with BrdU either 2 or 24 h before death (n≥4). BrdU is only bio-available for <2 h but will stay incorporated inside dividing cells effectively 'tagging' them, this allowed us to determine position of labelled cells following 24 h allowed migration and compare them to that of 2 h (Figure 3.6A). In order to quantify migratory ability of the epithelial cells in Apc2 deficient and *Apc2*$^{+/+}$ intestine, positions of BrdU labelled cells (Figure 3.6B) were scored. Intestinal epithelial cells migrate further/faster up the crypt-villus axis in *Apc2*$^{+/-}$ and more so in *Apc2*$^{-/-}$ mice compared to *Apc2*$^{+/+}$ mice, again indicating gene dose dependency. Plotting cumulative distribution represented graphically (Figure 3.6C) clearly demonstrated that Apc2 deficiency effects the normal distribution after 24 h of migration (Kolmogorov-Smirnov Z test p≤0.05 between *Apc2*$^{-/-}$ and *Apc2*$^{-/-}$ intestines).

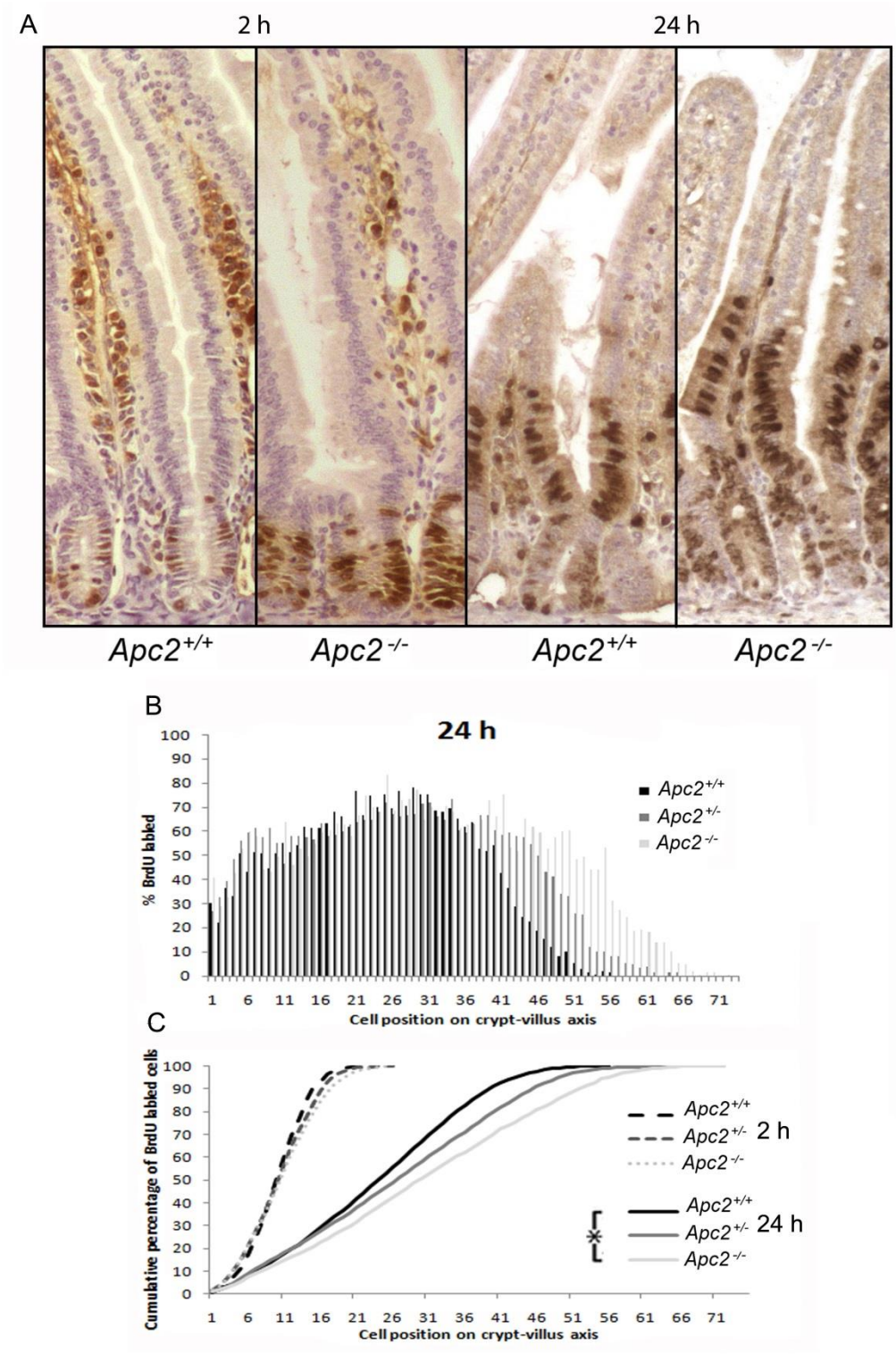

Figure 3.6 Loss of Apc2 led to an increase in cell migration along the crypt-villus axis. (**A**) Anti-BrdU stained intestinal tissue *Apc2*$^{+/+}$ vs *Apc2*$^{-/-}$, 2h and 24h after BrdU injection (**B**) Indicates cell position on crypt-villus axis vs percent chance BrdU positive, following 24h allowed migration (*Apc2*$^{+/+}$, *Apc2*$^{+/-}$ and *Apc2*$^{-/-}$). Note that cells deficient in Apc2 migrate further up the crypt-villus axis in a dose dependent manner. (**C**) Cumulative frequency plot of BrdU positive cells again show differences in BrdU positive cell distribution following 24h allowed migration (asterisk marks p≤0.05, Kolmogorov-Smirnov Z test, n≥4).

3.2.6 Apc2 loss leads to an increase in the number of crypt cells displaying nuclear localised β-catenin

As β-catenin is a key mediator of Wnt signaling, involved with phenotypes associated with intestinal Apc loss and can be negatively regulated by both Apc proteins, β-catenin status was assessed in intestines deficient for Apc2. Analysis of total levels of β-*catenin* mRNA expression using quantitative real-time PCR (qRT-PCR) revealed no global intestinal expression differences between any genotypes (Figure 3.7A, for all comparisons $p>0.2$, Mann-Whitney *U*-test, $n\geq4$). Intestinal sections from $Apc2^{+/+}$ and $Apc2^{-/-}$ mice were stained using an anti-β-catenin antibody to assess protein location (Figure 3.7B). β-catenin remained exclusively cytoplasmic or membrane associated in villus cells in $Apc2^{-/-}$ intestines. This contrasts to the situation observed in Apc deficient intestines (Sansom et al., 2004; Andreu et al., 2005), where cells display nuclear β-catenin regardless of crypt or villus position. However, a statistically significant increase in the number of cells possessing nuclear localised β-catenin did occur in the compartment towards the base of the crypt, in $Apc2^{-/-}$ crypts compared to $Apc2^{+/+}$ ($Apc2^{+/+}$ 2.32±0.47 and $Apc2^{-/-}$ 4.07±0.62 positive cells per crypt, $p\leq0.03$, Mann-Whitney *U*-test, $n\geq4$, Figure 3.7C).

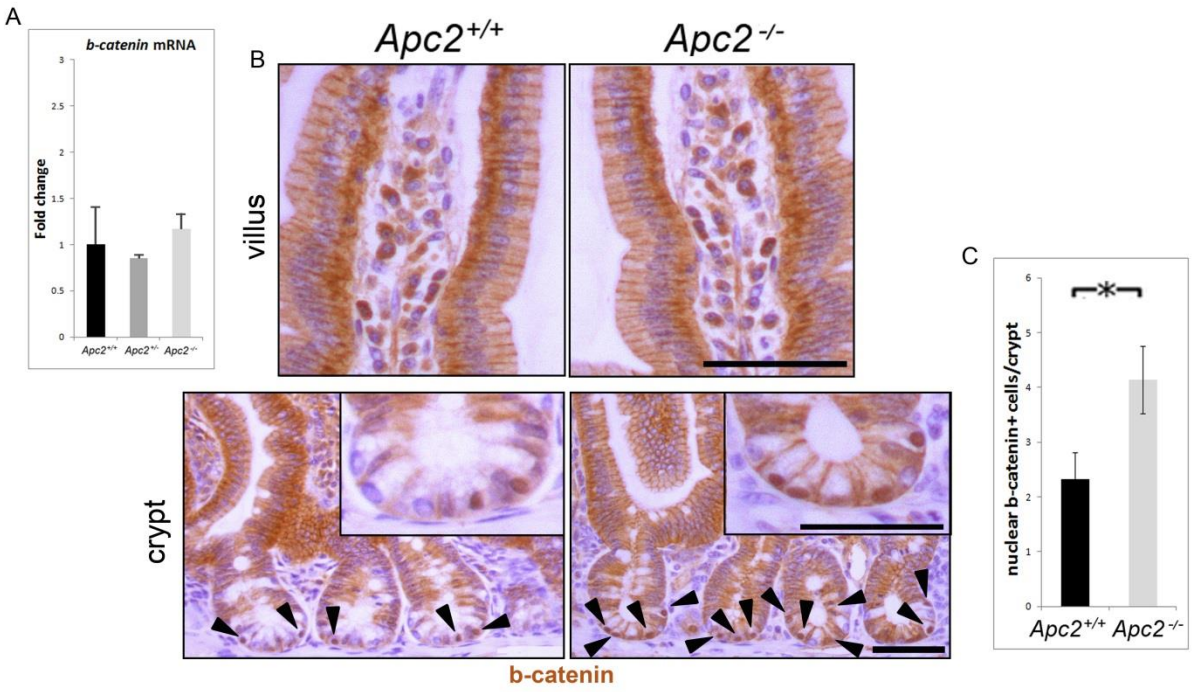

Figure 3.7 Increased nuclear translocation of the Wnt transduction mediator β-catenin resulting from loss of Apc2. (**A**) Quantitative RT-PCR revealed no difference in total expression levels of β-catenin between genotypes ($p>0.2$, Mann-Whitney *U*-test, $n\geq4$, error bars represent SEM of C_t values). (**B**) Sections from *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ intestines were stained with an anti- β-catenin antibody. β-catenin remained cytoplasmic/ membrane associated and indifferent from *Apc2*$^{+/+}$ within the villus region, following Apc2 loss. However, within the crypt region, more cells displayed nuclear localisation of β-catenin (black arrowheads indicate nuclear localised β-catenin, scale bar represents 50μm). (**C**) Quantitative analysis of cells displaying nuclear β-catenin per crypt revealed a statistical increase following Apc2 loss (error bars represent standard deviation, asterisk marks $p\leq0.01$, Mann-Whitney *U*-test, $n\geq4$).

3.2.7 Apc2 regulates intestinal Wnt signaling

To determine if Apc2 loss altered gene expression regulated by Wnt signaling within the intestinal epithelium, quantitative analysis of expression levels of a subset of Wnt target genes was performed. Total RNA from intestinal epithelial samples was extracted, subjected to reverse transcription and then compared using quantitative real-time PCR (qRT-PCR). *Apc2*$^{-/-}$ and *Apc2*$^{+/-}$ expression levels were directly compared to that of aged matched *Apc2*$^{+/+}$ samples (Figure 3.8). *Cyclin D1*, *Axin2*, *MIF* and *WIF1* displayed a statistical increase in expression in *Apc2*$^{-/-}$ compared to *Apc2*$^{+/+}$ control epithelia whilst *cMyc* and *CD44* also display a trend towards an increase, while *Ephrin B2* and *Ephrin B3* remained unaltered.

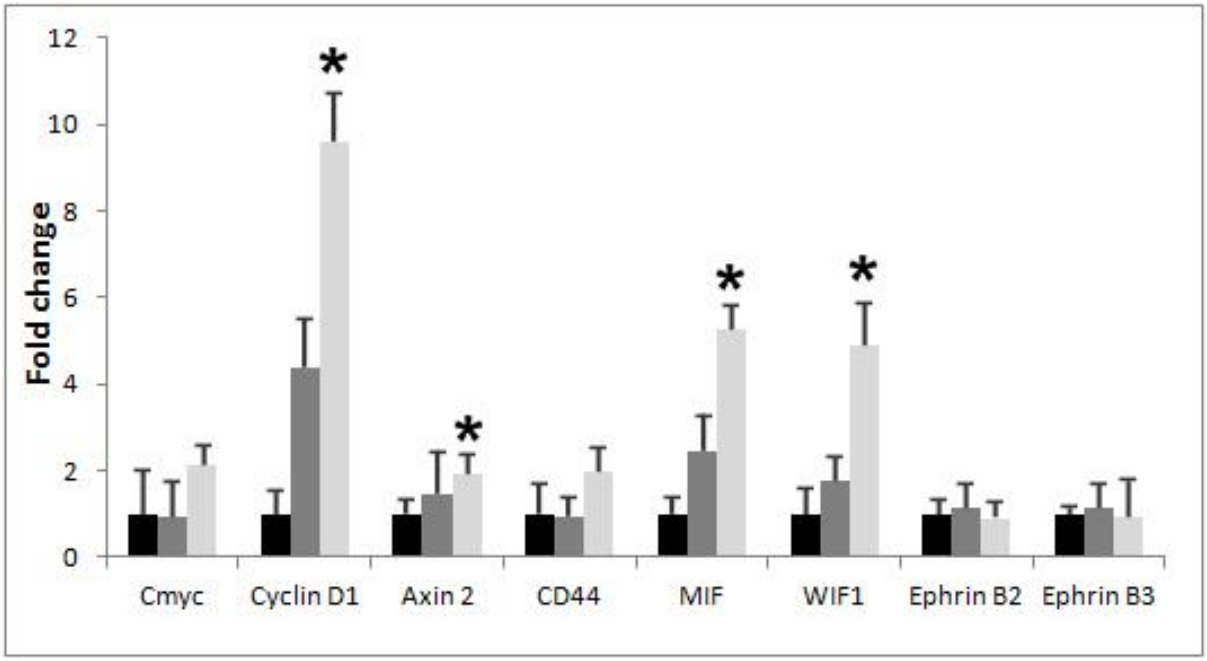

Figure 3.8 Apc2 loss activated Wnt target gene expression. Quantitative RT-PCR was used to compare expression levels of a panel of known Wnt target genes between *Apc2*$^{+/+}$ (black bars), *Apc2*$^{+/-}$ (dark gray bars) and *Apc2*$^{-/-}$ (light gray bars) intestinal epithelial extracts. Most genes showed a trend towards an increase in expression in a gene dose dependent manner excluding the Ephrins (asterisks mark the comparisons that were found to be significantly different from *Apc2*$^{+/+}$, p≤0.05, Mann-Whitney *U*-test, n≥5, error bars represent SEM of C_t values).

3.2.8 Apc2 plays a role in regulating intestinal stem cell populations

Given Wnt signalling plays a fundamental role in intestinal stem cell regulation (for review see Reya & Clevers, 2005) and markers for 'stemness' are increased following intestinal Apc loss (Jubb et al., 2006; Merlos-Suarez et al., 2011), analysis of the stem cell populations in intestines deficient for Apc2 was performed. Quantitative analysis examining the levels of known intestinal stem cell (ISC) markers; *Ascl2*, *Lgr5* and *Bmi1*, was performed using qRT-PCR (Figure 3.9A). Expression levels of *Ascl2* and *Lgr5* was elevated in intestines deficient for Apc2 (statistically significant between *Apc2*$^{-/-}$ compared to *Apc2*$^{+/+}$ samples, p≤0.05, Mann-Whitney *U*-test, n≥4). Conversely, *Bmi1* expression was significantly decreased (between *Apc2*$^{+/-}$ compared to *Apc2*$^{+/+}$, p≤0.05, Mann-Whitney *U*-test, n≥4). In order to assess localisation of ISC populations following loss of Apc2, '*in situ*' hybridisation analysis of *Ascl2* and *Olfm4* expression was carried out (performed by M. Young). Small intestinal sections from *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ intestines were stained using anti-*Ascl2* and anti-*Olfm4*

riboprobes (Figure 3.9B). Although an increase in staining intensity for both probes was apparent in *Apc2*$^{-/-}$ crypts compared to *Apc2*$^{+/+}$ controls, labelled cells still resided in the usual stem cell compartment towards the crypt base. The increase in staining intensity could represent either a higher percentage of positive cells or a comparable ISC number with increased ISC marker expression.

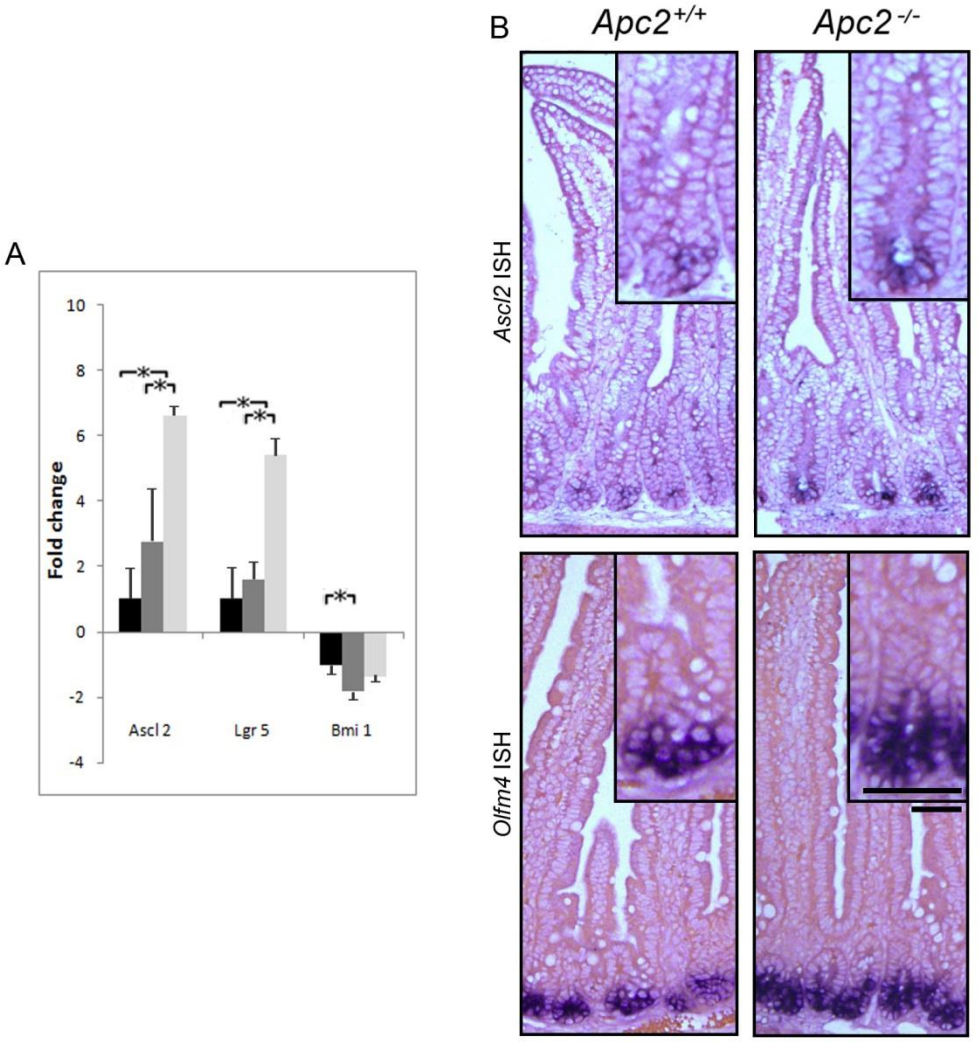

Figure 3.9 Apc2 loss modulates intestinal stem cell markers. (**A**) Quantitative RT-PCR expression analysis revealed a significant up-regulation of the stem cell markers *Ascl2* and *Lgr5* in the small intestinal epithelium of *Apc2*$^{-/-}$ (light gray bars) compared to *Apc2*$^{+/+}$ (black bars). *Bmi1* was significantly down-regulated in *Apc2*$^{+/-}$ (dark gray bars) compared to *Apc2*$^{+/+}$ (asterisks mark the pair-wise comparisons that were found to be significantly different, p≤0.05, Mann-Whitney *U*-test, n≥4, error bars represent SEM of C_t values). (**B**) *'In situ'* hybridisation analysis of *Ascl2* and *Olmf4* stem cell markers displayed an increase in staining intensity between *Apc2*$^{-/-}$ compared to *Apc2*$^{+/+}$. However, positive cells resided in the usual stem cell region towards the base of the crypt (scale bar represents 50μm).

3.2.9 Apc2 is dispensable for tumour suppression

Alterations in intestinal proliferation, Wnt signalling and ISC populations have been implicated with the tumour suppressor function of Apc. Given these features are also perturbed in the *Apc2*$^{-/-}$ intestines, long term survival and intestinal tumour burden of *Apc2*$^{-/-}$ was analysed. Cohorts of *Apc2*$^{+/+}$ (n=14) and *Apc2*$^{-/-}$ (n=20) which displayed no signs of hydrocephalus or brain trauma (non-existent beyond 100days of age) were established and aged until signs of ill health or death (Figure 3.10A). Survival analysis of the two cohorts demonstrated no significant differences between them (p=0.47 Log-Rank test, n≥14).

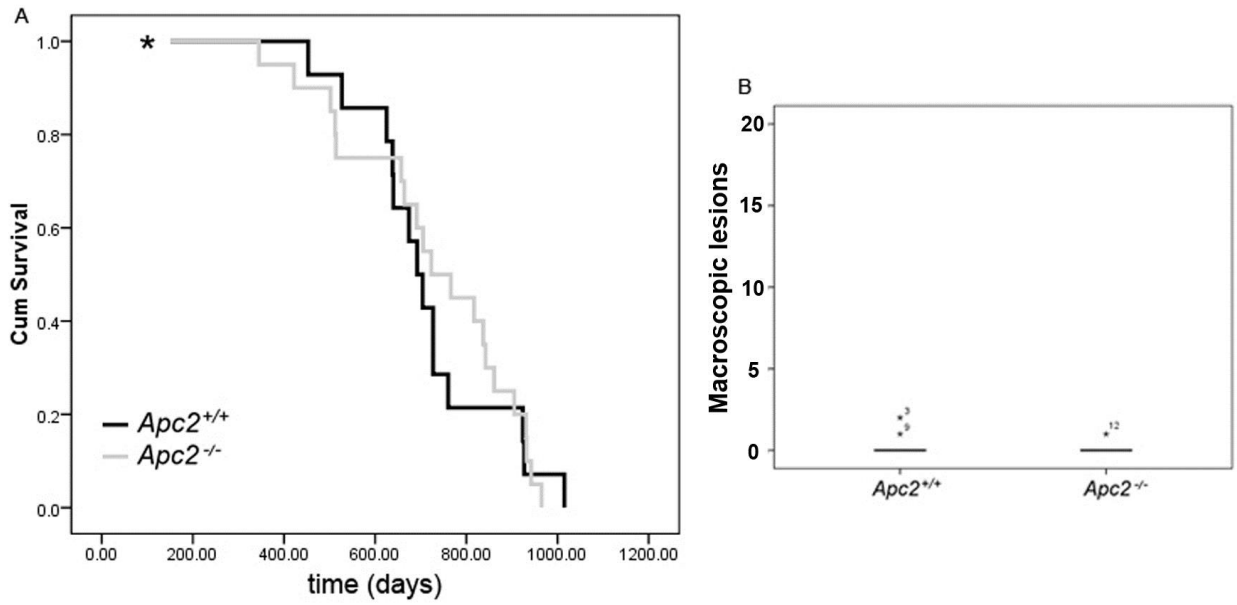

Figure 3.10 Apc2 alone has a limited role in tumour suppression in the murine intestine. (**A**)Cohorts of *Apc2*$^{+/+}$ (black line) and *Apc2*$^{-/-}$ (light gray line) mice were aged and sacrificed upon signs of ill health (survival presented as Kaplan-meier). No significant differences in overall survival were observed (Log-rank test, p>0.4, *Apc2*$^{+/+}$ n=14, *Apc2*$^{-/-}$ n=20). Asterisk indicates start of aging cohorts. No mice developed hydrocephalus beyond this point. (**B**) Macroscopic lesions were quantified in the small and large intestinal epithelium of *Apc2*$^{+/+}$ and *Apc2*$^{-/-}$ animals at time of death. Data are presented as a box plot. Loss of Apc2 did not induce intestinal tumour formation.

Notably, mice from both cohorts that were sacrificed due to ill health rarely displayed symptoms of intestinal tumorigenesis, such as pale feet or faecal blood. Most included symptoms such as piloerection, hunched posture, reduced movement and/or decreased

awareness which could be associated with general old age. Macroscopic tumour scoring revealed intestinal tumours were extremely rare in both genotypes (Figure 3.10B). Rare macroscopically identified intestinal tumours were usually identified microscopically as lymphoma in both cohorts. It was not possible to provide a precise diagnosis for the cause of death in the majority of cases, however, occasional non-tissue specific sporadic tumours and Lymphoma occurred with equal frequency in both cohorts. This, taken together with no overall lifespan changes in $Apc2^{-/-}$ mice, suggests Apc2 alone is dispensable for tumour suppressor function.

3.3 Discussion

Whilst APC's role as a tumour suppressor is undisputed, evidence implicating APC2 in tumourigenesis is mounting. APC2 has been shown to complement APC loss in human colon carcinoma cells (Nakagawa et al., 1998; Van Es et al., 1999). Further evidence includes epigenetic silencing, allelic imbalance, LOH and/or protein loss of APC2 extensively reported in various human cancer cell lines and primary tumours (Jarrett et al., 2001; Sobottka et al., 2000; Yang et al., 2004; Wang et al., 1999; Nakagawa et al., 1999; Schuebel et al., 2007; Hsieh et al., 2007; Chan et al., 2008; Montgomery et al., 2008; Mokarram et al., 2009; Kumar et al., 2009). Our own examination of human CRC samples revealed a reduction in *APC2* expression. However, it is important to note that our study, as is the case in most other studies, examined only advanced cancers, within which it is difficult to distinguish the importance and contribution of APC2 loss to tumour initiation / progression. As yet, it is unclear whether APC2 loss occurs as a passive event contributing little to tumourigenesis, or whether it possesses an initiating oncogenic potential or involvement through advancing tumour progression. Further studies to help delineate APC2's role in tumourigenesis would include expression levels correlated with cancer stage.

Apc has been extensively analysed and studies suggest the majority of its functions lie with its ability to regulate Wnt signaling (Hanson et al., 2005; Nathke, 2005; Polakis, 2000). Although Apc2 shares this characteristic (Nakagawa et al., 1998; Van Es et al., 1999) its importance in mammalian intestinal homeostasis has never been addressed. Apc heterozygosity (Apc^{min} mouse) mimics, to some extent, human mutation where mice are

predisposed to intestinal tumours (Moser et al., 1990). Immediate intestinal loss of Apc achieved through Cre-lox technology causes lethality within a few days. Small intestinal morphology is altered where villus cells display a 'crypt like' phenotype, parameters such as cell differentiation, division and migration are perturbed (Sansom et al., 2004; Andreu et al., 2005) and stem cell populations increased (Jubb et al., 2006; Merlos-Suarez et al., 2011). In this chapter I wished to pursue two aims by utilizing a transgenic mouse harbouring an *Apc2* mutation; Foremost, to elucidate Apc2's role in mammalian intestinal homeostasis focusing on above parameters usually altered following Apc loss and secondly to test if Apc2 loss can induce tumorigenesis.

Mice homozygous for constitutive *Apc2* mutation, did not die '*in utero*' and were viable, in stark contrast to that observed with *Apc* homozygous mutation (Moser et al., 1995). This demonstrated that fully functional Apc2 containing β-catenin and axin sites is dispensable for mouse development. However, a proportion of our *Apc2*$^{-/-}$ mice did develop hydrocephalus (unpublished data). It has been long known that Apc2 is highly expressed in the central nervous system (Yamanaka et al., 2002) with highest levels detected during foetal brain development (Van Es et al., 1999). Other studies have demonstrated an essential role for Apc2 in both foetal chick (Shintani et al., 2009) and mouse brain (Shintani et al., 2012). However, these studies utilised models with complete Apc2 loss as opposed to a truncation mutation as used in our study. It would be interesting to test the relevance of each domain of the Apc2 protein in regards to brain development. Some strained or incomplete functional redundancies relating to β-catenin and axin binding may exist between Apc homolog's in brain development, this could account for why only a proportion of our *Apc2*$^{-/-}$ mice developed hydrocephalus. Indeed, Apc is also highly expressed in the central nervous system during the embryonic period (Bhat et al., 1994) and deficiency can lead to neurological defects (Yokota et al., 2009; Ivaniutsin et al., 2009) which correlate with defects noted in brain specific β-catenin mutations (Chenn and Walsh, 2003; Wrobel et al., 2007; Machon et al.,2003). The role Apc proteins play in brain development and if functional redundancies exist are currently being pursued in collaboration with others. As surviving *Apc2*$^{-/-}$ mice appear healthy, do not display altered brain morphology, changes in behaviour or altered lifespan, we assume minimal brain defects unlikely to alter intestinal homeostasis or pathology.

Following Apc2 loss, mice did not display alterations in either macroscopic intestinal morphology or microscopic crypt-villus architecture and appeared healthy. Composition/ position of terminally differentiated cell types remained unaltered as did levels of apoptosis. Also, Apc2 loss did not impede intestinal cell migration, surprisingly it was increased in *Apc2*$^{-/-}$ mice (discussed in more detail later in discussion). These observations highlight major differences between the two Apc homologs in intestinal homeostasis. However, Apc2 loss did alter some attributes such as cell division and markers of intestinal stemness suggestive of an overlapping role in mediating Wnt signaling.

Analysis of the small intestinal epithelia between genotypes revealed that Apc2 does play a role in Wnt signal transduction. Although villus cells still retained membrane bound/ cytoplasmic β-catenin, in contrast to that observed following Apc loss (Sansom et al., 2004; Andreu et al., 2005), there was an increase in crypt cells displaying nuclear β-catenin. A likely explanation for this may lie with differential affinities for β-catenin destruction between Apc homologs (Nakagawa et al., 1998). Whilst Apc loss leads to nuclear translocation of β-catenin in both crypt and villus epithelia, villus cells are less sensitive to Wnt compared to crypt cells and Apc2 loss may lead to an increase in free β-catenin only able to surpass a threshold viable for nuclear translocation within the crypt region. In order to further test Apc2's effect in modulating Wnt signaling, examination of expression levels of known downstream gene targets of β-catenin/Wnt activation was carried out. Indeed, a subset of Wnt target genes was found to be up-regulated in Apc2 deficient epithelial cell extracts. *Cyclin D1*, *Axin2*, *MIF* and *WIF1* all displayed a statistical increase in expression, along with *cMyc* and *CD44* also displaying this trend. Interestingly, *Ephrin B2* and *Ephrin B3* were found to avoid this up-regulation, differing from a pattern normally seen following Apc loss (Sansom et al., 2004), the significance of this difference is discussed later.

Differences in Wnt gene expression could either indicate differential methods of Wnt repression between Apc homologs, for instance, Apc has been shown uniquely to directly block transcription of some Wnt target genes within the nucleus (Sierra et al., 2006) or that different levels of active β-catenin may activate particular Wnt target genes rather than homogenously activating the entire Wnt transcriptional program. Evidence towards the latter explanation include experiments that conclude that the location of Apc or Apc2 does not affect Wnt regulatory ability or rescue (Roberts et al., 2012) and different degrees of β-

catenin accumulation, achieved either directly (Hirata et al., 2012) or through different severities of Apc mutation (Kielman et al., 2002), can induce different patterns of Wnt target gene expression. It is important to note that whilst different levels of active β-catenin achieved through either Apc or Apc2 loss is a reasonable explanation for differences in Wnt target gene expression, we cannot rule out other possibilities, some of which could include; increases in expression or sensitivity of Wnt receptors occurring through developmental compensation, more effective Wnt repression/repressors or even indirect influences of non-epithelial tissue also deficient for Apc2.

Loss of Apc2 led to an increase in cell division. This can be explained through increased expression of Wnt target genes, some of which are renowned for inducing division. Also, coupled with increased Wnt signaling is an intestinal 'stemness' signature. Wnt signaling is imperative for intestinal stem cell renewal (Fevr et al., 2007) whilst over-activated Wnt, through Apc deletion, leads to an increase in stem cell marker expression (Jubb et al., 2006; Merlos-Suarez et al., 2011). Apc2 loss demonstrated increased expression of intestinal stem cell markers in our study. It came as no surprise that Apc2 had the ability to alter stem cell dynamics as previous work on *drosophila* revealed an intracellular role for Apc2 in stem cell renewal (Yamashita et al., 2003), and stable expression of APC2 in a human colon adenocarcinoma cell line (HT29) substantially reduced expression of the intestinal stem cell marker *Ascl2* (Jubb et al., 2006).

Importantly, whilst *Ascl2* and *Lgr5* expression was increased in Apc2 deficient intestinal epithelia, *Bmi1* was down-regulated. Whereas *Lgr5* marks a crypt base columnar stem cell population (Barker et al., 2007), *Bmi1* is thought to label a distinct sub-population of intestinal stem cells at the +4 region (Sangiorgi & Capecchi, 2008), although controversy exists as to whether these are true distinct populations (Barker et al., 2012; Munoz et al., 2012). Recent experiments demonstrate interplay between these two sub-populations whereby $Lgr5^{+}$ indicates a mitotically active population, which upon ablation or damage can be replaced by a normally quiescent $Bmi1^{+}$ population (Tian et al., 2011; Yan et al., 2012). Only the $Lgr5^{+}$ and not the $Bmi1^{+}$ intestinal stem cell population are sensitive to Wnt perturbations (Yan et al., 2012) which may explain why increases are only noticed in *Lgr5*

expression in Apc2 deficient intestines. The noticed decrease in *Bmi1* expression may specifically reflect a balance between *Lgr5*$^{+}$ and *Bmi1*$^{+}$ populations. In *Apc2*$^{-/-}$ intestines, cells positive for stem cell markers still resided in the usual crypt base position. Some studies suggest paneth cells constitute the important intestinal stem cell niche (Sato et al., 2011; Parry et al., 2013), whilst some argue they are dispensable for stem cell function (Kim et al., 2012). Mislocalisation of paneth cell associated with intestinal Apc loss (Sansom et al., 2004; Andreu et al., 2005) could contribute to abnormal stem cell labelling outside the crypt base position. Although markers of intestinal stemness increase in *Apc2*$^{-/-}$ intestines, correct location could be attributed to correctly positioned paneth cells.

Interestingly, although Apc2 loss possesses the ability to alter some attributes consistent with Apc loss, some parameters remained unaltered. Small intestinal morphology was unaltered as was terminally differentiated cell composition/position. As mentioned previously, this highlights major differences between the two Apc homologs in intestinal homeostasis. Correct intestinal architecture and number/position of physiologically relevant cell types could explain why *Apc2*$^{-/-}$ mice remain healthy and display no intestinal pathology. Mechanistically these differences could be explained a number of ways. Foremost, differential affinities for β-catenin destruction between Apc and Apc2 may affect either different regions or cells with different required threshold levels needed for Wnt induction or these different 'levels' may induce differential Wnt target gene programs. Interestingly, *Ephrin B2* and *Ephrin B3* were altered following Apc loss (Sansom et al., 2004) but unchanged in *Apc2*$^{-/-}$ intestines. Disruption of these ephrins has profound effects on cell differentiation and sorting/positioning (Batlle et al., 2002) and are proposed as a mechanism for some phenotypes associated with intestinal Apc loss (Sansom et al., 2004). Differences in gene target expression between the Apc homologs, with the ephrins being one example, could account for phenotypic differences. To further test this assumption microarray analysis could be employed to identify global gene expression differences between the Apc homologs.

One of the most interesting findings from this study was that Apc2 loss increased intestinal cell migration. This is in stark contrast to that observed following intestinal Apc loss where epithelial cell migration is abrogated (Sansom et al., 2004; Andreu et al., 2005). It is known that Apc has a prominent role in cell migration (reviewed in Etienne-Manneville, 2009) and this role can occur independent of β-catenin regulatory ability (Nelson et al., 2012). Loss of migratory ability may highlight a Wnt independent function unique to Apc. Although Apc2 has been suggested to have a role in directional neuronal migration (Shintani et al., 2012), our study shows that directional intestinal migration is not impaired. Intestinal epithelial cells deficient for Apc2 still migrate directionally in the same manner as control tissues albeit faster. An explanation as to why Apc2 deficient cells migrate faster may simply be as a consequence of altered cell density. The intestinal epithelial cells consist of a polarised monolayer and more epithelial cells being 'born' through increased cell division, could lead to cells being forced further up the crypt-villus axis. Although intestinal cell migration can still occur independent of cell division (Kaur & Potten, 1986) other models have shown reduced intestinal proliferation correlates with reduced migration (Simmen et al., 2007).

Upon aging mice from each genotype we can see that Apc2 loss does not induce intestinal tumour formation, or indeed tumour formation in any other tissue. This answers a very important question and suggests that Apc2 loss does not have initiating oncogenic potential. From these experiments we can hypothesise that the reduced APC2 expression noticed in human cancer is more than likely linked to tumour progression rather than initiation. As both Apc and Apc2 have the ability to alter intestinal Wnt signaling and homeostasis but only Apc loss has the potential to initiate tumour formation, it allows us to dissect out roles of Apc unique to tumour formation. Interestingly, increases in intestinal Wnt signalling, proliferation, and intestinal stem cell markers through Apc2 loss are not enough to disrupt intestinal physiology or induce tumour formation. However, this may just be a consequence of a weaker Wnt signal achieved with Apc2 loss compared to that of Apc. It is also known that Apc's role in tumour suppression is multifunctional (reviewed by Aoki and Taketo, 2007; Senda et al., 2007). Perhaps some of the non-canonical functions of Apc (Fodde et al., 2001; Kaplan et al., 2001; Näthke, 2006; Prosperi et al., 2009; Qian et al., 2008; Harris & Nelson, 2010) may be unique to Apc and play a more substantial role than previously thought.

Migratory ability may underline an essential and important anti-tumour quality in intestinal epithelium only abrogated following Apc loss. Micro-adenomas in *Apc*^{min} mice which progress to adenomas, initiate from a lack of directed cell migration (Oshima et al., 1997). These adenomas have gained a second mutation such that both copies of Apc are compromised. Several studies have suggested an overall reduction in intestinal migration in *Apc*^{min} mice is a contributing factor to tumour formation (Mahmoud et al., 1997; Javid et al., 2004). Interestingly, *Apc2*$^{-/-}$ intestinal epithelia which have retained migratory ability still maintain homeostasis although Wnt is perturbed and factors such as cell division are increased. This retained directional migration may substantially influence the lack of pathology noticed in *Apc2*$^{-/-}$ intestines.

To conclude, the findings presented here suggest that Apc2 does have a role in intestinal homeostasis through mediation of Wnt signaling. Loss of Apc2 can induce transcription of some Wnt target genes in a subset of cells which, in turn, can lead to increases in cell division and/or markers of stemness. Alone, Apc2 loss is not enough to induce tumorigenesis. This study highlights important attributes of Apc loss, such as lack of migration, unique to tumour formation.

Chapter 4

Investigating the effects of Apc2 loss in the context of intestinal *Apc* mutation

4.1 Introduction

In the previous chapter it was shown that murine Apc2 loss can partially modify intestinal Wnt signaling and alter the proliferative state of epithelial cells. However, although a number of human studies have linked APC2 loss with colorectal tumorigenesis (Schuebel et al., 2007; Hsieh et al., 2007; Chan et al., 2008; Mokarram et al., 2009; Kumar et al., 2009), Apc2 loss alone was insufficient to induce tumourigenesis. Perturbations of the Wnt signaling pathway occur in more than 90% of all human colorectal cancers (Giles et al., 2003) making it an attractive pathway for therapeutic intervention. Nevertheless, Wnt targeted therapies remain scarce and total understanding of the pathway remains incomplete. While both APC proteins share the ability to negatively regulate Wnt and both are highly evolutionally conserved (Van Es et al., 1999) only APC has been extensively studied in either humans and animal models of colorectal cancer (for review see Klaus & Brirchmeier, 2008). This in part may be due to the fact that APC2 was discovered relatively recently to APC and mutations in *APC2* are seen less frequently in cancer. Importantly, epigenetic silencing through promoter hypermethylation is now a highly accepted process of gene expression loss in cancer (reviewed in Herman and Baylin, 2003; Jones and Baylin, 2007) and *APC2* hypermethylation occurs remarkably frequently, being seen in 90-100% of human colorectal tumours (Chan et al., 2008; Mokarram et al., 2009; Kumar et al., 2009).

Although murine Apc2 alone loss is insufficient to induce tumourigenesis it has the potential to alter some attributes consistent with Apc loss. The dual influence of Apc and Apc2 in intestinal Wnt signaling, proliferation and stemness suggest some functional redundancies

may exist between mammalian Apc proteins. In *Drosophila*, Apc proteins do display co-operation in negative regulation of Wnt in a variety of tissues (McCartney et al., 1999; McCartney et al., 2006; Ahmed et al., 2002; Akong et al., 2002) and functional redundancies have been exposed through complementary rescue (Kunttas-Tatli et al., 2011; Roberts et al., 2012). In human colorectal cell lines with reduced APC, transfection with APC2 can rescue β-catenin destruction and reduce Wnt-responsive gene transcription (Van Es et al., 1999; Roberts et al., 2012). Together these experiments suggest that functional redundancies are present between Apc proteins and may be evolutionarily conserved. Thus, Apc and Apc2 could potentially co-operate in intestinal Wnt signal transduction.

In order to elucidate dual roles of the Apc proteins and deepen the understanding of this complex pathway in intestinal homeostasis and pathogenesis, transgenic mouse models were used to study concurrent intestinal loss of both Apc proteins. In this chapter I wished to pursue two aims; Foremost to expose any effects of additional Apc2 loss on immediate intestinal homozygous Apc loss, and secondly to delineate any role Apc2 may play in tumourigenesis in the context of a Wnt driven, Apc heterozygous tumour model.

4.2 Results

4.2.1 Induction of *Villin-Cre^{ER} Apc$^{fl/fl}$* mice causes Apc loss in the small intestinal epithelium

To conditionally knockout *Apc* from the intestinal epithelia a Cre/loxP transgenesis system was employed. The *Apc* allele used (*Apc$^{fl/fl}$*) was engineered with two loxP sites flanking exon 14. If exposed to Cre recombinase, exon 14 is spliced out introducing a stop codon to the open reading frame resulting in expression of a truncated, loss of function protein (Shibata et al., 1997). The *Villin-Cre^{ER}* transgenic mouse expresses Cre recombinase

conditionally and specifically in the intestinal epithelium following a tamoxifen induction regime (Colnot et al., 2004; Marjou et al., 2005; Andreu et al., 2005). *Villin-Cre*^{ER} *Apc*$^{fl/fl}$ mice were induced, intestinal sections collected and stained for Apc at different time points following tamoxifen induction. Fluorescent IHC using an antibody raised against the C-terminus end of the Apc protein was used to assess protein status (Figure 4.1). Pre induction (Day 0), Apc was expressed at its highest levels in differentiated villus cells with a decrease in staining intensity toward the crypt and at lowest detectable levels in crypt cells at the crypt base. Apc was mostly cytoplasmic and membrane associated. However, by Day 3 post induction, Apc staining intensity was considerably decreased in most villus cells. It was mostly nuclear associated, some residual membrane bound Apc remained however minimal cytoplasmic staining was apparent. Interestingly, at this time point clumps of crypt cells also expressed nuclear Apc. By Day 4, Apc staining was further reduced and by Day 5 staining was undetectable. Translocation and overall reduction of Apc by Day 3 is consistent with previous studies indicating a phenotypical onset 3 days following induction (Andreu et al., 2005). Progression of phenotype with time noted in these studies may correlate with increased reduction of Apc protein expression.

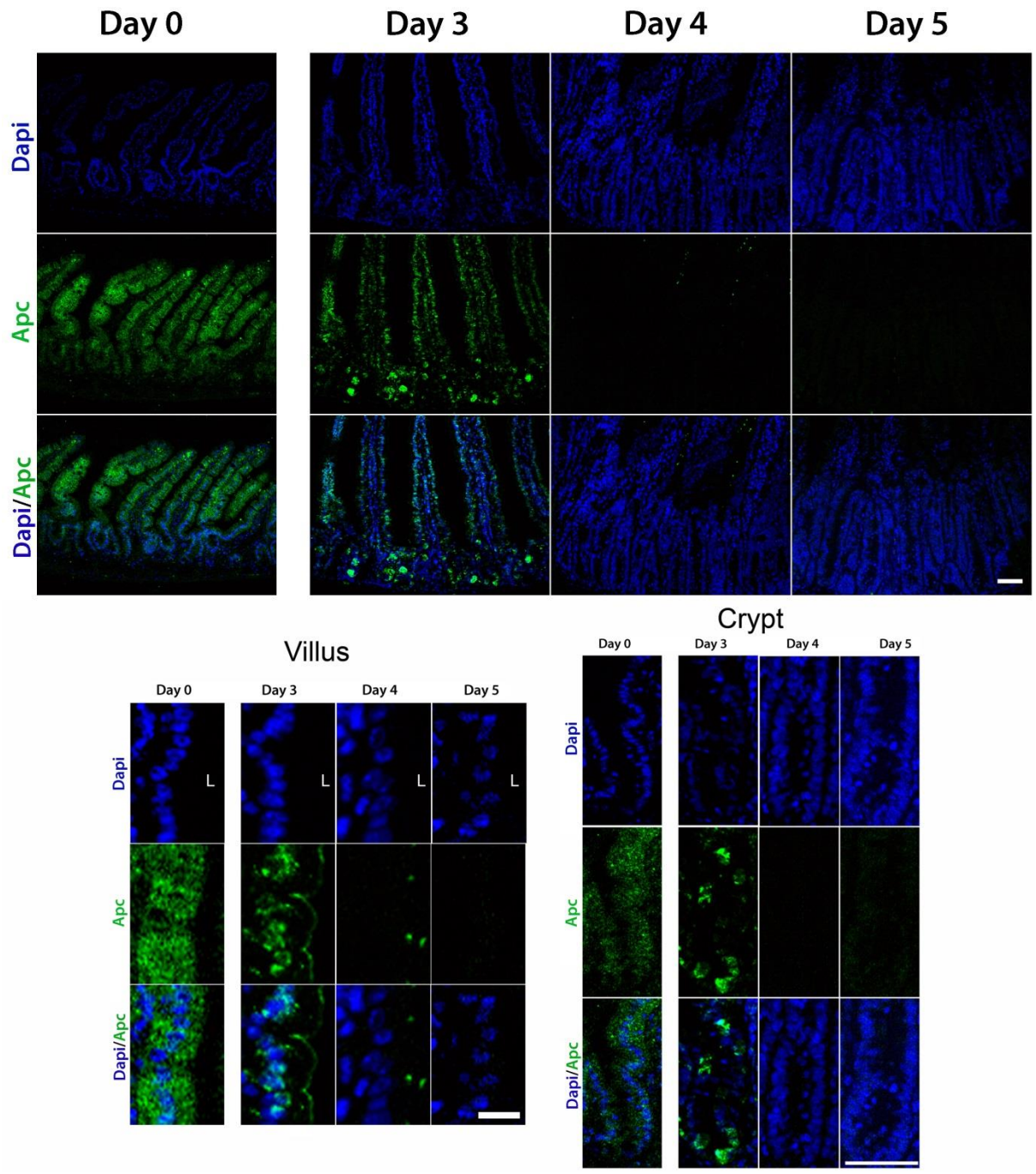

Figure 4.1 Induction of *Villin-Cre*^{ER} *Apc*$^{fl/fl}$ mice caused a loss of Apc protein in the small intestines. Fluorescence IHC using an antibody specific for Apc showed mis-localisation then depletion of Apc protein over time. At **Day 0** Apc was cytoplasmic and/or membrane associated and expressed at its highest intensity in differentiated villus cells. By **Day 3** Apc was partly depleted, expression was now mostly nuclear associated in the villus compartment with occasional clumps of cells displaying expression within the crypt. Through days **4** and **5** it became completely depleted and undetectable in all cells (scale bar represents 50μm). Bottom higher magnification of villus (L=lumen, scale bar represents 10μm) and crypt (scale bar represents 50μm) cells.

4.2.2 Apc2 deficiency delays onset of *Villin-Cre^{ER} Apc$^{fl/fl}$* morbidity

In order to elucidate any functional redundancies or interactions that may exist between Apc and Apc2 in intestinal homeostasis, *Villin-Cre^{ER} Apc$^{fl/fl}$* mice were crossed with *Apc2$^{-/-}$* mice. Cohorts were induced (see methods for regime) and inspected every 6 hours for signs of intestinal morbidity. Whilst un-induced animals and induced *Villin-Cre^{ER-}* animals displayed no signs of pathology over the study period (data not shown), all *Villin-Cre^{ER} Apc$^{fl/fl}$* (referred to as Apcfl hereafter) mice were culled due to signs of ill health with a median survival of 4.5 Days Post induction. Surprisingly, although *Villin-Cre^{ER} Apc$^{fl/fl}$ Apc2$^{-/-}$* (referred to as DKO hereafter) mice also developed signs of ill health, the addition of the Apc2$^{-/-}$ mutation delayed the onset of morbidity (median survival post induction, Apcfl=4.5 Days {n=8} vs DKO=6 Days {n=5}) (Figure 4.2). Overall analysis of survival probability revealed a statistical difference between the two cohorts (Log-rank test, p=0.002).

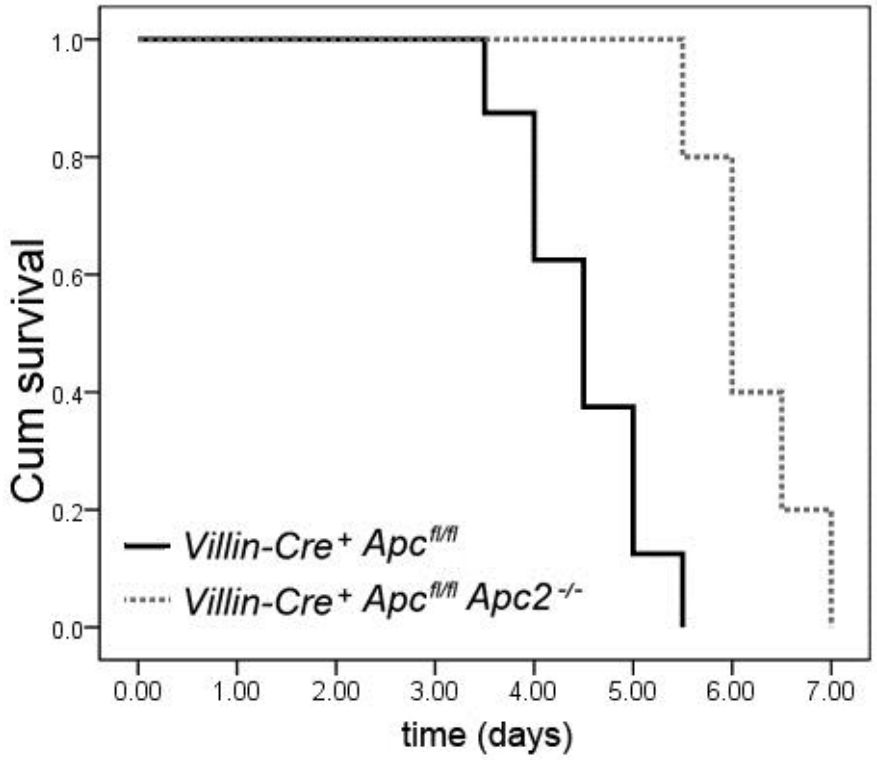

Figure 4.2 Addition of an *Apc2$^{-/-}$* mutation on a *Villin-Cre^{ER} Apc$^{fl/fl}$* background extended short term lifespan. Cohorts of *Villin-Cre^{ER} Apc$^{fl/fl}$* (black line) and *Villin-Cre^{ER} Apc$^{fl/fl}$ Apc2$^{-/-}$* (dotted gray line) mice were aged out and sacrificed upon signs of ill health (survival presented as Kaplan-meier). A significant difference between overall survival was observed between genotypes (Log-rank test, p=0.002, *Villin-Cre^{ER} Apc$^{fl/fl}$* n=8, *Villin-Cre^{ER} Apc$^{fl/fl}$ Apc2$^{-/-}$* n=5).

4.2.3 Additional loss of Apc2 in DKO mice delays/attenuates the 'crypt progenitor' phenotype associated with immediate Apc loss

The 'crypt progenitor' phenotype associated with immediate intestinal Apc loss has been extensively studied previously (Sansom et al., 2004; Andreu et al., 2005). Lack of differentiated intestinal cell types leading to inept water and nutrient uptake, along with increased intestinal 'bulk', either directly compressing/compromising other organs/systems or through indirect means are plausible explanations for health deterioration in mice following Apc loss. To expose any effects of Apc2 loss on the Apcfl crypt progenitor state, cohorts of each genotype were culled at 24h time intervals following induction and intestinal sections stained with H&E (Figure 4.3). The 'Crypt like' region was defined by cell

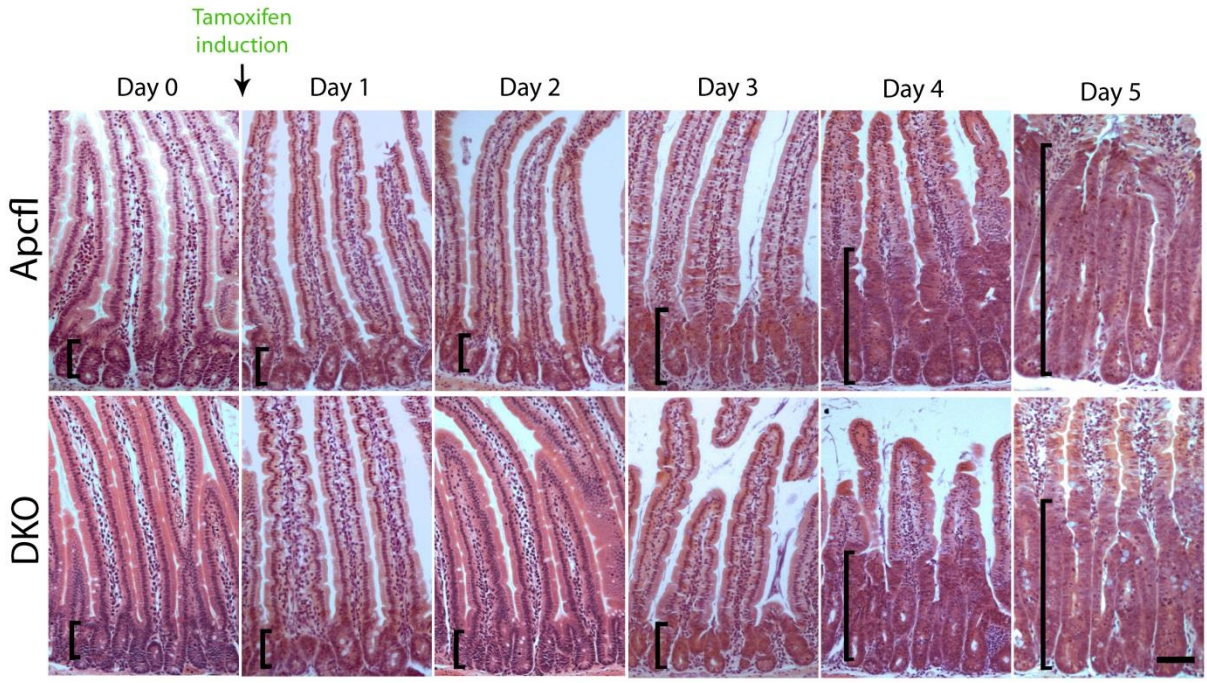

Figure 4.3 Loss of Apc led to a 'crypt progenitor' phenotype, which microscopically appeared delayed and attenuated in DKO. Intestinal sections from each genotype were taken at 24 hour intervals following tamoxifen induction and stained with H&E. Both **Day 1** and **Day 2** appeared phenotypically indifferent from un-induced mice (**Day 0**) in both genotypes. Crypt expansion was noticed in both genotypes through **Days 3-5** however, somewhat delayed and attenuated in DKO intestines. Apcfl (*Villin-Cre^{ER} Apc$^{fl/fl}$*), DKO (*Villin-Cre^{ER} Apc$^{fl/fl}$ Apc2$^{-/-}$*), scale bar represents 50μm.

morphology. In Apcfl intestines, consistent with previous studies (Andreu et al., 2005) both Day 1 & 2 were phenotypically indifferent from Day 0 (un-induced), this was also the case in DKO intestines. Both genotypes then displayed expansion of the 'crypt like' region through days 3 to 5 with an attenuated appearance in DKO intestines. Quantitative analysis of both crypt and total crypt-villus size was carried out and displayed schematically and graphically (Figure 4.4). At Day 3 there was an attenuation of crypt expansion in DKO mice compared to Apcfl intestines (27.40±1.41 vs 34.58±1.50 cell height per half crypt respectively {23.6 average crypt size in un-induced mice}, $p \leq 0.05$, Mann-Whitney *U*-test, n=4). There were no significant differences at Day 4. However, by Day 5 both crypt size (DKO 101.88±6.91 vs Apcfl 141.40±7.41 cell height per half crypt-villus structure {23.6 average crypt size in un-induced mice}, $p \leq 0.05$, Mann-Whitney *U*-test, n=4) and total crypt-villus size (DKO 141.40±7.41 vs Apcfl 101.88±6.91 cell height per half crypt-villus structure {98.6 average crypt-villus size in un-induced mice}, $p \leq 0.05$, Mann-Whitney *U*-test, n=4) were significantly reduced in DKO compared to Apcfl intestines. Taken together these results suggest that additional Apc2 loss attenuates 'crypt progenitor' zone expansion.

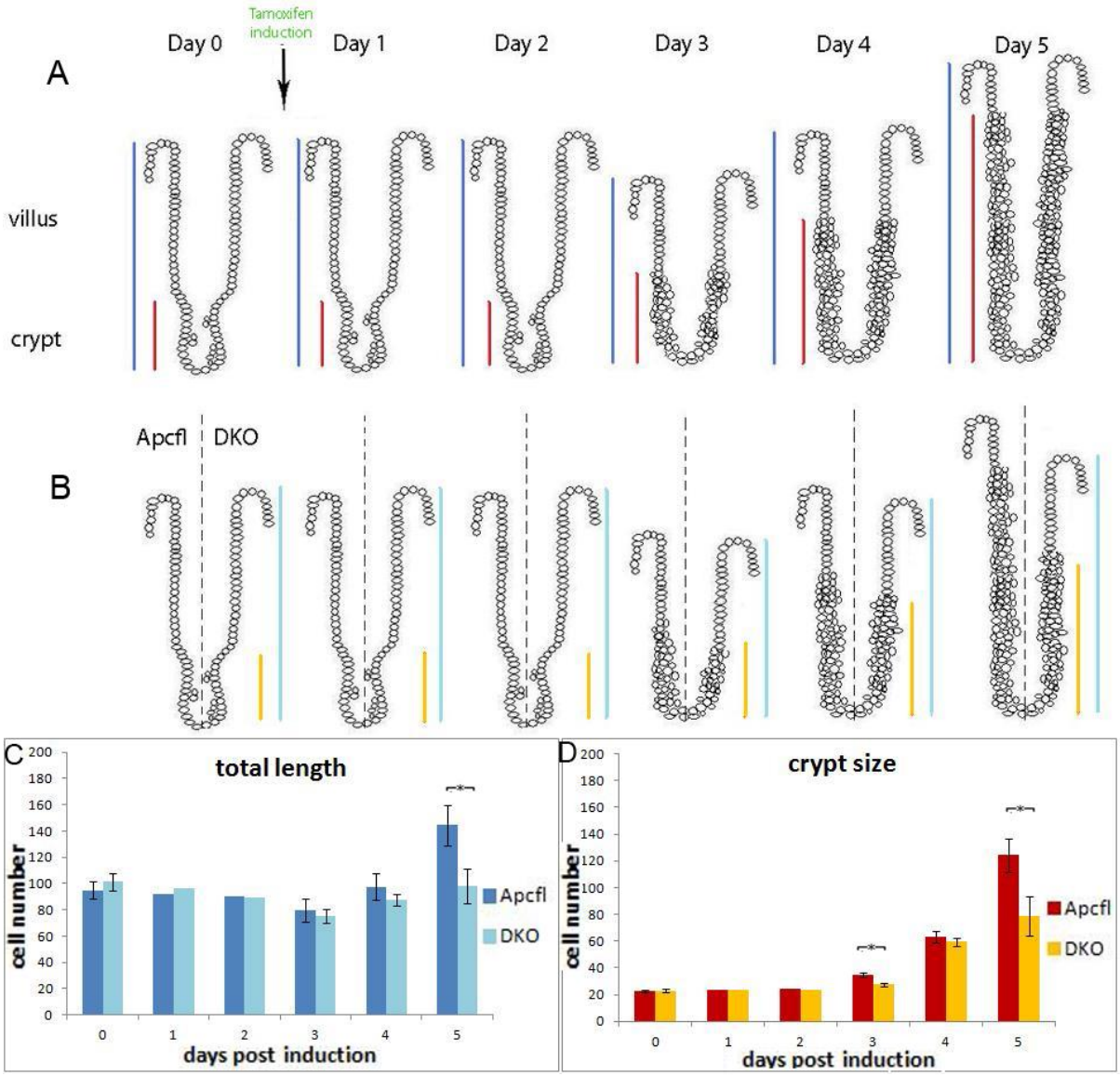

Figure 4.4 In DKO mice the Addition of an *Apc2*$^{-/-}$ mutation delayed and attenuated the crypt progenitor phenotype noticed in Apcfl intestines. Total crypt-villus length and crypt length were scored for each genotype at each time point (n=4 for each comparison). (**A**) Diagram of crypt size and total crypt-villus length for Apcfl over time. (**B**) Diagram demonstrating differences between DKO (right panels) and Apcfl (left panels) over time. Graphical representation of (**C**) total crypt-villus length and (**D**) crypt length. Note delay in increase of crypt size at **Day 3** in DKO intestines, and also statistical reduction of size in both crypt and total crypt-villus length in DKO intestines at **Day 5**. Blue= total crypt-villus length in Apcfl, light blue= total crypt-villus length in DKO, red= crypt length in Apcfl, yellow= crypt length in DKO. Asterisks mark statistically significant pair wise comparison, Mann-Whitney *U*-test, p≤0.05.

4.2.4 Apc2 deficiency alters Wnt pathway changes associated with immediate intestinal Apc loss

It is proposed that the majority of phenotypes associated with intestinal loss of Apc arise due to compromised β-catenin degradation, nuclear translocation and subsequent over activation of Wnt target gene expression (Sansom et al., 2004; Andreu et al., 2005). Apc2 shares this ability to degrade β-catenin (Nakagawa et al., 1998; Van Es et al., 1999) and its loss also activates certain Wnt targets within the intestines (Chapter 3). In order to investigate how additional Apc2 loss influences Wnt signaling in Apcfl intestines, quantitative analysis of Wnt target/repressor gene expression was performed on intestinal samples from DKO animals and compared directly against levels in Apcfl intestines at different time points (n=4). As no phenotype is observed in either genotype at Day 1 or 2 further analyses in this chapter is only carried out on Days 0, 3, 4, & 5. Total RNA was extracted from epithelial enriched samples of the small intestine and subjected to reverse transcription. Quantitative real-time PCR (qRT-PCR) was used to compare expression levels of a subset of Wnt associated genes between genotypes at different time points (Figure 4.5).

The intestinal phenotype of un-induced Apcfl mice was identical to that of *Wt,* consequently comparisons of gene expression levels on Day 0 effectively compare *Apc2*$^{-/-}$ and *Apc2*$^{+/+}$ genotypes (see chapter 3). DKO intestines did not display any significant increases in Wnt target gene expression in comparison to Apcfl at any post induction time point. On Day 3, qRT-PCR revealed reduced levels of Wnt target gene expression in DKO compared to Apcfl intestines for all analysed targets (statistically significant for *cMyc*, *Ephrin B2* and *Ephrin B3*, three out of four targets analysed {3/4}). On Day 4 fewer Wnt targets were significantly reduced in DKO compared to Apcfl intestines (*Cyclin D1 and CD44* {2/6}). By Day 5 there was no significant difference between genotypes in any of the Wnt target genes analysed {0/4}.

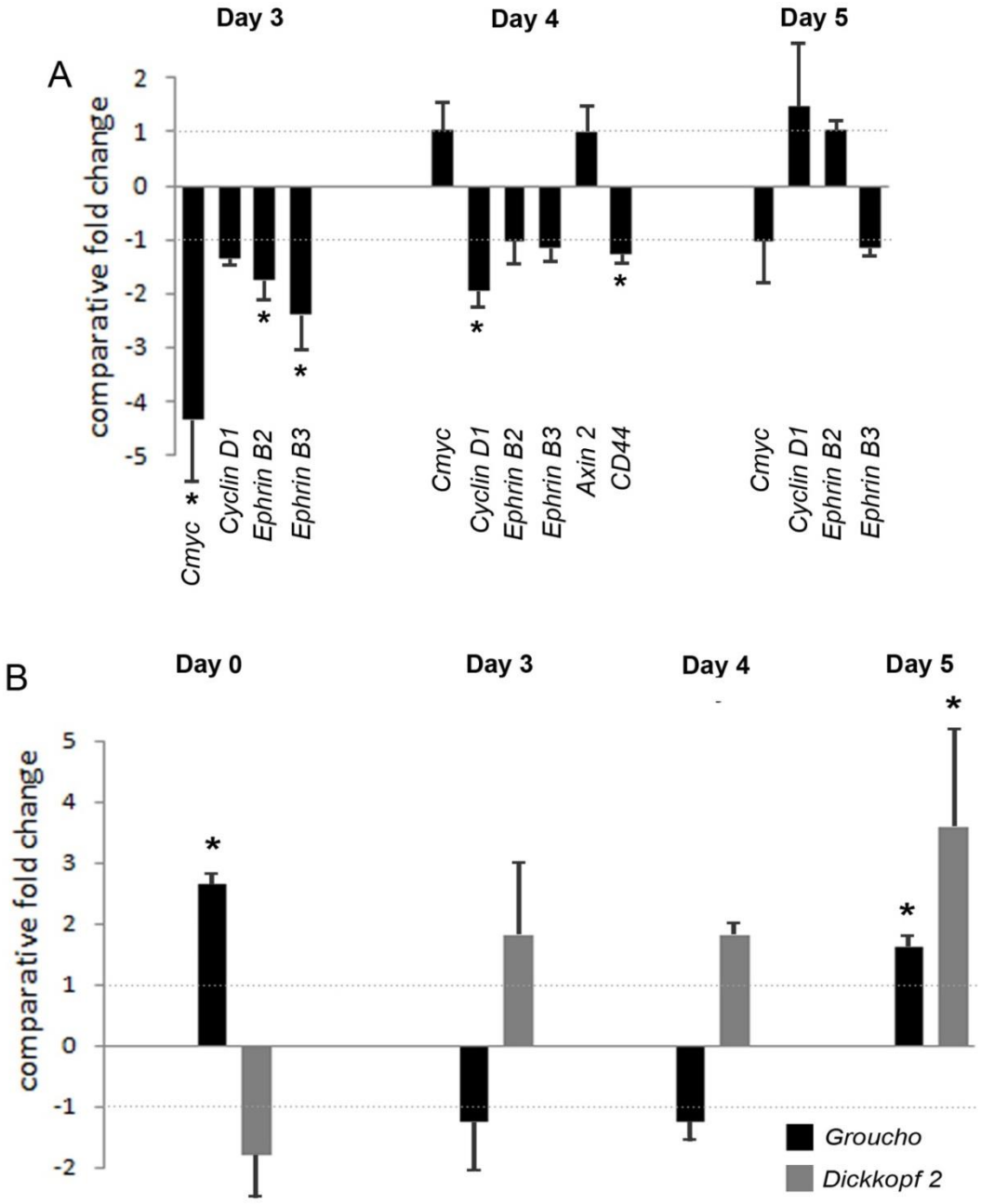

Figure 4.5 Addition of an *Apc2*$^{-/-}$ mutation altered Wnt target/Wnt repressor gene expression in Apcfl intestine. Quantitative RT-PCR was used to directly compare Wnt gene expression levels of DKO vs Apcfl intestinal cell extracts at different time points. (**A**) All Wnt target genes analysed were expressed at lower levels in DKO intestine on **Day 3**, most were expressed at lower levels on **Day 4** but by **Day 5** there were no significant differences in Wnt target gene expression. (**B**) The Wnt repressor *Groucho* was significantly increased in DKO intestines prior to induction (**Day 0**). On **Day 3** and **Day 4** although Wnt repressor *Dickkopf 2* shows a trend toward an increase in expression there were no significant differences. By **Day 5** both *Groucho* & *Dickkopf 2* displayed significantly increased expression in DKO intestines (asterisks mark the comparisons that were found to be significantly different in DKO intestines, $p \leq 0.05$, Mann-Whitney *U*-test, $n \geq 4$, error bars represent SEM of C_t values).

Interestingly, the Wnt repressor *Groucho* was significantly increased in DKO intestines pre induction (Day 0 {*Apc2*$^{-/-}$ vs *Apc2*$^{+/+}$}). No significant differences between genotypes were noticed in Days 3 or 4 in either Wnt repressor. However, by Day 5 both *Groucho* and *Dickkopf 2* displayed significant increases in expression levels in DKO intestines compared to Apcfl (for all significantly different comparisons $p \leq 0.05$, Mann-Whitney *U*-test, n=4). Together these results suggest that additional Apc2 deficiency initially inhibits Wnt gene activation, this inhibition becomes less apparent to nonexistent by Day 5 and Wnt repressors may play some role in this inhibition.

4.2.5 Additional Apc2 deficiency alters proliferative potential in Apcfl intestines

As intestinal cell division is closely coupled with Wnt signalling, BrdU labelling was used to compare composition of dividing cells between genotypes at days 3 & 4. Intestinal Apc loss has previous been shown to lead to substantial increases in cell division and proliferative compartment (Sansom et al., 2004; Andreu et al., 2005). To assess additional effects of Apc2 on Apcfl intestinal cell division, Apcfl and DKO mice were injected with BrdU 2 hours before death on Day 3 and Day 4. Cells incorporating BrdU were detected on intestinal sections using anti-BrdU antibody and quantitative analysis performed between genotypes (Figure 4.6). Surprisingly, at Day 3 whilst the 'crypt Progenitor' phenotype was attenuated in DKO intestines, BrdU distribution (proliferative compartment) and total number of BrdU positive cells were comparable between genotypes. On Day 4 there was a significant increase in the number of BrdU positive cells in DKO intestines (DKO 53.33±0.70 vs Apcfl 35.90±0.17 BrdU+ cells per half crypt-villus structure $p \leq 0.05$, Mann-Whitney *U*-test, n=4). Although 'crypt progenitor' phenotype/crypt sizes were now comparable at Day 4 between genotypes, the proliferative compartment was expanded in DKO intestines. Distribution of BrdU positive cells was significantly different (Kolmogorov-Smirnov Z test, $p \leq 0.05$, n=4). In DKO mice on both Day 3 & Day 4 villus epithelial cells were capable of incorporating BrdU outside of the 'crypt' region.

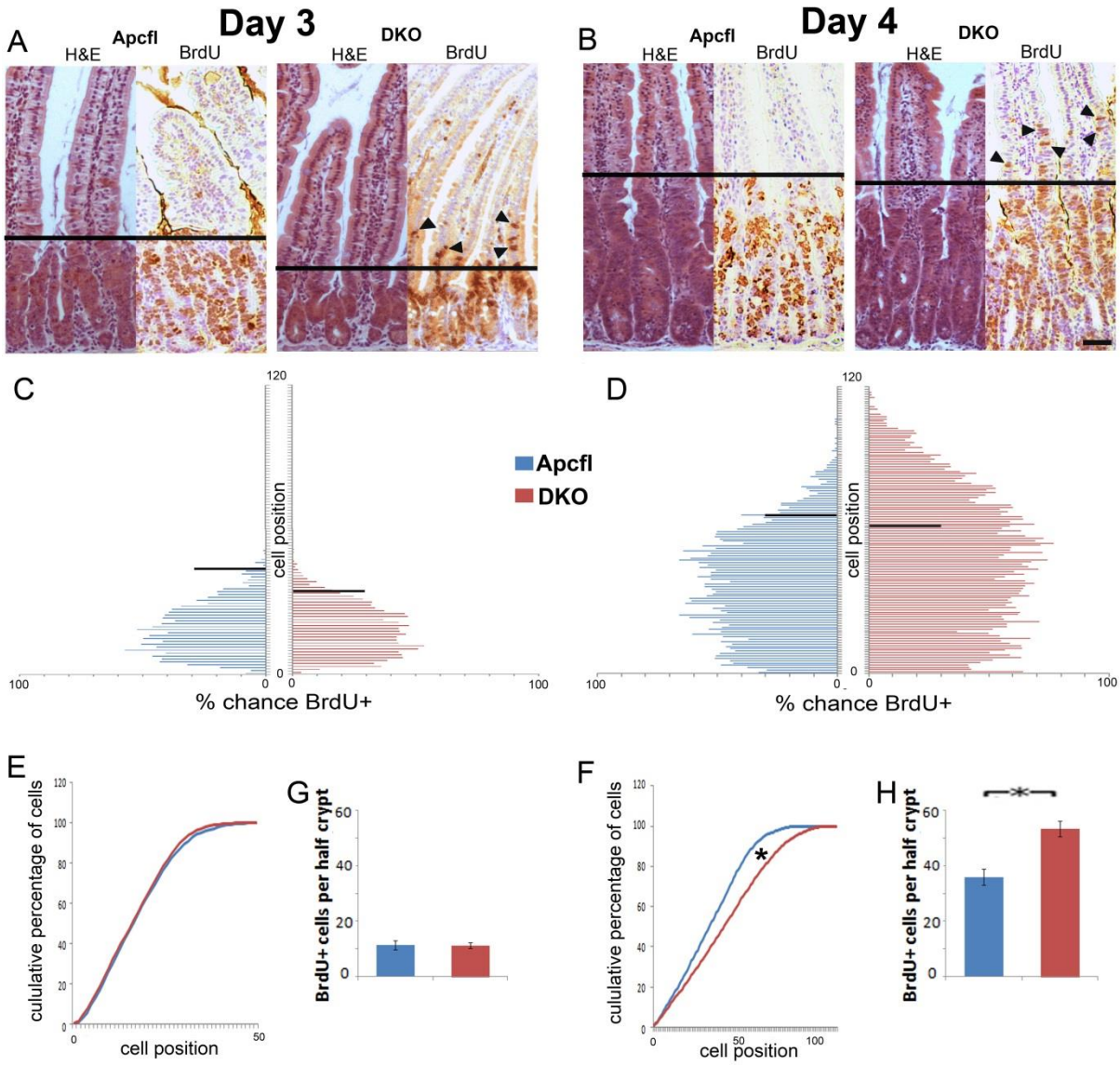

Figure 4.6 Addition of an *Apc2*$^{-/-}$ mutation increased the proliferative compartment of Apcfl intestine. Cohorts of mice from each genotype were injected with BrdU 2h before death. Intestinal sections from (**A**) **Day 3** and (**B**) **Day 4** where stained with an anti-BrdU antibody and compared next to corresponding H&E's. Villus cells outside the 'crypt' region had the potential to incorporate BrdU in DKO intestines at both time points (black line represents average crypt boundary assessed from H&E sections. Black arrowheads = BrdU positive cells outside 'crypt' region, scale bar represents 50μm. Positional analysis of BrdU positive cells was carried out at (**C**) **Day 3** and (**D**) **Day 4** (Black line represents average crypt boundary, see methods for graph description). Whilst at **Day 3** there were no obvious differences in distribution, at **Day 4** the proliferative compartment is expanded. A cumulative frequency plot of BrdU positive cells on (**E**) **Day 3** and (**F**) **Day 4** demonstrated a significant difference in distribution on **Day 4** (asterisk marks p≤0.05, Kolmogorov-Smirnov Z test, n=4). Total BrdU remained consistent between genotypes on (**G**) **Day 3** but statistically increased on (**H**) **Day 4** (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n=4).

4.2.6 Additional Apc2 loss alters the fate of BrdU labelled cells in Apcfl intestines

Previous studies have shown intestinal Apc loss abrogates epithelial cell migration (Sansom et al., 2004; Andreu et al., 2005). Experiments from the previous chapter demonstrate that Apc2 loss can effectively increase the rate of epithelial cell migration (Chapter 3). In order to analyse the effects of additional Apc2 loss on epithelial cell migration and fate in Apcfl intestines a BrdU pulse chase experiment was carried out and compared between genotypes. Cohorts of Apcfl and DKO mice were injected with BrdU on Day 4 (n=8). Half of each genotype where culled after 2h (n=4) and the remainders at 24h (n=4). BrdU is only bio-available for <2 h but will stay incorporated inside positively labelled cells effectively 'tagging' them. This allows us to determine the fate of these cells after 24 hours (Figure 4.7). In contrast to previous studies (Sansom et al., 2004; Andreu et al., 2005), scoring of BrdU positive cells in Apc deficient intestines (Figure 4.2.7B,D) did reveal statistical differences in distribution following 24 hours (Kolmogorov-Smirnov Z test, $p \leq 0.05$, n=4). This difference however was negligible compared to that observed in Wt intestines (Figure 3.6). There were a comparable number of BrdU positive cells in Apcfl intestines after 24 hours (Figure 4.7F). Conversely, in DKO intestine although no differences were observed in BrdU distribution after 24 hours (Figure 4.7C,E), there were significantly less BrdU positive cells (2h 53.33±0.46 vs 24h 31.90±0.37 BrdU+ cells per half crypt-villus structure $p \leq 0.05$, Mann-Whitney *U*-test, n=4). This decrease in number of BrdU cells after 24h is indicative of increased cell death.

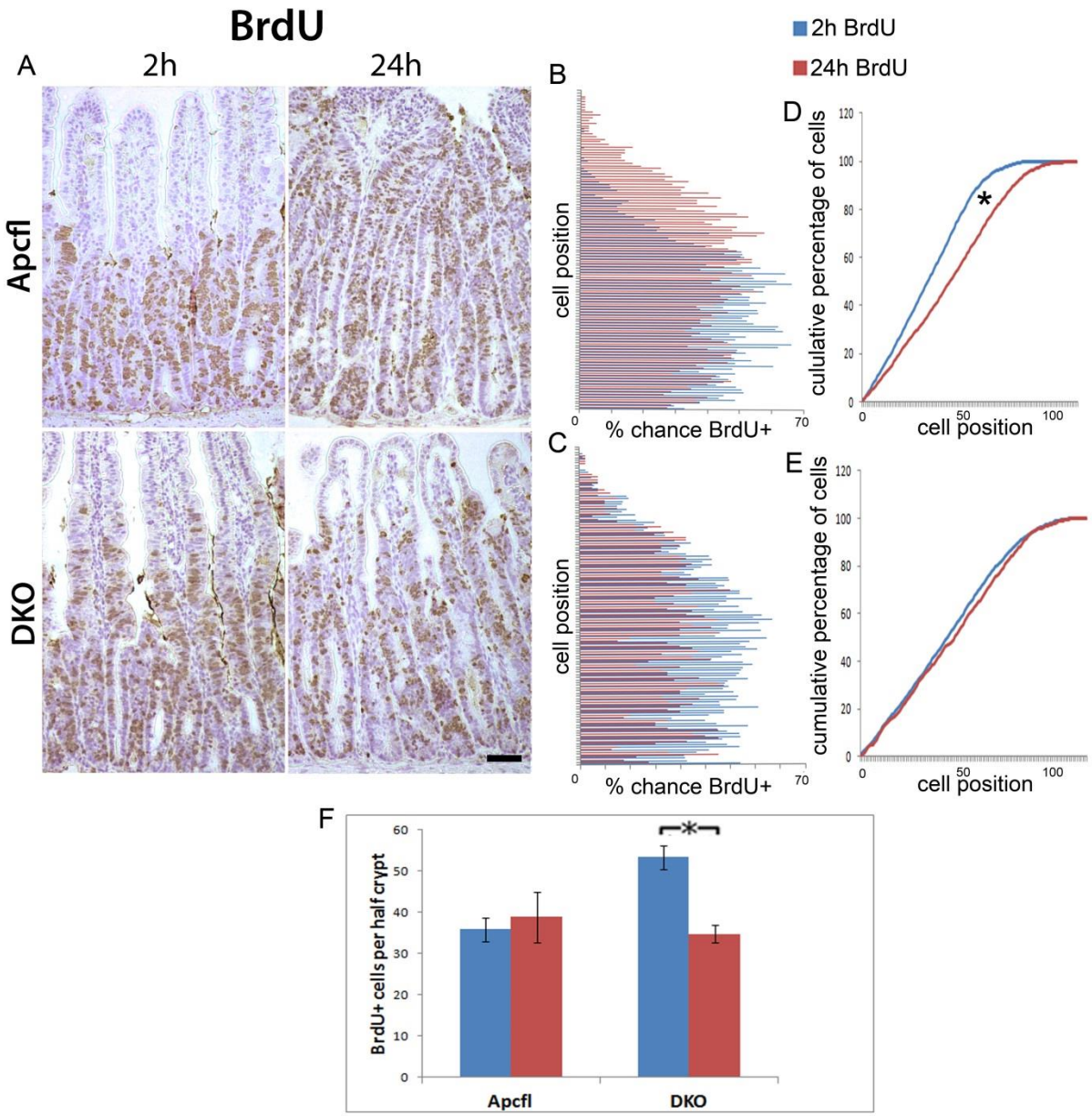

Figure 4.7 Altered fate of BrdU labelled cells in DKO intestines following 24 hour migration. Apcfl and DKO mice were injected with BrdU on **Day 4**, half of each group were culled at 2h and the remainder at 24h. (**A**) Anti-BrdU stained intestinal tissue from each group (scale bar represents 50μm). Positional analysis of BrdU positive cells 2h vs 24h for both (**B**) Apcfl and (**C**) DKO intestines and cumulative frequency plots (**D**) and (**E**) respectively, demonstrated a difference in position of BrdU positive cells following 24 allowed migration in Apcfl cells but not in DKO intestines (asterisk marks p≤0.05, Kolmogorov-Smirnov Z test, n=4). (**F**) Quantitative analysis of total numbers of BrdU positive cells showed a comparable numbers of labelled cells in Apcfl intestines after 24h however in DKO intestines there was a statistical reduction indicating loss of some of the BrdU positive cells following 24h (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n=4).

4.2.7 Additional Apc2 loss increases the mitotic and apoptotic indices in Apcfl intestines

H&E stained intestinal sections from each time point were used to quantify and compare mitotic figures (mitosis) between genotypes (Figure 4.8A). Both genotypes displayed increases in levels of mitosis with time. A statistical difference in indices of mitotic figures was observed between genotypes on Day 0, this difference is consistent with results from chapter 3 where Apc2 deficiency alone can increase cell division (Apcfl 0.87±0.21 vs DKO 1.59±0.21 percent of cells displaying mitotic figures, p≤0.05, Mann-Whitney *U*-test, n=4). Day 4 also displayed a statistically significant increase in levels of mitosis in DKO intestines (Apcfl 1.74±0.46 vs DKO 260±0.20 percent of cells displaying mitotic figures, p≤0.05, Mann-Whitney *U*-test, n=4). Days 3 & 5 displayed no statistical difference in levels of mitosis between genotypes.

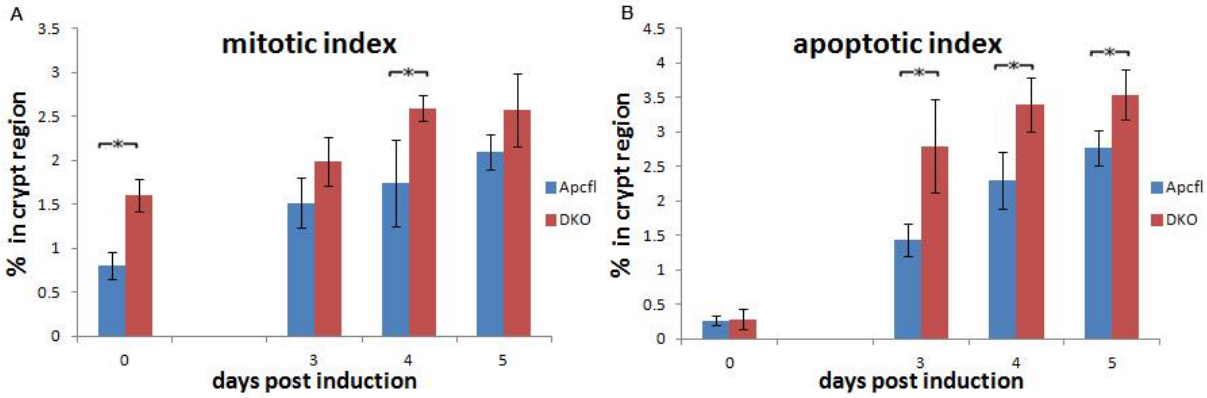

Figure 4.8 DKO mice displayed an increase in both mitosis and apoptosis at various time points compared to Apcfl intestines. H&E stained intestinal sections from each study group were scored for (**A**) mitotic indices and (**B**) apoptotic fragments. Quantitative analysis between genotypes revealed statistical differences in both mitosis and apoptosis at certain time points (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n=4).

Previous studies have shown an increase in levels of apoptosis following intestinal Apc loss (Sansom et al., 2004; Andreu et al., 2005) whilst Apc2 loss results in no apoptotic change (Chapter 3). H&E stained intestinal sections from each time point were used to quantify and compare apoptotic bodies (apoptosis) between Apc and DKO intestine (Figure 4.8B). Both

genotypes displayed increases in levels of apoptosis with time. At day 0 there are were no statistically significant differences in levels of apoptosis between genotypes. However, statistically significant increases were observed across all other time points in DKO intestines compared to Apcfl (Day 3= Apcfl 1.44±0.23 vs DKO 2.80±0.68, Day 4= Apcfl 2.30±0.41 vs DKO 3.40±0.38, Day 5= Apcfl 2.77±0.25 vs DKO 3.54±0.37, percent of cells displaying Apoptotic bodies, $p \leq 0.05$, Mann-Whitney *U*-test, n=4).

4.2.8 Additional Apc2 loss attenuates paneth cell disruption in Apcfl intestines

Apc deficient intestines displayed perturbed differentiation with disruptions in paneth cell differentiation and position (Sansom et al., 2004; Andreu et al., 2005). In order to analyse the effects of additional Apc2 loss on paneth cells within Apcfl intestines, sections from Apc and DKO mice from different time points were stained with lysozyme (Figure 4.9A). Quantification of paneth cell number per half crypt revealed increases in paneth cell number in both genotypes over time (Figure 4.9B). There were no significance differences between genotypes at any time point. To evaluate paneth cell composition in comparison to total cell number, paneth cell counts were normalized against crypt-villus structure size (Figure 4.9C). This would give a relative number of differentiated paneth cells independent of varying degrees of structure expansion. On Day 5 there were statistically more paneth cells in comparison to total cell number in DKO intestines compared to Apcfl (DKO 9.20±1.82 vs Apcfl 5.62±0.83 comparative paneth cell number, $p \leq 0.05$, Mann-Whitney *U*-test, n=4). Position analysis was performed (Figure 4.9D) also represented as cumulative distribution (Figure 4.9E). On Day 3, additional Apc2 loss rescued paneth cell mis-positioning in Apcfl intestines. On Day 4 the mis-positioning of paneth cells was attenuated. By Day 5 there were no significant differences (for statistical comparisons see Figure 4.9E).

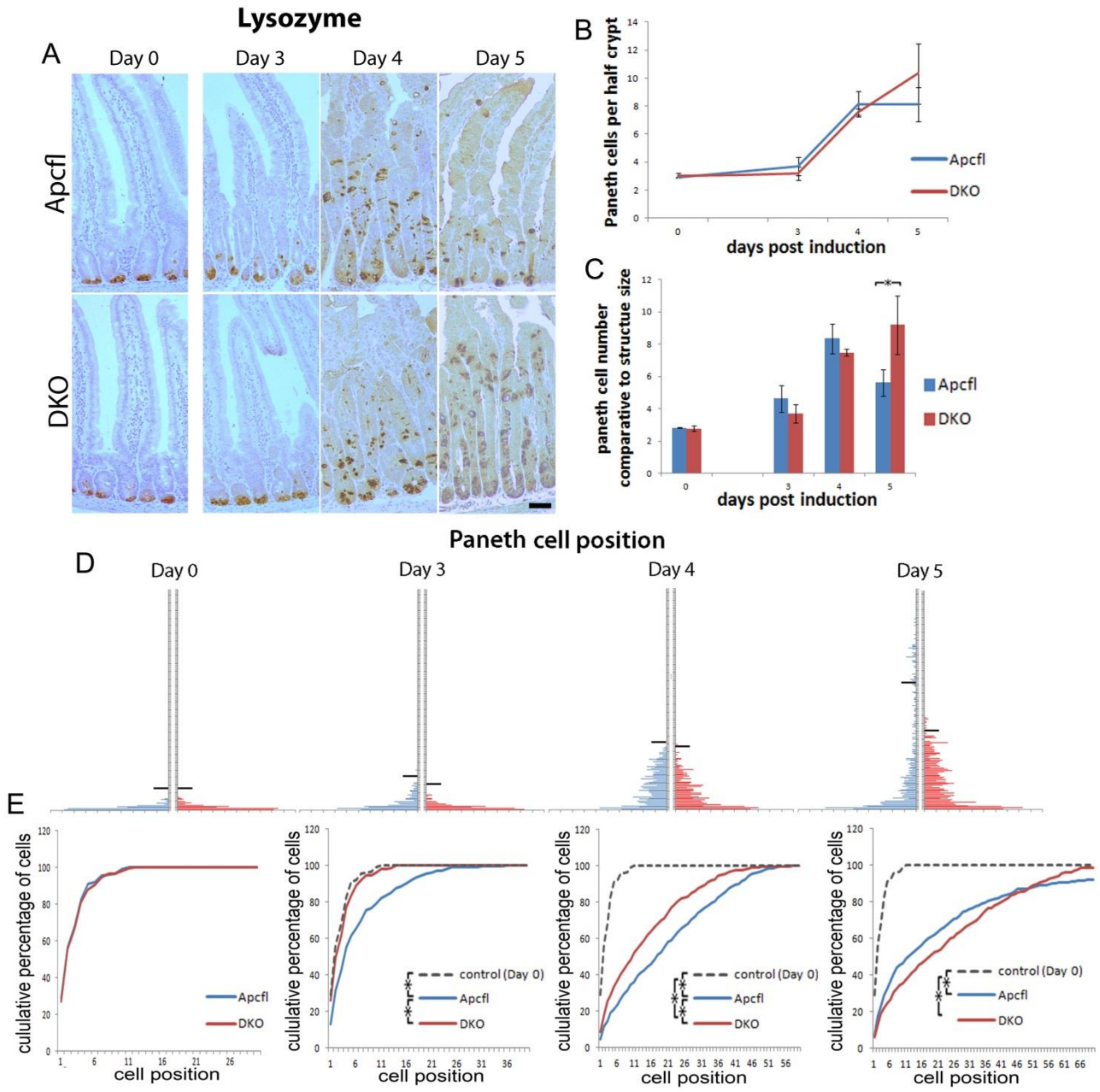

Figure 4.9 Attenuated paneth cell disruption in DKO intestines. (**A**) Intestinal sections from each genotype at the different time points were stained with lysozyme (scale bar represents 50μm). Quantitative analysis of paneth cell number per half crypt (**B**) demonstrated an increase in Paneth cell number from **Day 3** through to **Day 5** in both genotype but insignificantly different between genotypes. As each genotype displays different degrees of crypt-villus expansion, paneth cell number was normalized against structure size thus giving a value of paneth cells comparable to total cell number. (**C**)There was a statistical difference in comparative paneth cell number between genotypes at **Day 5** (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n=4). (**D**) Paneth cell position was plotted between genotypes at each time points (black line indicates average crypt boundary, blue=Apcfl, red=DKO) (**E**) also represented as cumulative frequency plots. In DKO intestines the Addition of an *Apc2*$^{-/-}$ mutation delayed paneth cell displacement noticed in Apcfl intestine (asterisks mark p≤0.05, Kolmogorov-Smirnov Z test, n=4).

4.2.9 Additional Apc2 loss rescues goblet cell depletion in Apcfl intestines

Previous studies have shown loss of intestinal Apc leads to depletion of goblet cells (Sansom et al., 2004; Andreu et al., 2005). In order to analyse the effects of additional Apc2 loss on goblet cells within Apcfl intestines, sections from Apc and DKO mice from different time points were stained with alcien blue (Figure 4.10A). Quantification of goblet cell number per half crypt (Figure 4.10B) revealed an initial increase in goblet cell number in both genotypes. By Day 5 there was a vast loss of goblet cells in Apcfl intestines, this loss was rescued in DKO mice (DKO 14.18±0.80 vs Apcfl 7.23±2.17 goblet cells per half crypt, $p \leq 0.05$, Mann-Whitney *U*-test, n=4). To evaluate goblet cell composition in comparison to total cell number, goblet cell counts were normalized against crypt-villus structure size (Figure 4.10C). This would give a relative number of differentiated goblet cells independent of varying degrees of structure expansion. On Day 5 there were statistically more goblet cells in comparison to total cell number in DKO intestines compared to Apcfl (DKO 12.65±0.71 vs Apcfl 5.01±1.88 comparative goblet cell number, $p \leq 0.05$, Mann-Whitney *U*-test, n=4). Position analysis was performed (Figure 4.10D) also represented as cumulative distribution (Figure 4.10E). Goblet cell distribution disruption was apparent by Day 4 however, there was no statistical difference between genotypes. By Day 5 there was an attenuation of goblet distribution disruption in DKO intestines (for statistical comparisons see Figure 4.10E).

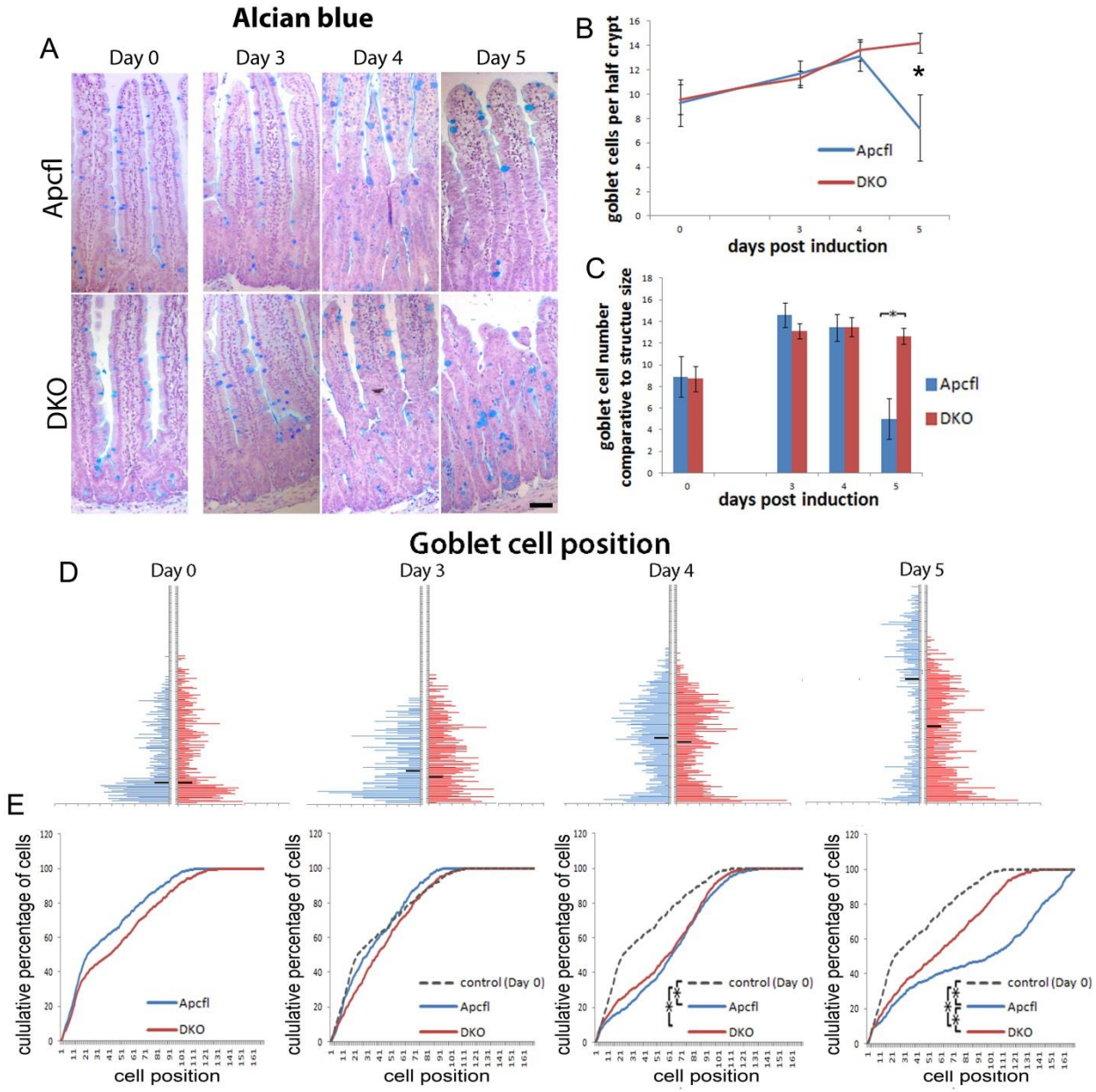

Figure 4.10 Attenuated goblet cell disruption in DKO intestines. (**A**) Intestinal sections from each genotype at the different time points were stained with alcien blue (scale bar represents 50μm). Quantitative analysis of goblet cell number per half crypt (**B**) demonstrated an increase in number from **Day 0** to **Day 4** comparable in both genotypes. However, there was substantial loss of goblet cells in Apcfl intestines at **Day 5** significantly different from DKO intestines (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n=4). As each genotype displays different degrees of crypt-villus expansion, goblet cell number was normalized against structure size thus giving a value of goblet cells comparable to total cell number (**C**). There was a statistical difference in comparative goblet cell number between genotypes at **Day 5** (error bars represent standard deviation, asterisks mark p≤0.05, Mann-Whitney *U*-test, n=4). (**D**) Goblet cell position was plotted between genotypes at each time points (black line indicates average crypt boundary, blue=Apcfl, red=DKO) (**E**) also represented as cumulative frequency plots. In DKO intestines the Addition of an *Apc2*$^{-/-}$ mutation attenuated goblet cell displacement noticed in Apcfl intestine on **Day 5** (asterisks mark p≤0.05, Kolmogorov-Smirnov Z test, n=4).

4.2.10 Additional Apc2 deficiency reduces expansion of the stem cell population in Apcfl intestines

Intestinal stem cells contribute to the normal repopulation of intestinal epithelium and are critically reliant on Wnt signaling (for review see Reya & Clevers, 2005). It has been reported previously that markers for intestinal 'stemness' increase following Apc loss (Jubb et al., 2006; Merlos-Suarez et al., 2011). As crypt expansion is more prominent in Apcfl intestine yet differentiated cell types were sparser in comparison to DKO, ISC composition was compared between genotypes. Day 4 was chosen as a good time point for comparison as crypt sizes were comparable. In order to compare state of ISC populations, '*in situ*' hybridisation analysis of *Ascl2* and *Olfm4* expression was carried out (performed by M. Young). Small intestinal sections from Day 0 (control), Apcfl and DKO intestines at Day 4 were stained using anti-*Ascl2* and anti-*Olfm4* riboprobes (Figure 4.11A). In control intestines both markers were expressed at the crypt base, consistent with the suspected stem cell compartment. Expansion of the stem cell compartment was evident in both genotypes following induction however, expression was sparse and intensity considerably reduced in DKO compared to Apcfl intestines, consistent with a reduction in stem cell population.

qRT-PCR was used to compare expression levels of ISC markers *Ascl2* and *Lgr5* between control (Day 0), Apcfl and DKO (both Day 4) intestinal RNA samples (Figure 4.11B). Expression levels of *Ascl2* and *Lgr5* were significantly elevated in both Apcfl and DKO compared to control intestines (Mann-Whitney *U*-test, $p \leq 0.05$, n=4), indicative of a stem cell expansion. However, Ascl2 expression levels were significantly reduced in DKO compared to Apcfl intestines (Mann-Whitney *U*-test, $p \leq 0.05$, n=4). *Lgr5* also displayed a trend toward reduced expression in DKO intestines however, statistical significance was not met. Reduced expression levels of ISC markers coupled with reduced intensity of '*in situ*' staining indicates an attenuation of ISC expansion in DKO mice compared to Apcfl. Taken together, these results suggest whilst Apc loss permits a suitable environment for ISC expansion, additional loss of Apc2 reduces this capacity.

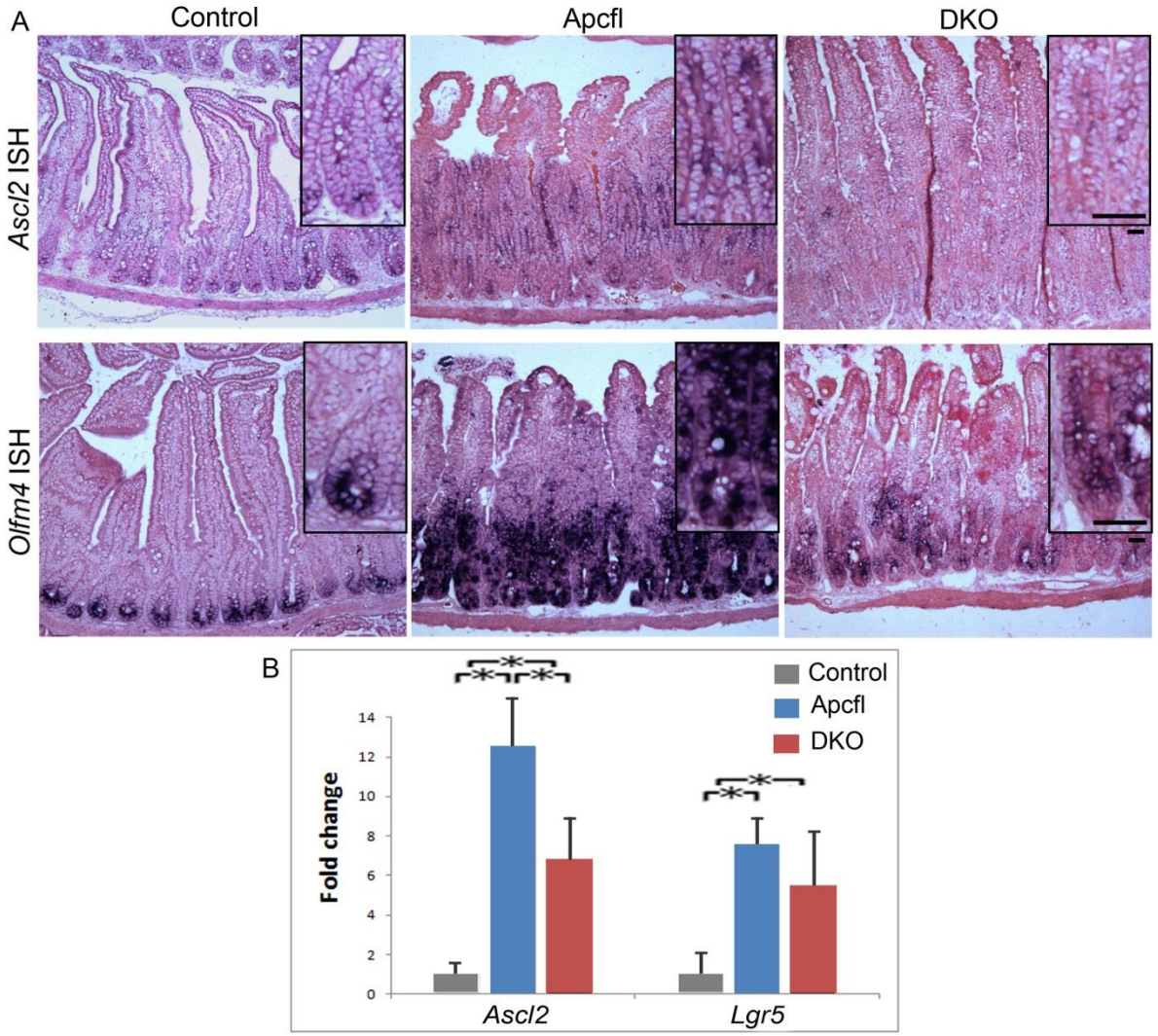

Figure 4.11 Additional Apc2 loss attenuated expansion of the small intestinal stem cell compartment seen in Apcfl intestines at **Day 4**. (**A**) '*In situ'* hybridisation analysis of *Ascl2* and *Olfm4* expression revealed a substantial expansion of the stem cell compartment in the intestinal epithelium of Apcfl (**Day 4**) mice compared to control animals (**Day 0**). Whilst expansion of a 'crypt progenitor' cell type was observed in the small intestine of DKO mice, expression levels of both *Ascl2* and *Olfm4*, appeared to be considerably reduced compared to that of Apcfl intestines (Scale bars represent 50 μm). (**B**) Quantitative RT-PCR expression analysis revealed a significant up-regulation of intestinal stem cell markers *Ascl2* and *Lgr5* in Apcfl (blue bars) and DKO (red bars) intestines compared to control (white bars) at **Day 4.** Expression levels of *Ascl2*, but not Lgr5 were found to be significantly reduced in the small intestine of DKO mice compared to that of Apcfl intestine(error bars represent standard deviation, asterisks mark pair wise comparison p≤0.05, Mann-Whitney *U*-test, n=4, error bars represent SEM of C_t values).

4.2.11 Apc2 deficiency does not alter survival or tumourigenesis in the context of a Wnt driven heterozygous *Apc* tumour model

Heterozygous inactivation of Apc is a well-established protocol for the induction of Wnt-driven tumourigenesis in the gastrointestinal tract of mice. Spontaneous mutation of the normal residing allele of *Apc* results in the development of aberrant crypt foci, which inevitably progress to adenomas. Additional Apc2 mutation has the ability to alter some attributes of immediate intestinal homozygous Apc loss, deemed significant for tumour formation such as stem cell control and cell death. In order to examine the effects of Apc2 deficiency on the survival of mice with heterozygous inactivation of Apc, previously established cohorts of mice expressing *Ah-Cre* recombinase and a targeted *Apc* allele carrying loxP sites (Sansom et al., 2004) were crossed with $Apc2^{-/-}$ mice to obtain cohorts of $Ah\text{-}Cre^{+}\ Apc^{+/fl}\ Apc2^{+/+}$ (Apc^{HET}) and $Ah\text{-}Cre^{+}\ Apc^{+/fl}\ Apc2^{-/-}$ ($Apc^{HET}\ Apc2^{-/-}$) mice. Heterozygous inactivation of Apc was achieved through induction with 3 intraperitoneal injections of 80 mg/kg *β*-naphthoflavone over 24 hours in both cohorts. Mice were aged and regularly inspected for signs of intestinal tumourigenesis. Upon signs of ill health, due to extensive tumour burden, mice were sacrificed (initiated by Dr. P. Shaw). Whilst very few un-induced or induced $Ah\text{-}Cre^{+}\ Apc^{+/+}\ Apc2^{+/+}$ mice displayed signs of ill health within the time frame of the experiment, both Apc^{HET} and $Apc^{HET}\ Apc2^{-/-}$ mice started developing signs of intestinal neoplasia, becoming ill as early as 120 days post induction. Analysis of the overall survival probability revealed no statistically significant difference between the two experimental cohorts (Figure 4.12A, median survival 243 and 212 days for Apc^{HET} and $Apc^{HET}\ Apc2^{-/-}$ mice respectively, Log-Rank test $p=0.65$, $n\geq16$).

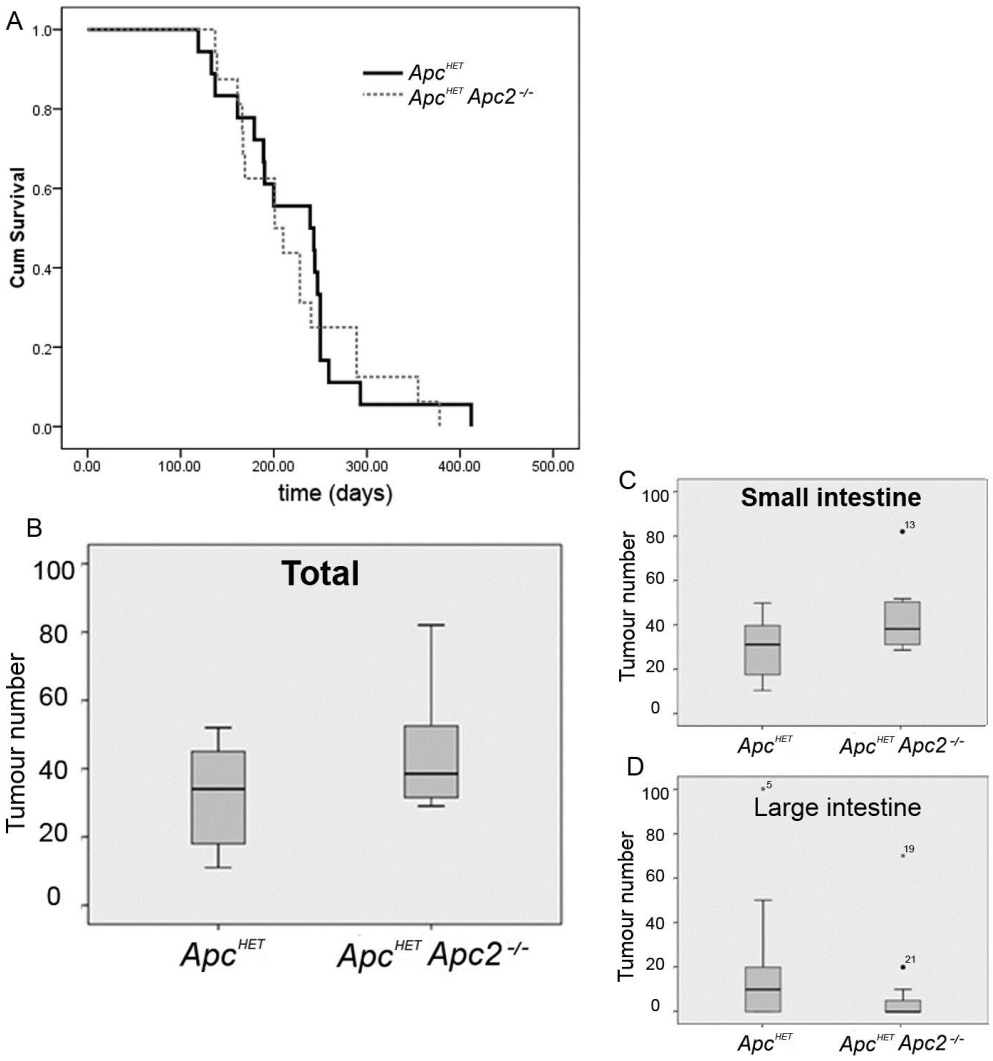

Figure 4.12 The Addition of an Apc2 mutation did not alter tumorigenesis in Apc heterozygous mice. (**A**)Cohorts of *Apc*^{HET} (black line) and *Apc*^{HET} *Apc2*$^{-/-}$ (gray dotted line) mice were aged out and sacrificed upon signs of ill health (survival presented as Kaplan-meier). No significant differences in overall survival were observed (Log-rank test, p=0.65, *Apc*^{HET} n=18, *Apc*^{HET} *Apc2*$^{-/-}$ n=16). Tumour burden was analysed in the intestinal epithelium of *Apc*^{HET} and *Apc*^{HET} *Apc2*$^{-/-}$ animals at the time of death. There were no statistical differences in (**B**) total tumour number or split down into (**C**) small or (**D**) large intestinal tumour counts in *Apc*^{HET} and *Apc*^{HET} *Apc2*$^{-/-}$ animals. Data are presented as a box plot (p>0.05, Mann-Whitney *U*-test, n≥15).

In order to analyse whether additional Apc2 deficiency affected tumourigenesis in mice heterozygous for Apc mutation, the alimentary tract of *Apc*^{HET} and *Apc*^{HET} *Apc2*$^{-/-}$ animals was dissected at the time of sacrifice and analysed for tumour burden. Statistical analysis revealed no significant difference in total, small or large intestine between the two cohorts (Figure 4.12B,C,D). Microscopic examination of H&E stained intestinal sections from each genotype identified tumours as adenomas. No differences in tumour morphology,

progression or grade between genotypes were observed. Together with the lack of difference in survival, these observations indicated that Apc2 loss neither attenuated nor accelerated intestinal tumourigenesis in Apc^{HET} mice.

4.3 Discussion

The effects of Apc2 loss on intestinal homeostasis were analysed in the previous chapter. Despite evidence linking APC2 loss with human tumourigenesis, murine Apc2 loss alone failed to initiate tumours. However, it did possess the potential to alter some attributes consistent with Apc loss. Previous studies in *Drosophila* have suggested functional redundancies exist between the two *Apc* homologs (McCartney et al., 1999; McCartney et al., 2006; Ahmed et al., 2002; Akong et al., 2002; Kunttas-Tatli et al., 2011; Roberts et al., 2012). This, along with the fact that induced APC2 over-expression can rescue Wnt activation achieved through reduced APC expression in human colorectal cancer cell lines (Van Es et al., 1999; Roberts et al., 2012) suggests these proteins may have overlapping roles in mammalian intestines. In this chapter I utilised mouse transgenics to concurrently delete both Apc proteins from the intestinal epithelium and pursue two aims; foremost to expose any effects of additional Apc2 loss on immediate homozygous Apc loss and secondly to delineate any role Apc2 may play in tumorigenesis in the context of an *Apc* heterozygous tumour model. Data presented in this chapter revealed that additional Apc2 loss in the context of intestinal homozygous *Apc* deletion initially attenuates Wnt over-activation and associated phenotypes. Crypt progenitor expansion was reduced although cells 'marginal' to the crypt progenitor displayed increased proliferative capacity. Increases in cell death and attenuated expansion of the ISC pool most likely implement this reduction of crypt progenitor expansion. Although additional Apc2 loss had profound effects on immediate intestinal homozygous Apc loss, Apc2 deficiency neither attenuated nor accelerated overall tumorigenesis in a Wnt driven Apc heterozygous intestinal tumour model.

Apc is indispensable for normal mouse development (Moser et al., 1995) so a conditional, inducible Cre-lox system was employed to conditionally delete Apc from the murine intestines (previously used by Andreu et al., 2005). Upon induction, Apc depletion only

started manifesting a phenotype after 3 days and complete loss took up to 5 days. This is most likely due to time required for tamoxifen absorption, Cre mechanics and dynamics of Apc RNA and protein turnover. Interestingly, by Day 3 where Apc levels are being substantially reduced, its cellular location was altered from being mostly cytoplasmic to exclusively nuclear and membrane bound. Apc protein can usually be found in a variety of subcellular locations (for review see Bienz, 2002) consistent with its multifunctional role. As Apc protein is reduced, relocation to the nucleus and retained membrane binding may highlight the most vital roles of Apc. The intrinsic ability of Apc to shuttle to and from the nucleus is essential for its role as negative regulator of Wnt signaling (Henderson, 2000). Further experiments demonstrate that Apc directly blocks transcription of some Wnt target genes within the nucleus (Sierra et al., 2006). Relocation of Apc to the nucleus may highlight this location as the most effective location in blocking Wnt in an Apc depleted setting. Apc is also found in adherens junctions and is essential for cellular adhesion (for review see Bienz and Hamada, 2004). Apc's membrane location noticed in an Apc depleted setting may also highlight its role in cellular adhesion as a critical one. Interestingly, at Day 3 post induction occasional clumps of crypt cells displayed high levels of nuclear Apc staining which were subsequently lost by Day 4. These cells could represent progeny of un-recombined cells unable to survive in an Apc deficient background or cells recently exiting quiescence un-affected by *Villin-Cre*^{ER} until Days 3-4.

Previous studies have demonstrated morbidity in mice following homozygous intestinal Apc deletion (Sansom et al., 2004; Andreu et al., 2005; Sansom et al., 2007). The aberrant phenotypes are thought to occur primarily as a consequence of over-activation of the Wnt signaling pathway. Interestingly, although loss of either Apc or Apc2 possesses the ability to disruptively activate Wnt, combined loss loss delayed the onset of morbidity associated with intestinal Apc loss. Morbidity in mice with intestines deficient for Apc could, in part, be accounted for by the lack of differentiated cell types and/or the vast increase in intestinal cell numbers causing an increased intestinal 'bulk'. Less comparatively differentiated cell types with relevant physiological function in the intestines would hinder the organs main role of water/nutrient uptake leading to morbidity. Increased intestinal 'bulk' could induce morbidity a number of ways; either directly through reduced peristalsis or compression of other organs/systems or indirectly through means such as sequestering of blood. Additional

loss of Apc2 delayed and attenuated crypt progenitor expansion reducing intestinal 'bulk' and also helped retain more comparatively differentiated intestinal cell types.

Loss of Apc within the intestines has profound effects on intestinal differentiation (Sansom et al., 2004; Andreu et al., 2005). Reduction or mis-positioning of terminally differentiated cell types undoubtedly will affect intestinal function and lead to morbidity in affected mice. Interestingly, additional Apc2 mutation attenuated perturbations in terminally differentiated cell types, noticed in Apc deficient intestines. Paneth cells function both as a secretor of antimicrobial peptides (Ganz, 2003; Wehkamp et al., 2005) and as an essential stem cell niche, providing essential Wnt, EGF and Notch signals (Sato et al., 2011; Parry et al., 2013). Wnt signaling is essential for terminal maturation of Paneth cells (Van Es et al., 2005) and loss of Apc leads to perturbation in paneth cell number and positioning (Sansom et al., 2004; Andreu et al., 2005). Whilst this study also confirmed those findings, additional Apc2 loss delayed and attenuated perturbations in paneth cell positioning. This can be explained by attenuated *cMyc* and subsequently *Ephrin B2* and *Ephrin B3* expression in intestines deficient for both Apc proteins.

The ephrin system is responsible for creating tissue boundaries, defining paths of cell migration and maintaining proper cellular positioning (reviewed in Crosnier et al., 2006). Disruptions in ephrin signaling and increases in expression of *Ephrin B2* and *Ephrin B3* are responsible for paneth cell disruption in intestines deficient for Apc (Sansom et al., 2004; Andreu et al., 2005). A model with concurrent intestinal deletion of *cMyc* and *Apc* rescued most attributes of Apc loss including paneth cell positioning (Sansom et al., 2007). This was partly attributed to attenuated *Ephrin B2* and *Ephrin B3* expression. Interestingly, additional Apc2 loss at early time points in our experiment, drastically attenuated *cMyc* expression. This was concomitant with highest levels of *Ephrin B2* and *Ephrin B3* attenuation and rescued paneth disruption. This confers with the consensus that effects of intestinal Apc loss are cMyc dependent (Sansom et al., 2007) and modulation of Cmyc may be a good therapeutic approach in colorectal cancer prevention.

Goblet cells secrete mucins into the intestinal lumen which form the mucus layer. This layer is indispensable for digestion and acts as an essential barrier between the luminal contents

and the epithelial surface (Shirazi et al., 2000). Goblet cells are another terminally differentiated cell type affected by Apc loss (Sansom et al., 2004; Andreu et al., 2005). In our study, Apc loss led to the depletion of goblet cells by Day 5. This was attenuated by additional loss of Apc2. Although Notch signaling is mostly associated with goblet cell differentiation (Fre et al., 2005; Van Es et al., 2005), crosstalk between Wnt and Notch in intestinal homeostasis exists (Nakamura et al., 2007; Fre et al., 2009). Increases in intestinal Wnt through Apc loss may inadvertently affect Notch, disrupting goblet cell differentiation. Attenuated Wnt expression in intestines deficient for both Apc proteins may indirectly rescue goblet cell disruption through Notch signaling. This is an avenue for further research.

ISC's are critically reliant on Wnt signaling and contribute to the normal repopulation of intestinal epithelium (for review see Reya & Clevers, 2005). It has been reported previously that markers for intestinal 'stemness' increase following Apc loss (Jubb et al., 2006; Merlos-Suarez et al., 2011). Crypt progenitor expansion noticed in Apc deficient intestines could, in part, be attributed to increases in the ISC pool and subsequently born progenitors. Upon analysis of stem cell markers it was revealed that additional loss of Apc2 attenuated ISC expansion. This proposes that whilst Apc loss permits a suitable environment for ISC expansion, additional loss of Apc2 reduced this capacity. As ISC's are reliant on high Wnt, attenuated Wnt signaling may account for a reduced ISC pool. Conversely, increased cell death and/or promotion of terminal differentiation of ISC's may be causative factors in reduced Wnt signaling observed in intestines deficient for both Apc proteins. Regardless of mechanism, reduced ISC/precursor cells and comparatively more terminally differentiated cells types would contribute to an extended post induction survival.

Analysis of proliferative potential between genotypes using BrdU revealed some interesting findings. In Apc deficient intestines, positively labelled cells resided within the expanded crypt/progenitor zone, however, intestines deficient for both Apc proteins displayed positively labelled cells outside this region. These cells marginal to the crypt progenitor region were capable of incorporating BrdU but displayed normal villus cell histology. This suggests that additional Apc2 loss in Apc deficient intestines has the potential to activate a proliferative program within cells higher up the crypt-villus axis at a lower gradient of secreted Wnt, and this can occur independently to the crypt progenitor phenotype. As Wnt

target gene expression was attenuated in intestines deficient for both Apc proteins at the studied time points, the mechanisms behind increased BrdU labelling remain unclear. Although increased cell division and expansion of the proliferative compartment were noticed in intestines deficient for both Apc proteins compared to that of just Apc deficiency, there was a reduction in survival of labelled cells, indicative of cell death. In contrast to previous reports (Sansom et al., 2004; Andreu et al., 2005; Sansom et al., 2007), Apc deficient intestines did display a mild positional difference in labelled cells following 24h allowed migration. Surprisingly, although intestines deficient for both Apc proteins displayed an overall attenuated phenotype, no positional differences were apparent in labelled cells following 24h. This suggests a more severe migrational retardation following intestinal loss of both Apc proteins. There are two caveats to this interpretation. Foremost, higher rates of crypt expansion were noted in intestines deficient for Apc, this may force cells higher up the crypt-villus axis rather than reflecting true migration. Furthermore, the increased cell death noted in intestines deficient for both Apc proteins may well complicate the interpretation of migration.

The majority of alterations in Apc deficient intestines are thought to act through increased expression of Wnt target genes (Sansom et al., 2004; Andreu et al., 2005). Apc2 deficiency also shares this ability to modify Wnt signaling (Chapter 3). Interestingly, cumulative loss initially attenuated Wnt target gene expression (*cMyc*, *Cyclin D1, CD44, Ephrin B2* and *Ephrin B3*) in comparison to Apc loss alone. This attenuation of Wnt target gene expression concomitant with an increase in cell death are a possible explanation for phenotypical differences between genotypes. This prompts the question as to how additional Apc2 loss induces cell death and attenuation of Wnt activation in Apc deficient intestines. Three plausible hypotheses seem relevant; more effective Wnt repression, overall reduction due to death of selective Wnt hyper-active cells, and/or overall reduction due to selective death of cells by Wnt independent means.

Apc2 loss is achieved through a constitutive mutation (Van der Meer et al., 2001). Re-adjustment of Wnt repression due to insults on Wnt may have occurred as a program of developmental compensation. The recruitment of Groucho to TCF/LEF gene regulatory

sequences results in transcriptional repression (Cavallo et al., 1998; Levanon et al., 1998; Buscarlet & Stifani, 2007). Indeed *Groucho* was up-regulated before induction in intestines deficient for Apc2. Other pre-established methods of Wnt repression, with molecular machinery already in place could also contribute to attenuation of Wnt target gene expression. A further explanation as to why intestines deficient for both Apc protein display reduced Wnt signalling, may lie with increases in negative feedback signals. Dickkopf 2 is a secreted antagonist of the Wnt signaling pathway, inhibiting binding of Wnt ligands to the LRP5/6 receptor (Kawano Y, et al., 2003; Niehrs et al., 2006). Murine loss of Dickkopf 2 causes intestinal increases in Wnt target gene expression (Li at el., 2012). At Day 5 post induction, *Dickkopf 2* and *Groucho* were both significantly up-regulated in intestines deficient for both Apc proteins, representing both extra and intra-cellular means of Wnt repression, respectively. Stronger negative feedback signals would explain greater increases in expression of Wnt repressors. To further elucidate other potential mechanism of Wnt repression or potential increases in negative feedback signals microarray technology could be employed to study global transcriptome changes.

Another possible explanation for the attenuated phenotype may lay with selective cell death due to hyper-activation of the Wnt signaling pathway. A 'Just right' hypothesis regarding levels of Wnt signaling has been suggested for oncogenesis and neoplastic growth in colorectal cancer cell lines (Lamlum et al., 1999; Albuquerque et al., 2002; Lucero et al., 2010) Interestingly, although moderate increases in Wnt signaling lead to incurred growth advantage, higher increases induce cell death (Lazarova et al., 2004; Bordonaro et al., 2008). In intestines deficient for both Apc proteins there was an increase in cell death across all post induction time points. Cells with higher levels of Wnt (Wnt^{high}), such as ICS's may be selected against, due to accumulative Wnt hyper-activation caused by loss of both Apc proteins. Indeed, a reduction in markers for stemness was observed in intestines with combined loss. Selective death of Wnt^{high} cells due to Wnt Hyper-action in intestines deficient for both Apc proteins, whilst permitted survival of Wnt^{high} cells in Apc deficient intestines could explain global Wnt intestinal differences between genotypes.

A third explanation as to why additional Apc2 loss attenuates phenotype and Wnt target gene expression in Apc deficient intestines may lay with disrupted functional redundancies

which are Wnt independent. Apc's role is multi-functional (reviewed by Aoki and Taketo, 2007; Senda et al., 2007) and non-canonical functions of Apc are being intensively reported (Fodde et al., 2001; Kaplan et al., 2001; Näthke, 2006; Prosperi et al., 2009; Qian et al., 2008; Harris & Nelson, 2010). As Apc2 shares structural homology to Apc (Vans Es et al., 1999), it undoubtedly too has Wnt independent functions which may overlap with those of Apc. Both Apc and Apc2 loss have been reported to induce Wnt independent cytoskeletal defects in mouse neurons (Yokoto et al., 2008; Shintani et al., 2012). Compromised Wnt independent redundancies in intestines deficient for both Apc proteins may indirectly affect Wnt transduction, ISC dynamics or induce cell death in particular cell types leading to an attenuated phenotype.

Results regarding immediate intestinal homozygous Apc loss indicate additional Apc2 loss attenuates this phenotype and delays the onset of morbidity. This appears to be achieved through a combination of reduced perturbations in differentiated cell types, reduced ISC/progenitor expansion and increased cell death. From these findings, one would postulate that additional Apc2 loss would exert some effect on tumorigenesis caused by Apc loss. To test this assumption, mice with and without Apc2 were analysed in the context of a heterozygous deletion of *Apc*. Initiation of tumours in this validated model, develop due to a spontaneous mutation of the normal residing *Apc* allele. Surprisingly, analysis of tumour burden and survival probability failed to detect differences in tumour incidence and progression between *Apc* heterozygous mice with or without Apc2 deficiency. Taken together, this indicates that Apc2 deficiency provides no selective advantage or disadvantage in Wnt driven intestinal tumourigenesis.

A potential explanation for this surprising finding may come from a detailed examination of the tumour driving mutations found in the *Apc* gene. Evidence from human data suggests that although most colorectal cancer patients contain constitutively activating mutations of the Wnt pathway, tumours frequently retain a certain degree of pathway regulation (Le et al., 2008; Vermeulen et al., 2010; Sousa et al., 2011). Germline mutation of *APC* in FAP patients influence the nature of the second hit/mutation of the normal residing *APC* allele (Lamlun et al., 1999) and this never results in complete loss of function of the protein (Albuquerque et al., 2002). Together, these results suggest that some residual β-catenin

degradation function is needed and selected for in tumours, and this fine-tuned balance of Wnt activity optimal for cell transformation. Interestingly, Apc2 loss has the ability to alter Wnt levels and phenotype in Apc deficient intestines. As different Apc mutations can result in differing levels of Wnt transduction (Gaspar & Fodde, 2004), it would be of significant interest to see how additional Apc2 loss altered the nature/position of the second hit/Apc mutation in our model. One could assume, to obtain an 'optimum' Wnt level needed for tumour formation, the position or pattern may change in intestines with additional Apc2 loss. This may explain why no overall differences in tumorigenesis between groups were observed.

To conclude, the findings presented in this chapter revealed that additional Apc2 loss in the context of intestinal homozygous Apc deletion initially attenuated Wnt over-activation and associated phenotypes. Crypt progenitor expansion was reduced although cells marginal to the crypt progenitor displayed increased proliferative capacity. Increases in cell death and attenuated expansion of the ISC pool most likely implement this reduction of crypt progenitor expansion. Although additional Apc2 loss had profound effects on immediate intestinal homozygous Apc loss, Apc2 deficiency neither attenuated nor accelerated overall tumorigenesis in a Wnt driven Apc heterozygous intestinal tumour model.

Chapter 5

Investigating the combined effects of Apc and Apc2 loss in the mammary epithelium

5.1 Introduction

In the previous chapters it was shown that in the intestine, Apc2 has the ability to partially modify Wnt signaling and can influence the phenotype associated with intestinal Apc loss. However, a range of studies have implicated Apc2 in other tissues. It is known that both Apc proteins are expressed in a range of mammalian tissues (Van Es et al., 1999) and work in *Drosophila* point toward a co-operation in negative regulation of Wnt in a variety of organs (McCartney et al., 1999; McCartney et al., 2006; Ahmed et al., 2002; Akong et al., 2002). Functional redundancies in Wnt control have been exposed through complementary rescue in both *Drosophila* and human tissue (Kunttas-Tatli et al., 2011; Roberts et al., 2012; Van Es et al., 1999), although the role Apc protein play in the mammary gland and/or how they contribute to Wnt signaling is undetermined. Aberrant activation of Wnt signaling pathway is among the most common signaling defect in human cancers (Reya & Clevers, 2005) however, much controversy exists over its role in human breast cancer (Smalley & Dale, 2001; Howe & Brown, 2004). Some studies suggest dysregulation of the Wnt signaling pathway is an important contributor to the initiation of breast cancer (Mohinta et al., 2007), whilst others suggest a more prominent role in cancer progression (Khalil et al., 2012). Although activating mutations in *CTNNB1* (*β-catenin*) are rare in breast cancers (Geyer et al, 2010), both elevated levels of β-catenin (Lin et al., 2000) and reduced levels of membrane expression are common and associated with poor prognosis (Dolled-Filhart et al., 2006; Khalil et al., 2012). This suggests that defects are exerted though inappropriate localization and regulation.

Both APC and APC2 can regulate β-catenin/Wnt signaling (Van Es et al., 1999; Roberts et al., 2012) and both are expressed in human mammary epithelium (Kashiwaba et al., 1994*;* Schlosshauer et al., 2000; Jarrett et al., 2001). Reduction of APC has been reported in breast

cancers through loss of heterozygosity (Kashiwaba et al., 1994; Thompson et al., 1993), promoter hypermethylation (Jin et al., 2001; Ho et al., 1999; Schlosshauer et al., 2000; Sarrio et al., 2003; Van der Auwera et al., 2008) and somatic mutation (Furuuchi et al., 2000). Reduced APC2 has also been implicated in breast cancer through loss of heterozygosity (Sobottka et al., 2000; Yang et al., 2004; Wang et al., 1999), allelic imbalance (Jarrett et al., 2001) and promoter hypermethylation (Chan et al., 2008). Interestingly, *APC2* is located within a chromosome region lost with Peutz-Jeghers syndrome (PJS). Patients with PJS are more susceptible to certain cancers including those of the breast (Mehenni et al., 1998; Bignell et al*.,* 1998). Although loss of the *LKB1* gene is responsible for most of the symptoms of PJS, this gene is not mutated in sporadic cancers suggesting the existence of another tumour suppressor within this region. Importantly, combined status of both APC proteins in breast cancer has never been addressed.

Transgenic models altering the Wnt signaling pathway in the murine mammary have also led to controversy. Expression of an activated form of β-catenin has been reported to cause mammary gland hyperplasia, precocious development and tumour formation in some studies (Michaelson et al., 2001; Imbert et al., 2001). However in others, active β-catenin resulted in metaplasia but failed to induce neoplasia (Miyoshi et al., 2002). This confusion also extends to mammary Apc loss. It is known that Apc^{Min} mice, along with a predisposition to intestinal tumorigenesis, also have a higher incidence of mammary tumours (Moser et al., 1993). Some studies targeting Apc mutation specifically to the mammary led to tumorigenesis (Kuraguchi et al., 2009), whilst others fail to observe tumour formation unless combined with other oncogenic mutations (Gallagher et al., 2002; Meniel et al., 2005).

As both APC proteins regulate Wnt, both are expressed in mammary epithelium and loss of either has been linked with breast cancer, there is a possibility redundancies exist between them. In this chapter I aimed to test the importance of Apc proteins in the murine mammary epithelium. Utilising mouse transgenics, I aimed to study the consequence of Apc loss, Apc2 loss and concomitant loss of both to elucidate possible functional redundancies.

The mammary gland represents a unique organ whereby the majority of development occurs postnatally (Figure 5.0). Whilst the ductal epithelial network can lay relatively

quiescent in mature virgins, pregnancy and associated hormone fluxes increase and transform this tissue into a milk producing organ capable of nourishing offspring.

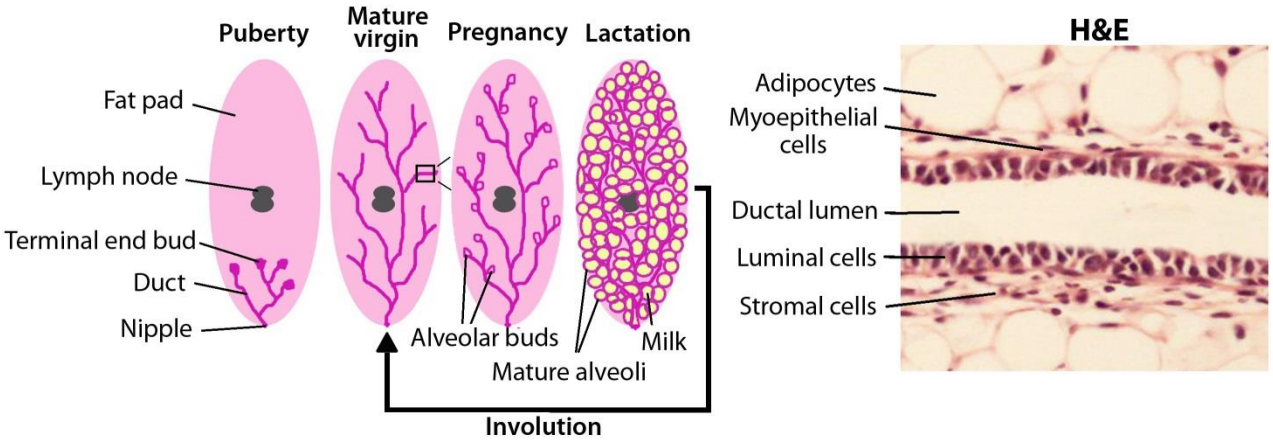

Figure 5.0 Mammary gland development. During puberty a ductal epithelial network extends from the nipple growing into and occupying the fat pad. Mature virgin mammary glands display a fat pad filled with this ductal network though the tissue remains quiescent (H&E stained cross section through one of these ducts reveals the tissue architecture). The mammary gland will remain in this state throughout adulthood unless pregnancy is attained. During pregnancy and through to lactation a plethora of hormones induce epithelial changes that prompt formation of milk producing alveola. This milk is released into the remaining duct structures and can be suckled through the nipple by the offspring. Following weaning the mammary gland tissue reverts back to a stage reminiscent of that of a mature virgin gland through the process of involution.

5.2 Results

5.2.1 Both Apc proteins are expressed in murine mammary epithelial tissue

Mammary glands were dissected from adult wild type (*Wt*) virgin mice and sections stained using fluorescent IHC with antibodies raised against Apc and Apc2 (Figure 5.1). Both Apc proteins displayed overlapping expression in mammary epithelial cells. As both of the antibodies used were raised against the C-terminus tails of Apc and Apc2 respectively where they share the least homology, this was less likely to be the effect of cross reactivity.

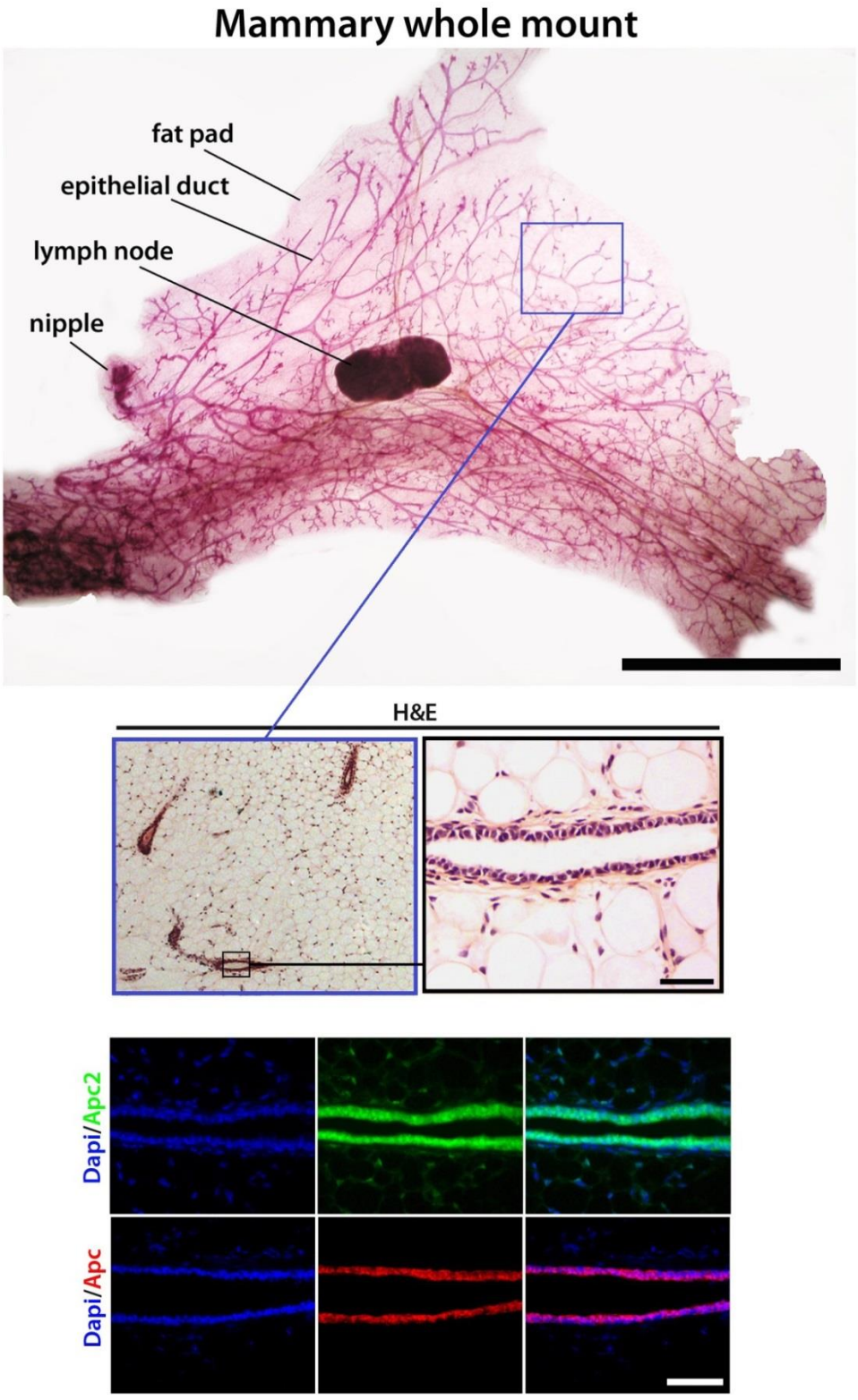

Figure 5.1 Both Apc and Apc2 proteins are expressed in the mammary epithelium. The top image represents a whole mounted mouse mammary gland, cleared for fat and stained with carmine alum (scale bar represents 1cm). Note ductal network of epithelial cells. The middle images represents H&E stained mammary gland section. Fluorescent IHC on Sections through ducts revealed both Apc and Apc2 are expressed in mammary epithelial cells (scale bar represents 50µm).

5.2.2 Targeting loss of Apc proteins in the mammary epithelium

To achieve Apc2 loss in the mammary epithelium, the constitutive *Apc2*$^{-/-}$ mouse was used (as described in previous chapters). Fluorescent IHC revealed comprehensive loss of Apc2 protein from *Apc2*$^{-/-}$ mammary epithelium (Figure 5.2).

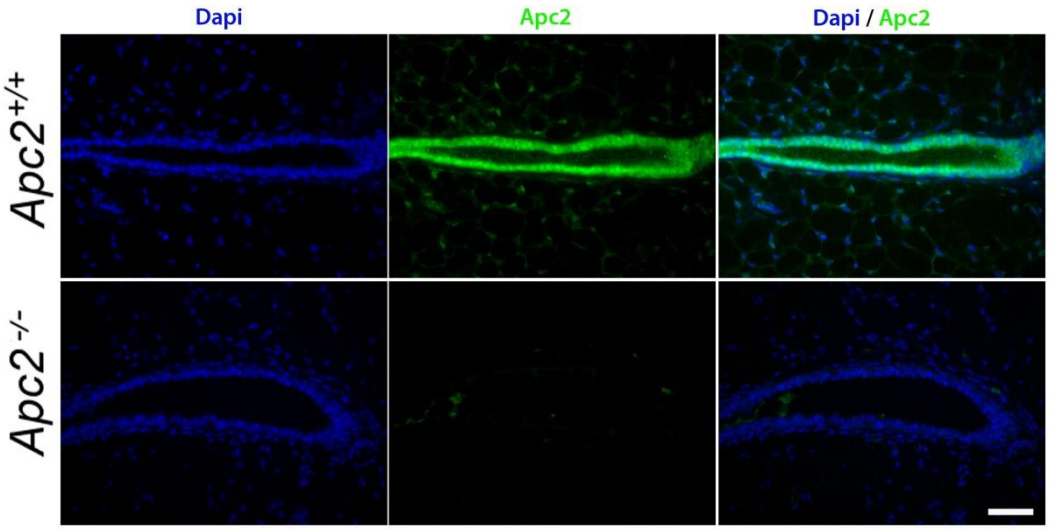

Figure 5.2 Apc2 expression is lost in *Apc2*$^{-/-}$ mammary epithelium. Fluorescent IHC was used to confirm Apc2 loss in the mammary epithelium (scale bar represents 50µm).

Constitutive Apc loss is embryonic lethal (Moser et al., 1995), so a Cre/loxP transgenesis system was employed to delete Apc from the mammary epithelium. The *Apc*$^{fl/fl}$ allele was used (described in Chapter 4) targeted by the *Blg-Cre*$^{+}$ promoter (Selbert et al., 1998). *Blg-Cre*$^{+}$ mice were crossed with a Cre *ROSA26* reporter strain (Soriano et al., 1999) to quantify cre mediated recombination in the mammary gland. Expression of the Cre recombinase resulted in recombination of the loxP flanked stop-cassette thus allowing the expression of the β-*galactosidase* gene. Mammary glands from 10wk old virgin *Blg-Cre*$^{+}$ *ROSA26*$^{+}$ mice were dissected and incubated with X-gal (a substrate for β-galactosidase). LacZ positivity indicated the recombination events had occurred in epithelial cells in a heterogeneous manner (Figure 5.3A). Fluorescent IHC with an anti- β-galactosidase antibody revealed recombination events in both luminal and myoepithelial cells in a mosaic fashion (Figure 5.3B). Fluorescent IHC against Apc on sections of *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mammary glands validated this heterogeneous pattern of loss in epithelial cells (Figure 5.3C). Epithelial cells retaining Apc expression showed nuclear localisation.

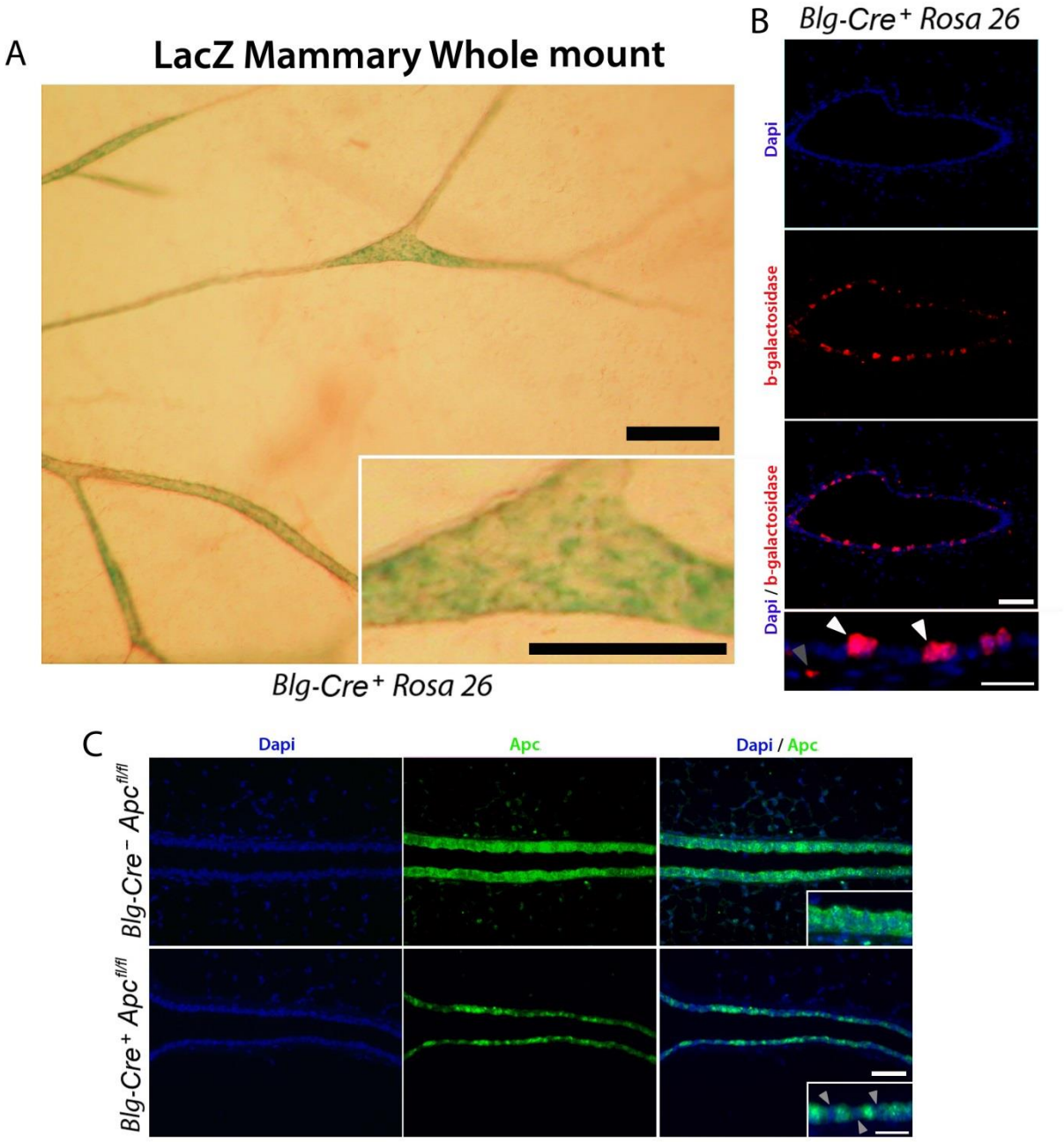

Figure 5.3 Apc deletion is driven heterogeneously using the *Blg-Cre* transgene. (**A**)Virgin *Blg-Cre*$^{+}$ *Rosa26*$^{+}$ mice were sacrificed at 10wks of age. Mammary whole mounts incubated with X-gal revealed a heterogeneous pattern of Cre recombination (scale bar 2mm). (**B**) *Blg-Cre*$^{+}$ *Rosa26*$^{+}$ mammary sections stained with a β-galactosidase antibody displayed recombination events in both luminal and myoepithelial cells, again in a mosaic manner (white arrowheads=recombination in luminal cells, grey arrowhead=recombination in myoepithelial cells, scale bar represents 50µm in main image, 25µm in higher magnified image). (**C**) IHC for Apc in *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice revealed a heterogeneous pattern of loss in epithelial cells. Epithelial cells retaining Apc expression showed nuclear localisation. (grey arrowheads indicate cells which have lost Apc expression, scale bar represents 50µm in main image, 25µm in inlet).

5.2.3 Combined loss of Apc and Apc2 induces loss of epithelial integrity and atypical ductal hyperplasia

In order to test the functions of Apc proteins in mammary homeostasis, four cohorts were generated (*Wt*, *Apc2*$^{-/-}$, *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ {termed *DKO* hereafter}). Virgin animals from each genotype were culled at 10wks of age (n≥3) and mammary glands examined. Following a 2h fixative period, mammary glands were stained overnight with carmine alum and cleared for fat (Figure 5.4A). Whole mount and H&E stained sections revealed both Apc and Apc2 alone are dispensable for epithelial integrity. Ductal branching, polarity and epithelial structure remained similar to *Wt* glands. Apc loss has previously been reported to show minimal effects on epithelial structure in virgin mice (Gallagher et al., 2002; Meniel et al., 2005). Strikingly, concurrent loss of both Apcs led to severe epithelial disruption. Carmine alum stained *DKO* whole mounted glands revealed an epithelial thickening, reduced branching and disruption of epithelial structure. This occurred with 100% penetrance in all glands of *DKO* mice (4 additional H&E stained biological replicate gland sections are displayed in Figure 5.4B). H&E stained sections of *DKO* glands displayed an increase in epithelial filled fat pad, increase in peri-ductal stroma and appearance of aggregates of 'ghost cells'. These have been reported extensively in a variety of human tumours and consist of anucleate 'cells' which stain positive for hard keratins and are most likely necrotic (Tanaka et el., 2007). Also, the epithelium had lost cohesion along with severe defects in polarity. Furthermore, an inflammatory response was also observed with macrophage giant cells being present.

Upon histopathological inspection (performed in collaboration with Prof. Geraint T. Williams) *DKO* lesions were classed as atypical ductal hyperplasia. As no mammary phenotype was observed in *Blg-Cre*$^{+}$ *Apc*$^{+/fl}$ *Apc2*$^{-/-}$ mice (data not shown), these phenotypic changes are thought to arise due to homozygous Apc loss in conjunction with Apc2 deficiency. Quantification of epithelial and stromal cells was carried out between genotypes by counting cell types per field of view (Figure 5.4C). Whilst loss of either Apc or Apc2 alone did not give rise to changes in cell number, combined loss induced statistically significant increases in both epithelial and stromal cell types per field of view (statistically significant verses all other genotypes $p \leq 0.001$, Mann-Whitney *U*-test, n≥3).

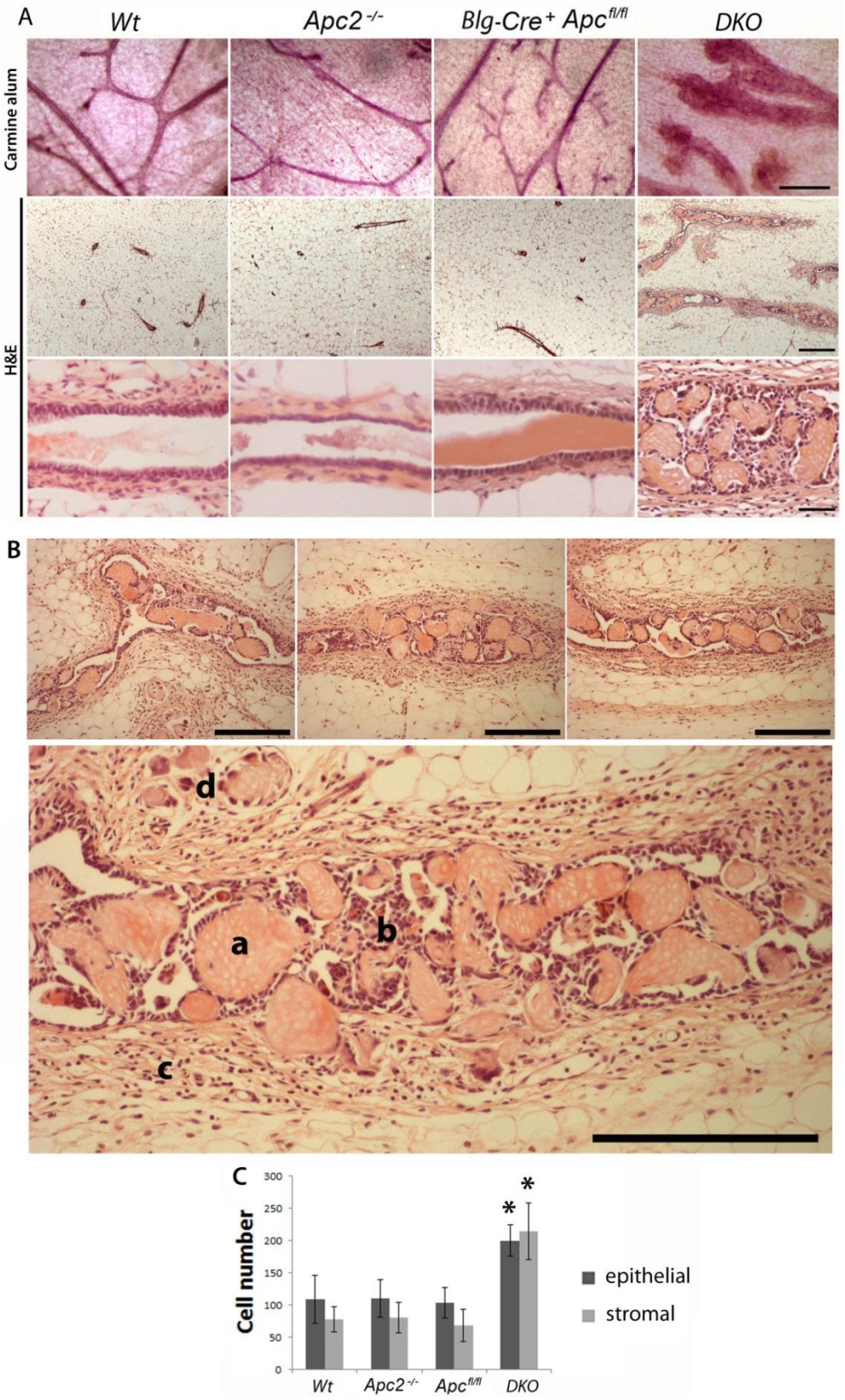

Figure 5.4 Combined loss of both Apc and Apc2 caused severe epithelial disruption, appearance of

'ghost' cells and increased epithelial and stromal cell number. (**A**) Top image displays comparative carmine alum stained whole mount glands and H&E stained sections from 10wk old virgin mice of each genotype. Whilst loss of either Apc protein alone is tolerated, combined loss caused severe defects in ductal branching and epithelial thickening (scale bar represents 2mm). H&E stained sections of mammary tissue from each genotype revealed epithelial disruptions in glands deficient for both Apc proteins (middle images=scale bar represents 1mm, bottom images=scale bar represents 50µm). (**B**) 4 additional H&E stained *DKO* biological replicate mammary epithelial sections;- **a**= 'ghost cell' aggregate, **b**= ductual thickening/hyperplasia/loss of polarity, **c**= increase in peri-ductual stroma and immune response, **d**= loss of epithelial cohesion/adhesion (scale bar represents 200µm). (**C**) Epithelial and stromal cells from each genotype were scored and quantified. Loss of either Apc or Apc2 had no effect on cell populations. However, combined loss lead to statistical increases in both epithelial and stromal cells (error bars represent standard deviation, asterisks mark $p \leq 0.001$ compared against all other genotypes, Mann-Whitney *U*-test, $n \geq 4$).

Rates of cell turnover are relatively low in the mammary epithelium (Neese et al., 2002) especially compared to that of the intestines (Reya & Clevers, 2005), a tissue grossly affected by Apc loss (Sansom et al., 2004). Mitotic figures were readily observable in *DKO* epithelium whilst seldom in other genotypes, consistent with a hyperplastic phenotype. To confirm increases in proliferation, mammary sections from each genotype were labelled using an anti-Ki-67 antibody (Figure 5.5A). Quantification of positively labelled cells revealed a statistical increase in percent of dividing cells in *DKO* mammary epithelium compared to all other genotypes (statistically significant *DKO* verses all other genotypes $p<0.01$, Mann-Whitney *U*-test, $n \geq 3$). Interestingly, *Apc2*$^{-/-}$ displayed a significant decrease in proliferation compared to *Wt* mammary epithelium ($p \leq 0.05$, Mann-Whitney *U*-test, $n \geq 3$).

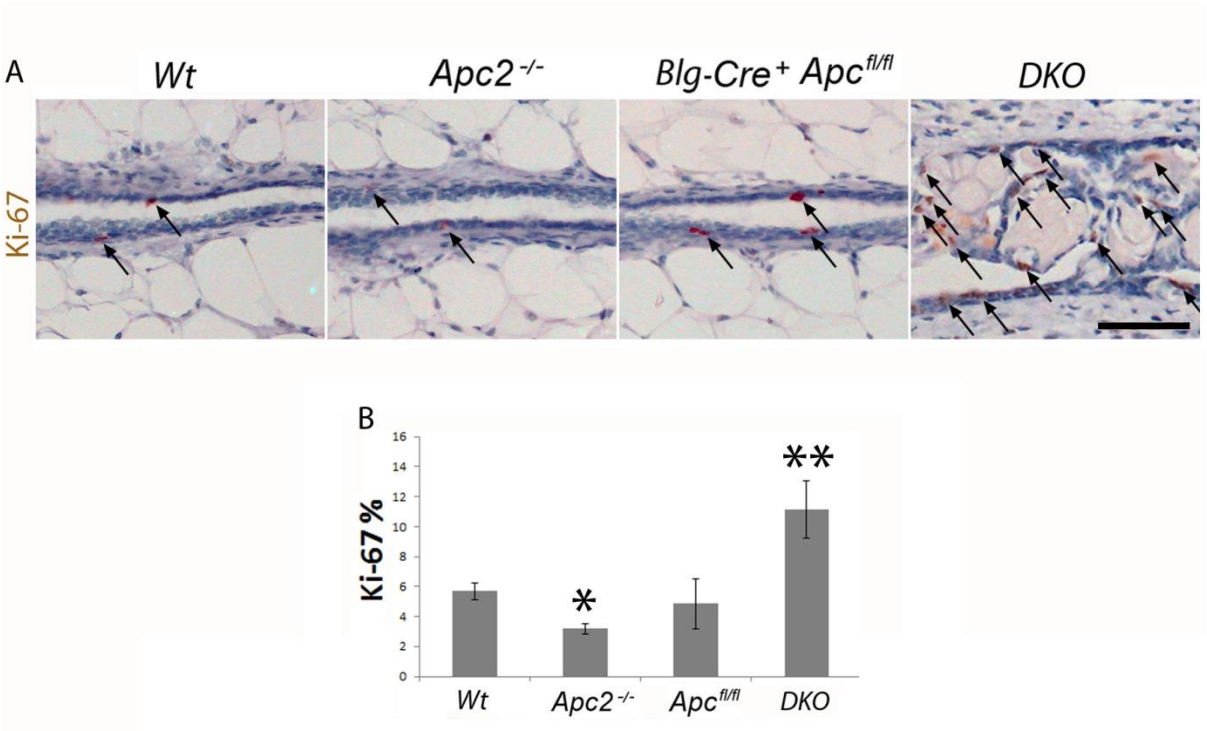

Figure 5.5 Combined loss of Apc and Apc2 induced proliferative increases. (**A**) Sections from each genotype where stained with an anti-Ki-67 antibody. An increase in labelled cells was noticed in epithelium deficient for both Apc proteins (arrows indicate positively labelled cells, scale bar represents 50µm). (**B**) Quantification of positively labelled cells revealed a reduction in proliferation in *Apc2*$^{-/-}$ compared to *Wt* epithelium. However, a statistical increase was noticed in epithelium deficient for both Apc proteins verses all other genotypes (error bars represent standard deviation, * marks p≤0.05 verses *Wt*, ** marks p≤0.01 compared against all other genotypes, Mann-Whitney *U*-test, n≥3).

5.2.4 Functional redundancies exist between Apc proteins in mammary epithelial Wnt transduction

The majority of intestinal phenotypes associated with Apc loss are thought to occur due to its influence on Wnt signaling (Hanson et al., 2005; Nathke, 2005; Polakis, 2000). Both Apc proteins share the ability to mediate Wnt signaling through β-catenin degradation (Nakagawa et al., 1998; Van Es et al., 1999) and activating β-catenin mutations have been previously reported to induce mammary gland hyperplasia (Michaelson et al., 2001; Imbert et al., 2001), thus this pathway was addressed. Mammary epithelium from each genotype

were labelled using IHC with an anti- β-catenin antibody. Mammary epithelium from Apc2$^{-/-}$ and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ displayed no difference in β-catenin protein location in comparison to *Wt* (Figure5.7). β-catenin status was compared using fluorescent IHC between *DKO* epithelium and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ as a control (Figure 5.6). β-catenin in *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ epithelium displayed a cytoplasmic and membrane bound expression pattern, similar to that observed in *Wt* tissue. The highest expression was detected on the apical surface in a polarised manner (Figure 5.6A). Additional loss of Apc2 (*DKO* epithelium) disrupted this staining pattern in a heterogeneous manner. Some cells displayed a generalised expression pattern where all cellular locations were labelled (Figure 5.6B). Other single cells and clumps of cell displayed up-regulation including classical nuclear β-catenin staining (Figure 5.6C). Unexpectedly, some regions appeared to lose detectable β-catenin from all cell locations (Figure 5.6D). 'Ghost cells' labelled for neither β-catenin nor nuclear material (Figure 5.6E).

Serial section from each genotype at 10wks of age were labelled using IHC for Apc, β-catenin, cMyc and CD44 (Figure 5.7). Loss of Apc2 or Apc alone did not display changes in β-catenin localisation or detectable changes in cMyc or CD44 expression. However, combined loss in certain areas, induced increased staining intensity along with nuclear translocation in mammary epithelium. cMyc was also detectable in cells displaying nuclear β-catenin consistent with an activation of the Wnt signaling pathway, However, CD44 remained undetectable in cells deficient for both Apc proteins. Taken together, these results suggest that Apc proteins co-operate in Wnt signal transduction within the mammary epithelium however, that this pattern is complex and does not lead to uniform activation of the Wnt pathway in the majority of cells.

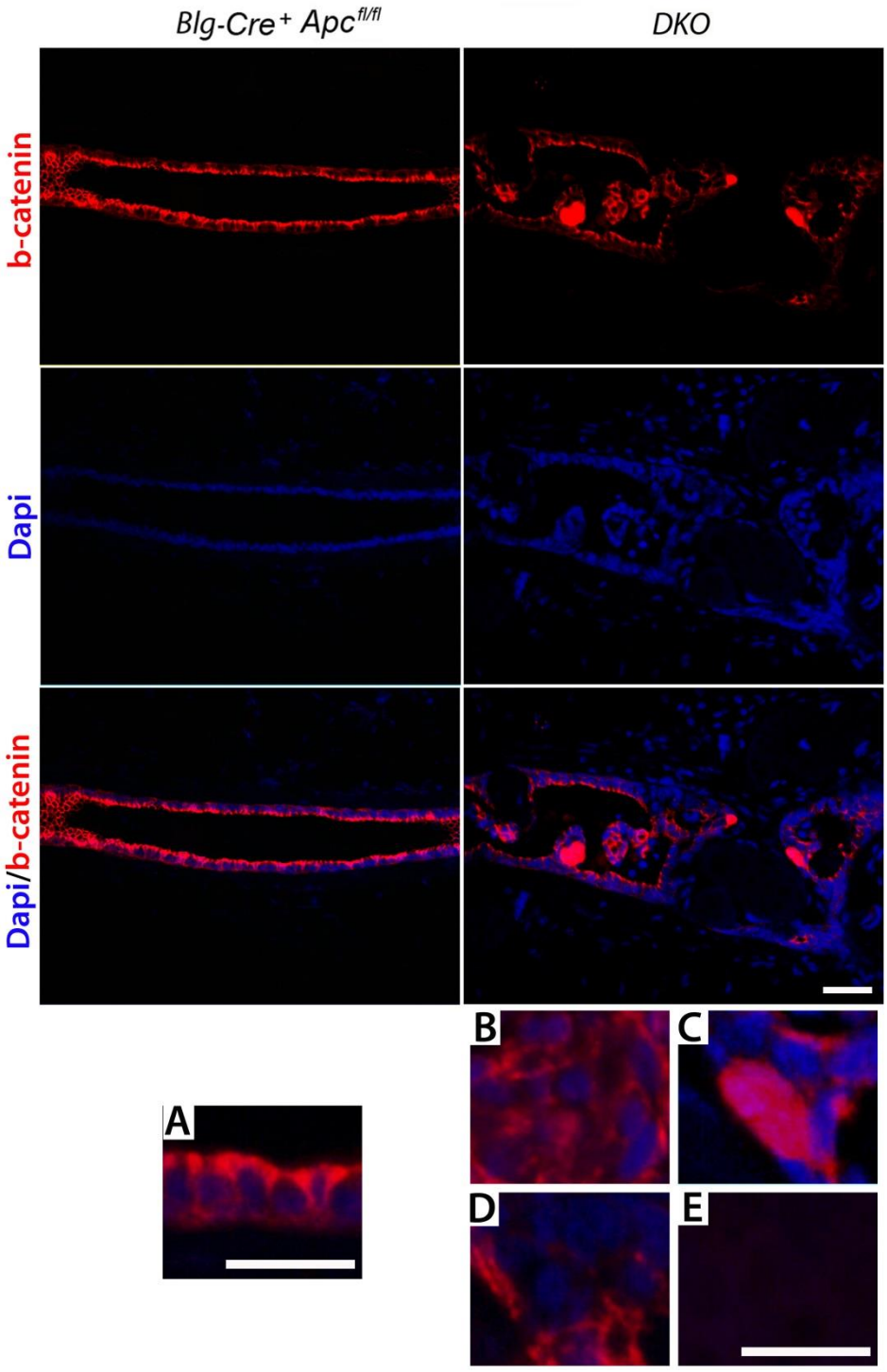

Figure 5.6 Combined loss of both Apc proteins disrupted β-catenin. Sections from *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ and *DKO* mammary epithelium were stained fluorescently with an anti-β-catenin antibody and compared. Higher magnification images of top image displayed below. (**A**) Epithelium deficient for Apc alone displayed a cytoplasmic and membrane associated staining pattern consistent with other genotypes. Staining was at its highest intensity towards the apical surface in a polarised manner. In mammary tissue deficient for both Apc proteins, epithelial β-catenin was disrupted. Certain areas displayed (**B**) mis-localized, (**C**) strong nuclear or even (**D**) absent epithelial β-catenin staining. (**E**) Ghost cells display no Dapi or β-catenin staining (scale bar represents 50µm in top image, 25µm in bottom image).

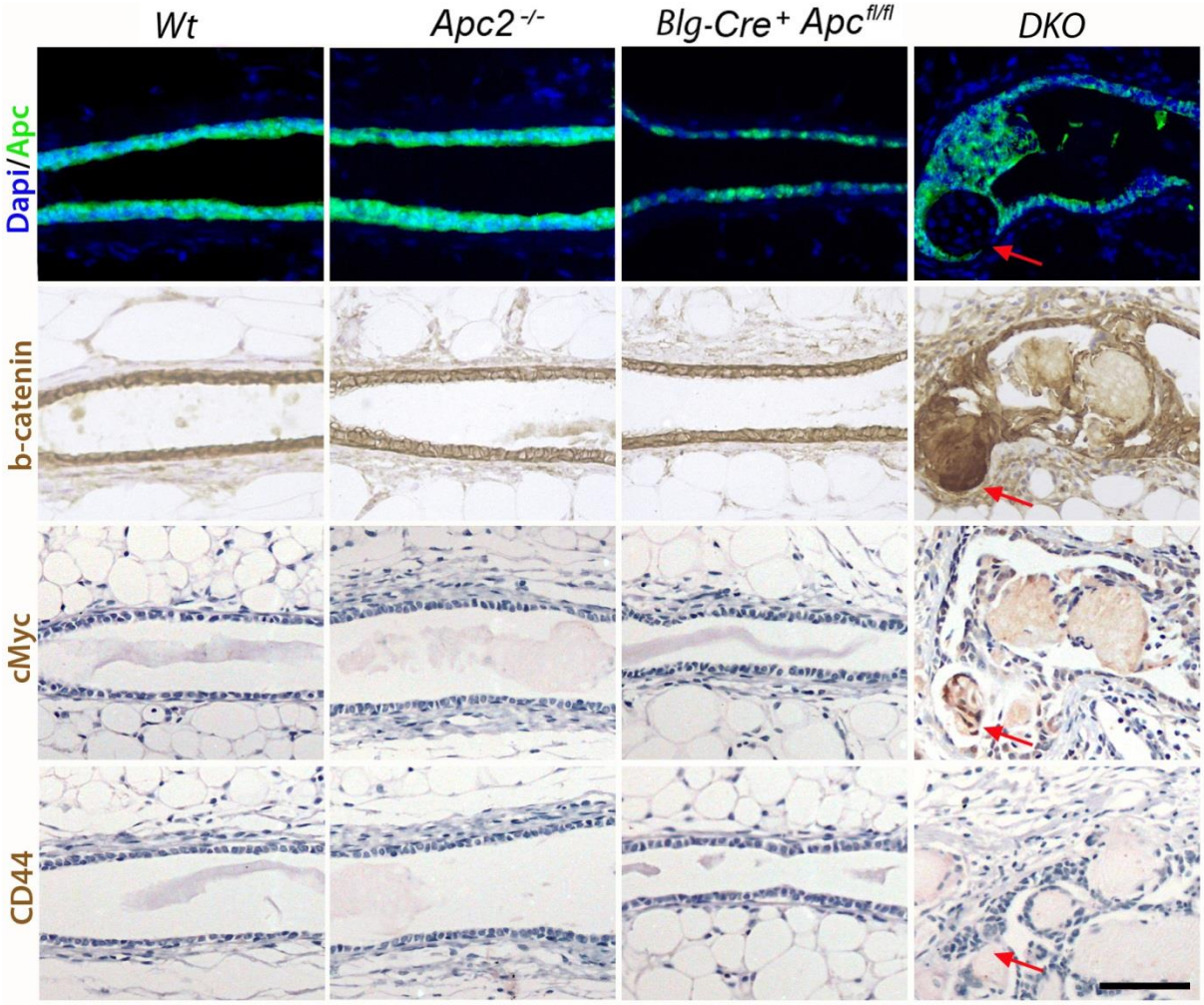

Figure 5.7 Combined loss of Apc and Apc2 caused activation of the Wnt signaling pathway in a subset of cells. Serial sections of mammary epithelium from each genotype were labelled with Apc, β-catenin, cMyc and CD44. Inactivation of either Apc protein alone did not induce changes in β-catenin localisation or status of Wnt targets. Combined loss induced up-regulation & nuclear translocation of β-catenin and activated expression of the Wnt target gene cMyc. CD44 expression was undetectable in any genotype (arrow indicates area deficient for Apc, scale bar represents 50μm).

5.2.5 Combined Apc and Apc2 loss induce ghost cell formation and increases in apoptosis

'Ghost cells' have been reported extensively in a variety of human tumours and consist of anucleate 'cells' which stain positive for hard keratins (hair proteins) and are most likely necrotic (Tanaka et el., 2007). Principally, tumours containing these cells arise in the head and neck region (Hong et al., 1991; Marrogi et al, 1992; Kumaran et al., 2006) although occurrence has been reported in the breast (Raman et al., 2000; Rosner et al., 2002;

Hernanz et al., 2012). A previous report has suggested aberrant Wnt signaling may influence ghost cell formation in odontomas (Tanaka et el., 2007). Ghost cells occur in all *DKO* mammary epithelium indicative that Wnt signaling likely plays some role, at least in the context of mammary epithelial origin. Sections of *DKO* mammary glands stained for H&E occasionly revealed areas of apparent transition between normal seeming epithelium through to structures with a ghost cell appearance (Figure 5.8). As cells move through transition apoptotic bodies become perceptible, the cells start expressing a pink material reminiscent of keratin and become increasingly anucleate. Serial sections through

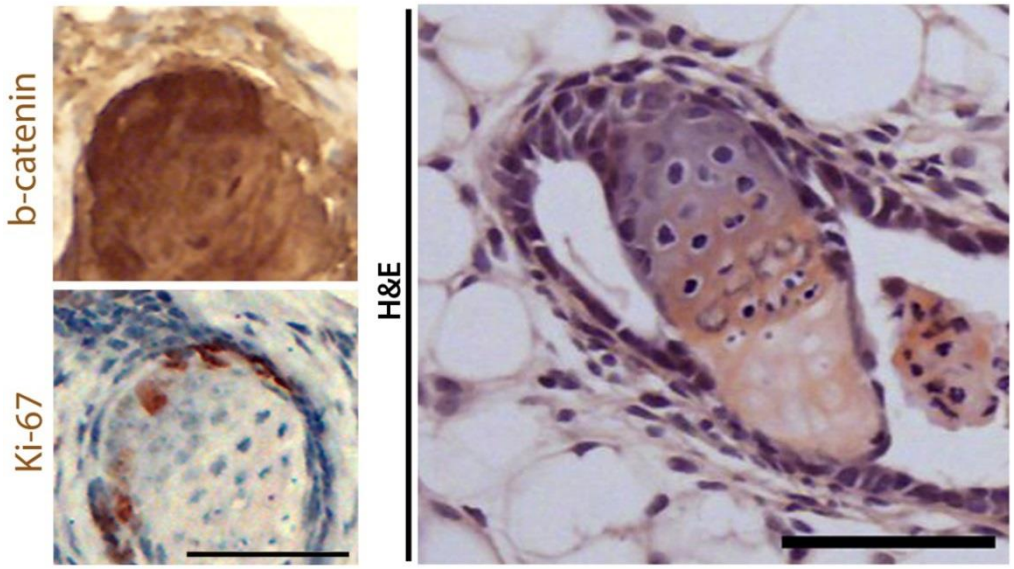

Figure 5.8 Ghost cells appear associated and may arise from Wnt activated cells. A foci of cells stained with H&E display transition from epithelial to a ghost cell appearance. Cells become anucleate during transition with an increase in apoptotic fragments. IHC on serial sections revealed nuclear translocation and up-regulation of β-catenin in the in the early stages before transition, concurrent with Ki-67 staining. As cells leave this region they gradually adopt a ghost cell appearance (scale bar represents 25μm).

these foci suggest in the early stages of possible transition Wnt signaling is activated and the cells are cycling. Taken together these results suggest combined loss of Apc and Apc2 leads to activation of the Wnt signaling pathway, hyper-proliferation of epithelial cells and an ultimate transition to a ghost cell morphology, at least in a subset of cells. To assess the extent of cell death, mammary gland sections from each genotype where stained with an

anti-cleaved caspase-3 antibody (Figure5.9A). Positive cells were seldom observed in *Wt* epithelium or epithelium deficient for either Apc protein. However, in glands deficient for

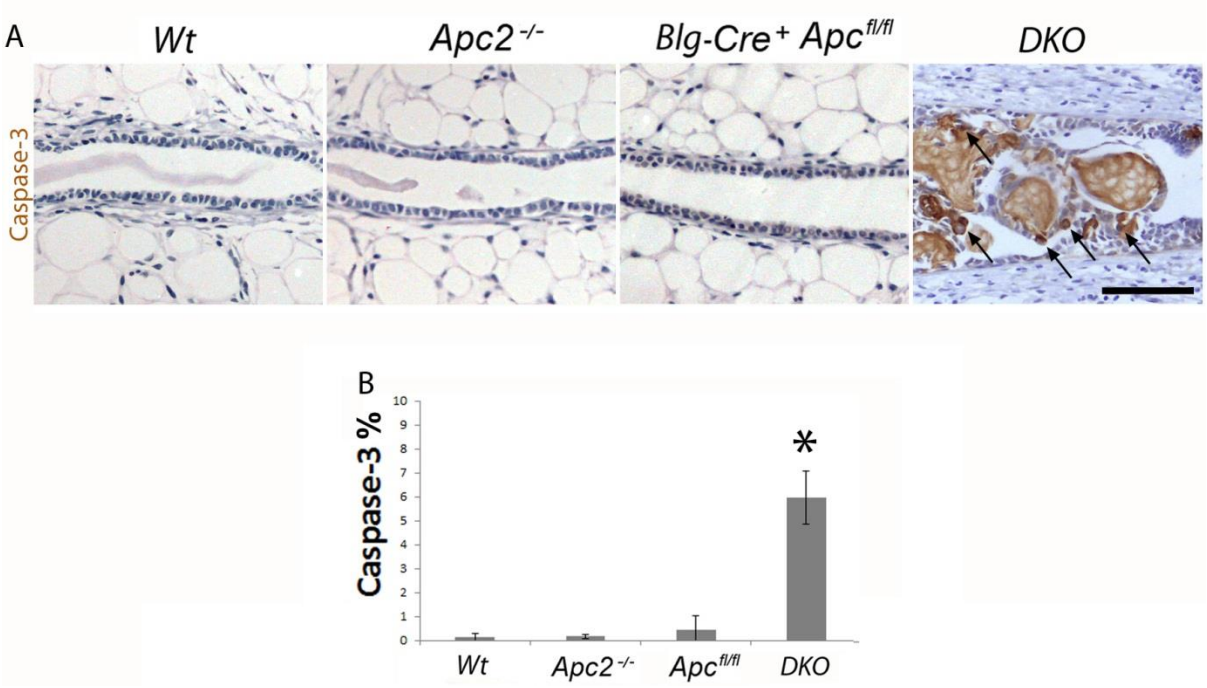

Figure 5.9 Loss of both Apc proteins led to an increase in cell death. (**A**) Sections from each genotype were stained with an anti-caspase-3 antibody. *Wt* cells or epithelial cells deficient for either Apc or Apc2 alone, very rarely labelled for caspase-3. In *DKO* epithelium there was an increase in labelled cells (arrows indicate positively labelled cells, scale bar represents 50µm). (**B**) Quantification of positively labelled cells revealed a statistical increase in apoptosis in epithelium deficient for both Apc proteins (error bars represent standard deviation, asterisks marks p≤0.001 compared against all other genotypes, Mann-Whitney *U*-test, n≥3).

both Apc proteins there was a vast increase in labelled cells. Quantification of these cells (Figure 5.9B) revealed a statistical increase in *DKO* tissue compared against all other genotypes (statistically significant for *DKO* verses all other genotypes p≤0.001, Mann-Whitney *U*-test, n≥3).

5.2.6 Apc proteins co-operate in maintaining epithelial polarity

Along with hyperplasia and ghost cell formation at 10 weeks of age, *DKO* epithelium display defects in polarity (Figure 5.10). *Apc*$^{-/-}$ and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ glands appear indifferent from *Wt.* Subsequent analyses were therefore performed between *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ and *DKO* mammary glands to compare the effects of additional Apc2 loss in the context of *Apc* deletion. Sections from *DKO* and *Blg-Cre*$^{+}$ *Apc*$^{fl/f}$ were double labelled for cytokeratin 8 (luminal cell marker) and cytokeratin 5 (myoepithelial cell marker) using fluorescent IHC. In *Blg-Cre*$^{+}$ *Apc*$^{fl/f}$ epithelium cells were organised in a highly polarised manner consistent with that observed in *Wt* tissue. Loss of both Apc proteins induced severe disruption of polarity. Luminal and basal (myoepithelial) cells lost positional identity and were located haphazardly. E-cadherin is the principle component of adheren junctions imperative in cell-cell adhesion (for review see Gumbiner, 1996). Zo-1 is a component of tight junctions critical for maintaining barrier function (Umeda et al., 2006) Together these proteins help establish and maintain epithelial polarity (Ando-Akatsuka et al., 1999). In *Blg-Cre*$^{+}$ *Apc*$^{fl/f}$ epithelium, Zo-1 is normally maintained in an apical position and E-cadherin at a position consistent with cell-cell contacts. However, in *DKO* epithelium Zo-1 was almost completely diminished along with cells displaying a loss of E-cadherin. Together these results show functional redundancies between Apc proteins in the maintenance of polarity and tight junction functionality.

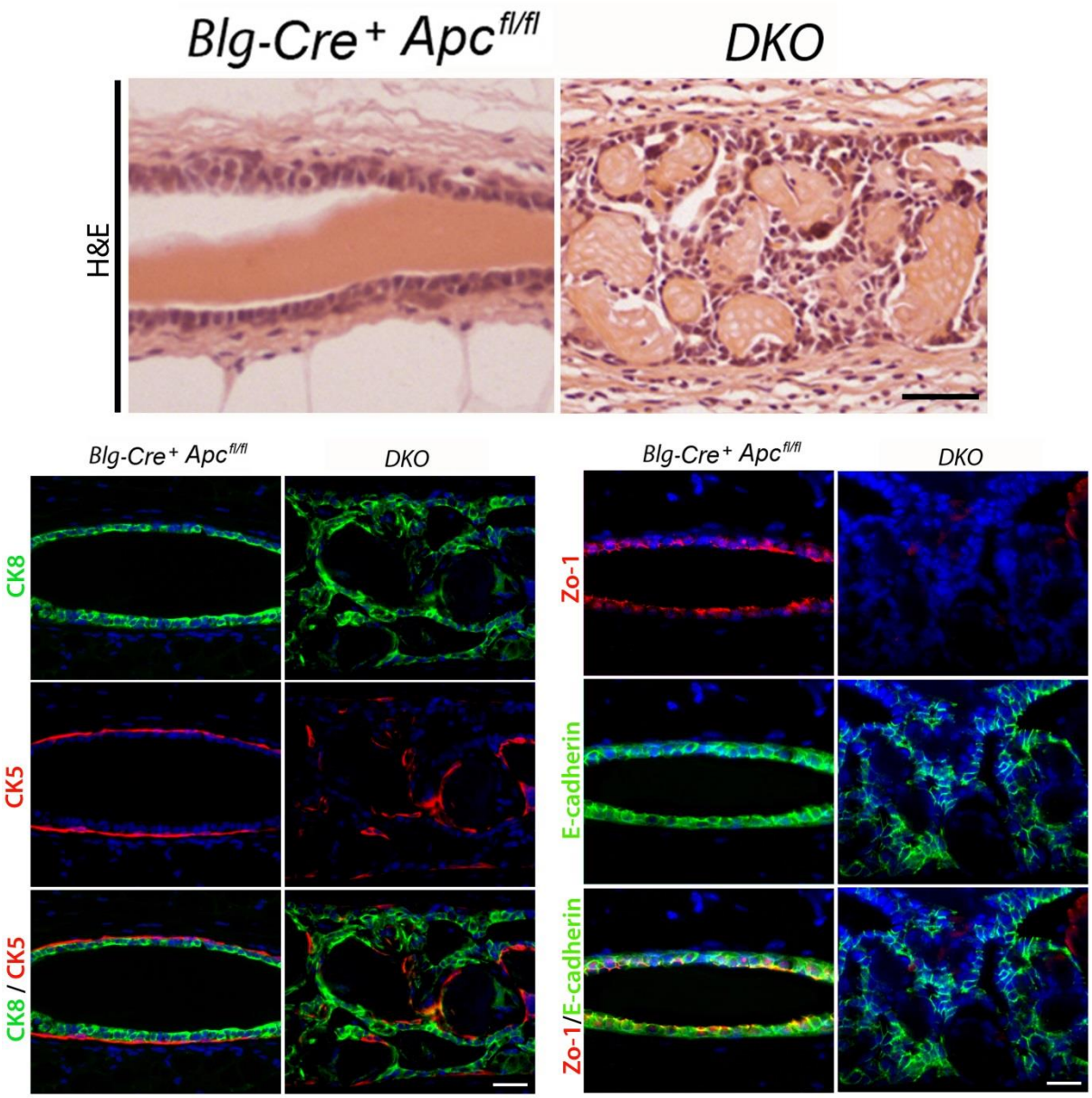

Figure 5.10 Combined loss of both Apc proteins led to polarity defects. Top image represents H&E stained mammary sections from epithelium with Apc loss compared to epithelium deficient for both Apc and Apc2. Bottom left image represents the same comparison with sections double labelled using fluorescent IHC for cytokeratin 8 (CK 8, luminal cell marker) and cytokeratin 5 (CK 5, basal cell marker). Note disruption in polarisation of epithelium deficient for both Apc proteins. Bottom right image represents again the same comparison but double labelled using fluorescent IHC for both Zo-1 and E-cadherin. Note loss of polarity marker Zo-1 and E-cadherin in mammary epithelium deficient for both Apc proteins (scale bar represents 50µm).

5.2.7 Combined epithelial loss of both Apc proteins leads to local tissue invasion and characteristics reminiscent of EMT

Upon further examination of H&E stained *DKO* mammary sections, occasional epithelial cells appeared to lose continuity with neighbouring epithelium and 'invade' local surrounding tissue (Figure 5.11). 'Invading' cells lost β-catenin expression but some still retained residual cytokeratin 5 and/or 8 labelling indicating an epithelial lineage origin. These cells may occur as a consequence of duct rupture caused by a build-up of ghost cell aggregates. However, cell damage was not overtly obvious and nor was death of these cells. Indicators of epithelial-mesenchymal transition (EMT) further corroborate the hypothesis that these cells have acquired invasive capabilities. Whilst *Blg-Cre*$^{+}$ *Apc*$^{fl/f}$ epithelium retained E-cadherin expression and were negative for vimentin, some *DKO* mammary epithelial cells displayed a loss of E-cadherin coincident with strong vimentin expression (Figure 5.12). Snail expression showed no correlation with these cells or with β-catenin status (data not shown).

Given both Apc and Apc2 possess the ability to directly interact with the cytoskeleton and microtubules (Näthke, 2006; Shintani et al., 2012), microtubule status was provisionally assessed in both *Blg-Cre*$^{+}$ *Apc*$^{fl/f}$ and *DKO* mammary sections using an anti-α-tubulin antibody (Figure 5.12). In *Blg-Cre*$^{+}$ *Apc*$^{fl/f}$, epithelium staining displayed a tightly bound pattern consistent with a maintained regulation. However, in *DKO* mammary epithelium, staining revealed a disorganised pattern consistent with a disruption of microtubules. High magnification images comparing *Blg-Cre*$^{+}$ *Apc*$^{fl/f}$ and *DKO* epithelial cells reveal Apc loss in the context of Apc2 deficiency can lead to β-catenin loss, as oppose to nuclear accumulation (Figure 5.13A). Cells also lost E-cadherin and Zo-1 expression suggesting a non-canonical route of cytoskeletal disruption. Confocal images comparing α-tubulin between genotypes revealed a reduction of microtubules (Figure 5.13B). Together these results suggest some phenotypes associated with combined loss of both Apc proteins may be exerted through compromised functional redundancies relating to cytoskeletal regulation. This is consistent with the consensus for a multi-functional role for Apc proteins (Aoki and Taketo, 2007; Senda et al., 2007; Hanson et al., 2005; Nathke, 2005; Polakis, 2000).

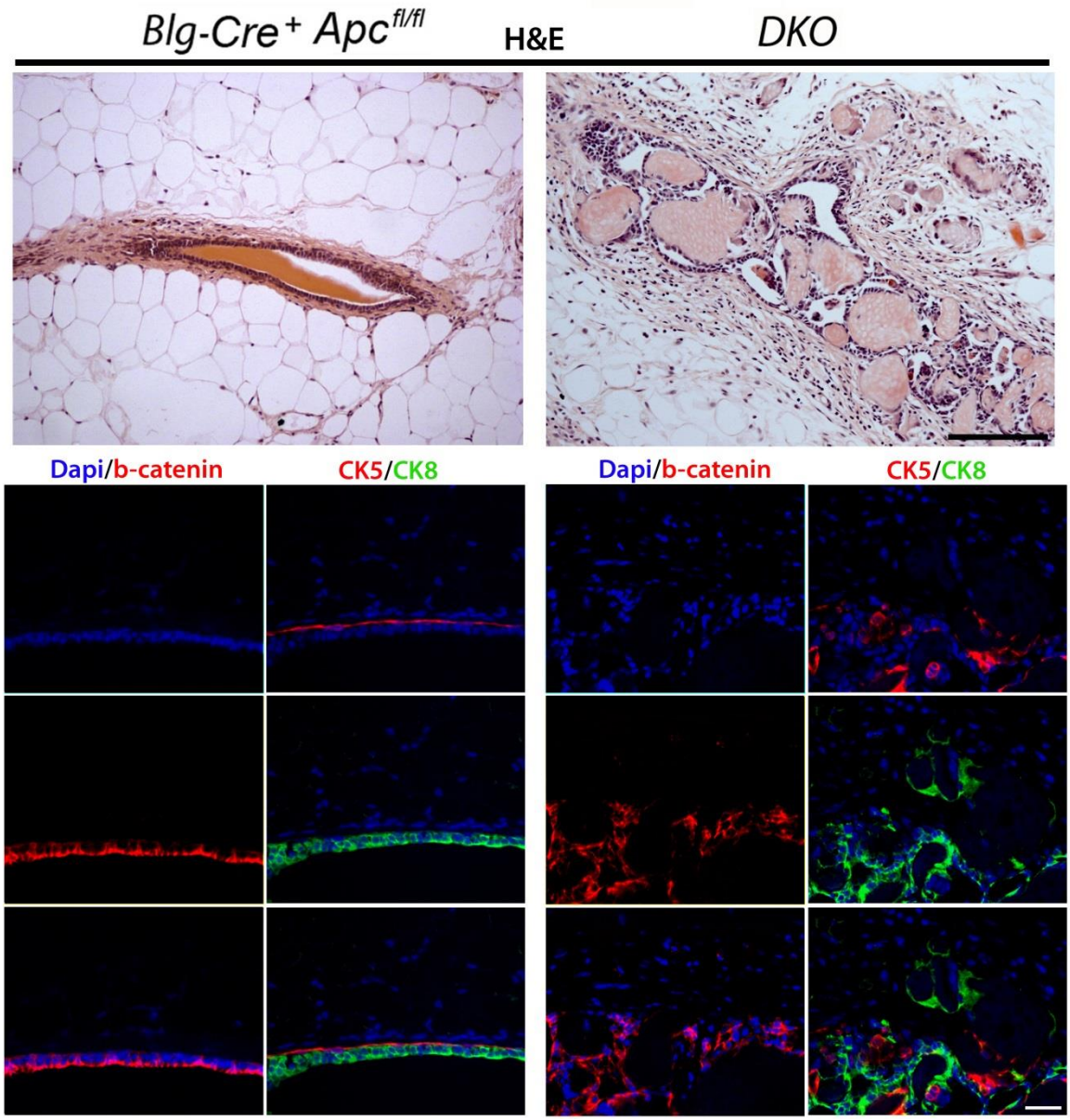

Figure 5.11 Apc loss in the context of Apc2 deficiency leads local tissue invasion. Top image represents H&E stained mammary sections from epithelium with Apc loss compared to epithelium deficient for both Apc and Apc2 (scale bar represents 150µm). Note cells 'floating' in extracellular matrix with epithelial appearance in glands deficient for both Apc proteins. Bottom image represents epithelium deficient for Apc on left and both Apc and Apc2 on the right. Serial sections were labelled using fluorescent IHC for β-catenin and double labelled for both CK 5 and CK 8. In glands deficient for Apc, β-catenin remained membrane bound with strong apical localisation, consistent with maintained epithelial architecture. In mammary glands deficient for both Apc proteins, β-catenin was disrupted along with disrupted epithelial architecture. Occasional cells lost β-catenin expression and detached from the epithelial structure maintaining some cytokeratin 5 and/or 8 expression (scale bar represents 50µm).

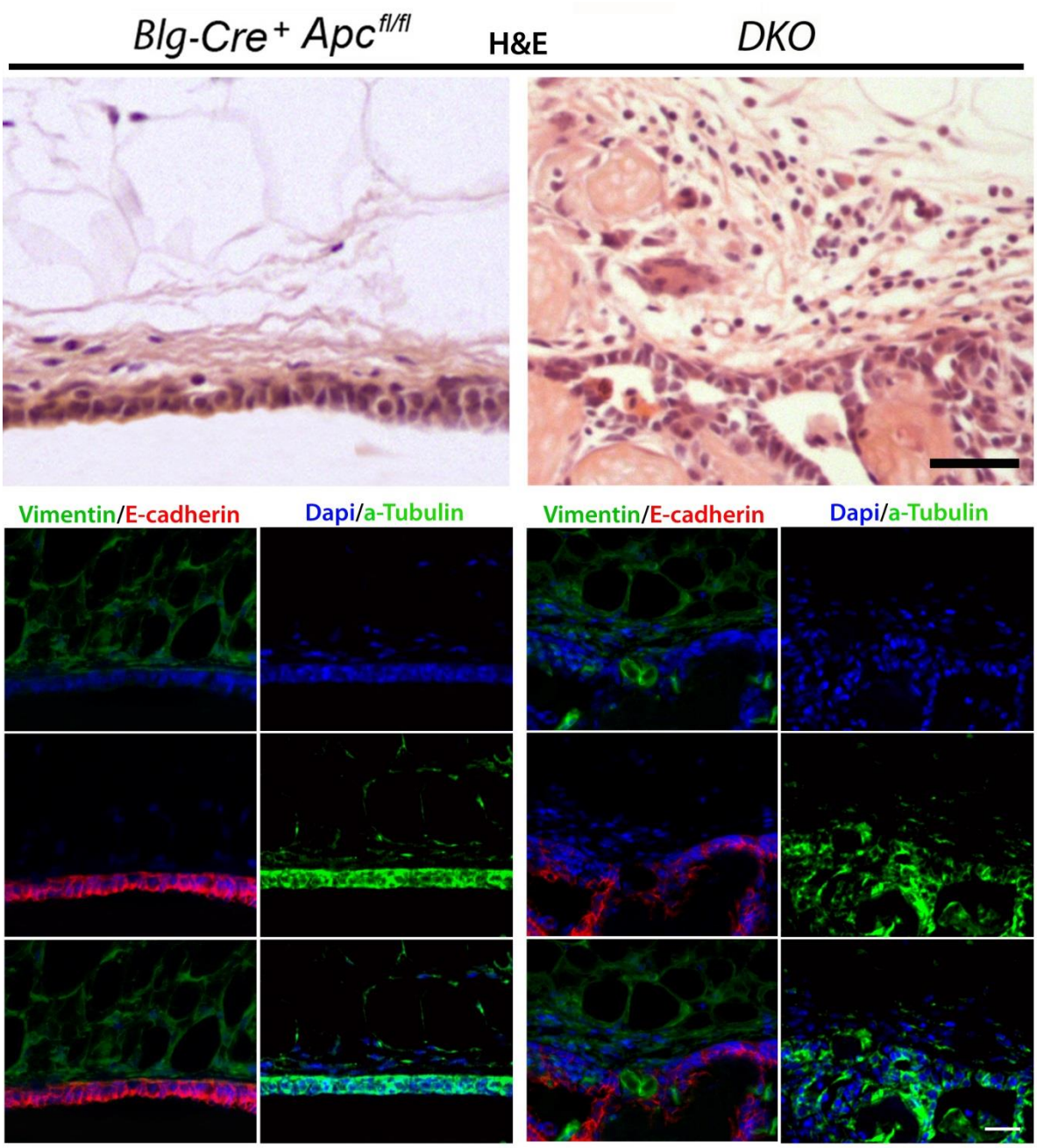

Figure 5.12 Epithelial cells deficient for both Apc proteins displayed evidence of EMT and disruption in microtubules. Images on the left represent glands deficient for Apc and on the right glands deficient for both Apc proteins. In epithelium with Apc loss, E-cadherin was expressed in all cells and mostly membrane associated. Combined loss led to a disruption in E-cadherin expression and a loss in some cells. Cells negative for E-cadherin now stain positive for vimentin, consistent with EMT. Staining with α-tubulin revealed a disruption in microtubules in epithelium deficient for both Apc proteins (scale bar represents 50μm).

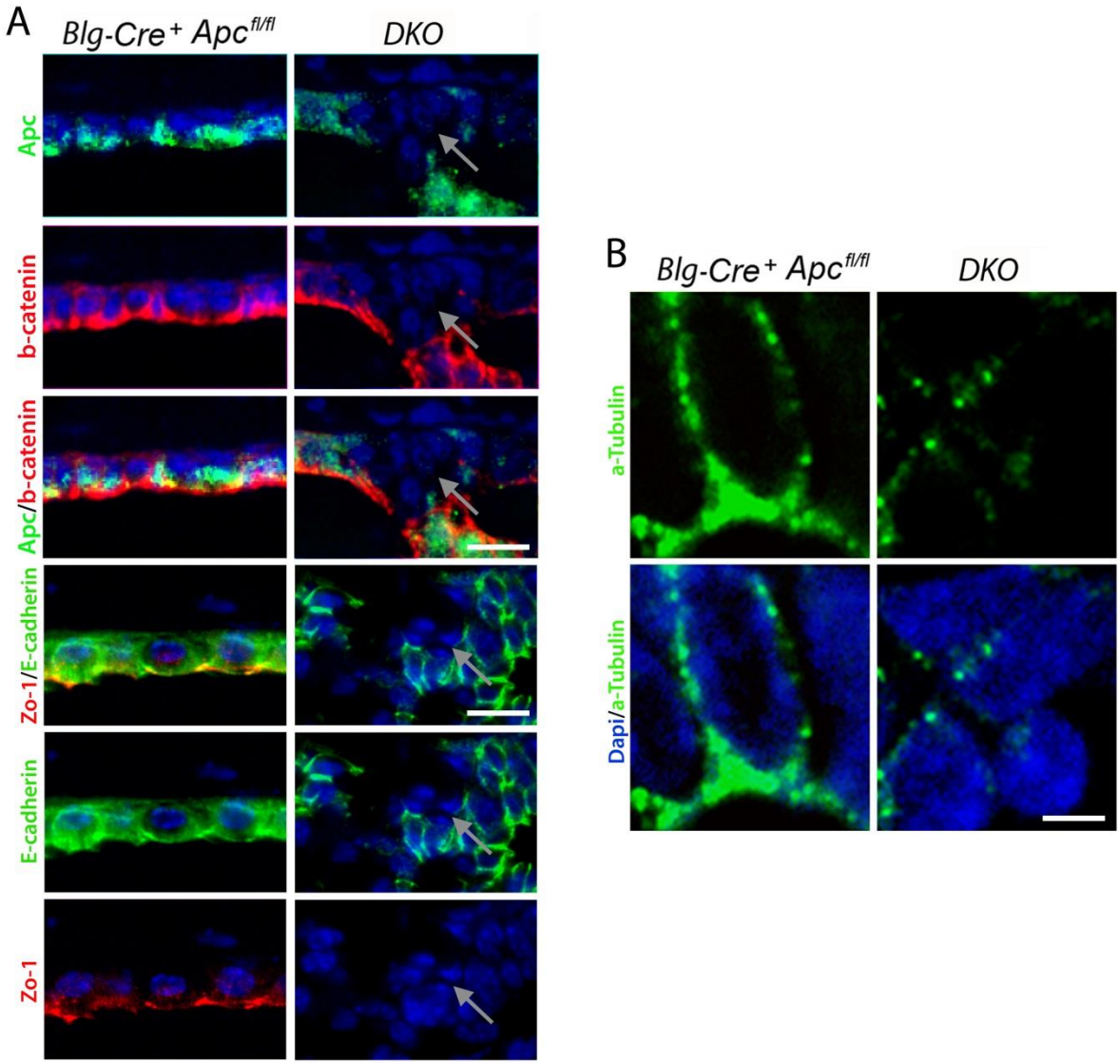

Figure 5.13 Apc loss in the context of Apc2 deficiency caused cytoskeletal disruptions. (**A**) Sections of epithelial cells labelled for Apc, β-catenin, Zo-1 and E-cadherin compared between glands deficient for Apc and glands deficient for both Apc and Apc2. Loss of Apc in addition to Apc2 deficiency caused loss of β-catenin, E-cadherin and Zo-1 (arrows represents cells with additional Apc loss, scale bar represents 15 µm). (**B**) Confocal images display disrupted microtubules in epithelial cell deficient for both Apc proteins (scale bar 5µm).

To further explore the consequences of combined mammary epithelial Apc and Apc2 loss whilst excluding the influence of Apc2 deficient non-epithelial cells, mammary epithelial culture was employed. Mammary epithelium from *Apc*$^{fl/f}$ and *Apc*$^{fl/f}$ *Apc2*$^{-/-}$ mice were separated from other cell types and grown in culture (see methods). Apc2 deficiency did not adversely affect epithelial cells or affect the ability of these cells to form a polarised monolayer. An adenovirus containing a Cre-recombinase expression construct (Ad-CMV-Cre) was used to infect epithelial cells and target Apc loss. Previous studies have proven a high rate of recombination in infected mammary cultures (Rijnkels & Rosen., 2001; Naylor et al., 2005). High rates of recombination were also observed with our chosen MOI (multiplicity of infection) of 50 gauged though titre experiments (data not shown). Infection of *Apc*$^{fl/fl}$ epithelial cultures and subsequent Apc loss caused negligible differences and these cells still maintained their ability to form a polarised monolayer. However, in infected cultures permitting combined Apc and Apc2 loss, cells lost their ability to grow in monolayer and cell-cell contact was perturbed consistent with defects in adhesion and polarity. In addition to this, cell shape changes arose, with transformation from cuboidal epithelial towards a more fibroblastic appearance. Together these changes are consistent with an EMT event. Furthermore, these '*in vitro*' experiments demonstrate functional redundancy exists between the two Apc proteins within the mammary epithelial cells, in order to maintain cell polarity, adhesion and prevent EMT. The role of supportive non-epithelia cells, which would also be deficient for Apc2 in *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice does not seem to be important or compensatory for this element of functional redundancy noticed within the '*in vitro*' cultured epithelial cells.

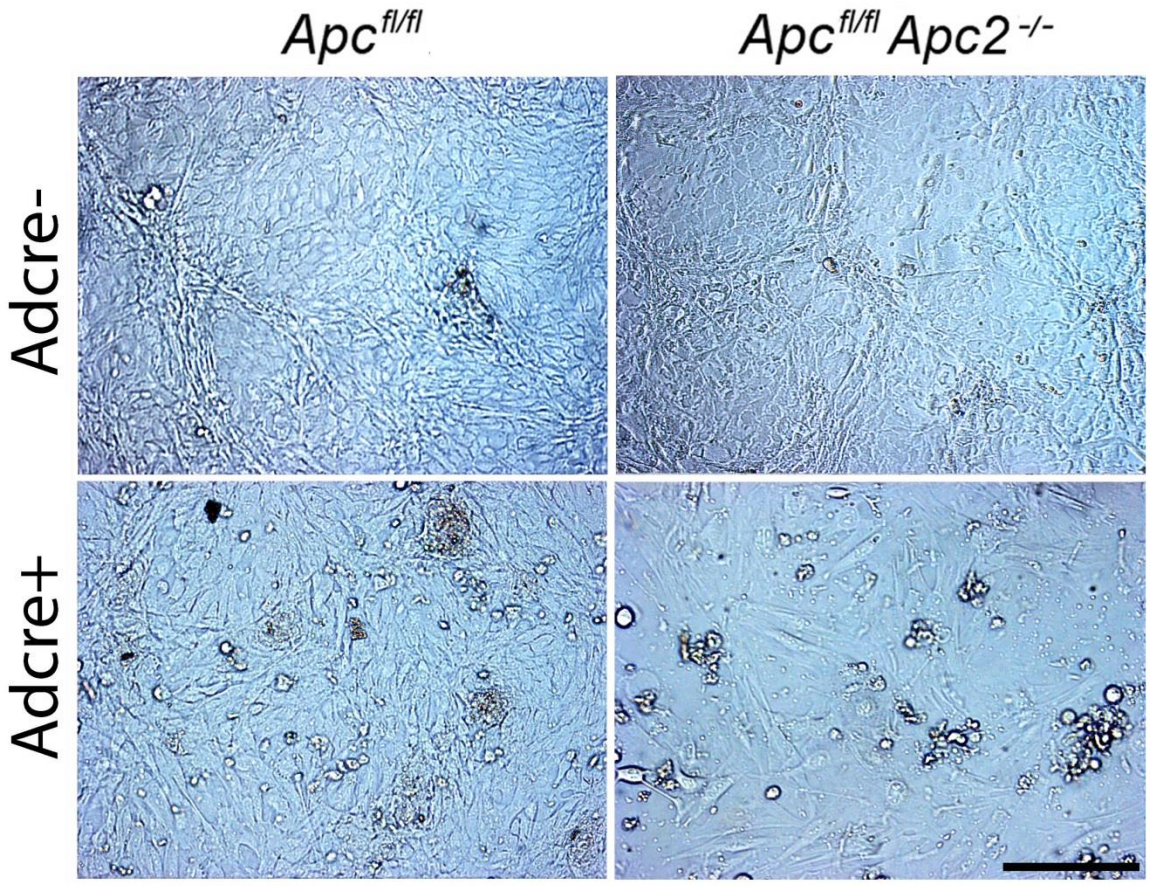

Figure 5.14 Combined Apc and Apc2 deficiency induced loss of adhesion, polarity and cell shape changes reminiscent of EMT. *Apc$^{fl/fl}$ and Apc$^{fl/fl}$ Apc2$^{-/-}$* mammary epithelial cells were harvested and grown in culture. Infection with an Adenoviral-Cre to delete *Apc* induced cellular changes only in conjunction with Apc2 deficiency. Cells lost their ability to grow in monolayer, indicative of polarity loss. Cells also displayed reduced adhesion and shape changes characteristic of EMT (scale bar represents 100µm).

5.2.8 Apc proteins co-operate in tumour suppressor function

The altered characteristics observed in mammary epithelium deficient for both Apc proteins at a short term time point (10 weeks of age) are physiognomies usually altered in tumourigenesis. Thus, to examine the role of both Apc proteins in tumourigenesis, cohorts containing different combinations of mutations were aged and sacrificed upon signs of ill health (Figure 5.15A). These cohorts were *Wt*, *Apc2$^{-/-}$*, *Blg-Cre^{+} Apc$^{fl/fl}$*, *Blg-Cre^{+} Apc$^{fl/fl}$ Apc2$^{+/-}$* and *Blg-Cre^{+} Apc$^{fl/fl}$ Apc2$^{-/-}$*. Mice deficient for Apc2 or Apc alone displayed no differences in

survival from *Wt* however, additional Apc2 loss in the context of *Apc* mutation led to reduced survival. Further reduced survival was observed in *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ compared to *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{+/-}$ mice, indicating an *Apc2* gene dose dependent effect in the context of Apc loss. Mice with combined epithelial loss of both Apc proteins developed mammary tumours (Figure 5.15B). Upon histopathological inspection (Figure 5.15C) (performed in collaboration with Prof. Geraint T. Williams), tumours were deemed invasive with 'spiky' edges (Figure 5.15D). Ghost cells were still apparent (Figure 5.15E) and some areas displayed squamous differentiation with areas of keratin production prominent (Figure 5.15F). The majority of mammary tumours analysed displayed similar characteristics and together were classified as Squamous carcinoma. Importantly, these findings demonstrate that functional redundancies exist between Apc proteins in tumour suppressor function.

Given *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{+/-}$ mice also developed tumours, mammary tissue was examined at an early time point (10wks of age). Whole mount and histological analyses revealed abnormalities similar to that in *DKO* glands although the severity was somewhat reduced (Figure 5.16). This suggests that Apc2 has a gene dose effect in maintaining epithelial integrity in the context of Apc loss. Mammary tumour incidence was recorded at the time of death for all genotypes (Figure 5.17A). Whilst *Wt*, *Apc2*$^{-/-}$ or *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ glands showed no signs of tumour formation, 46% of *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{+/-}$ and 80% of *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice had tumour tissue in one or more of their mammary glands. This suggests that Apc2 has a gene dose effect in tumour suppression in the context of Apc loss.

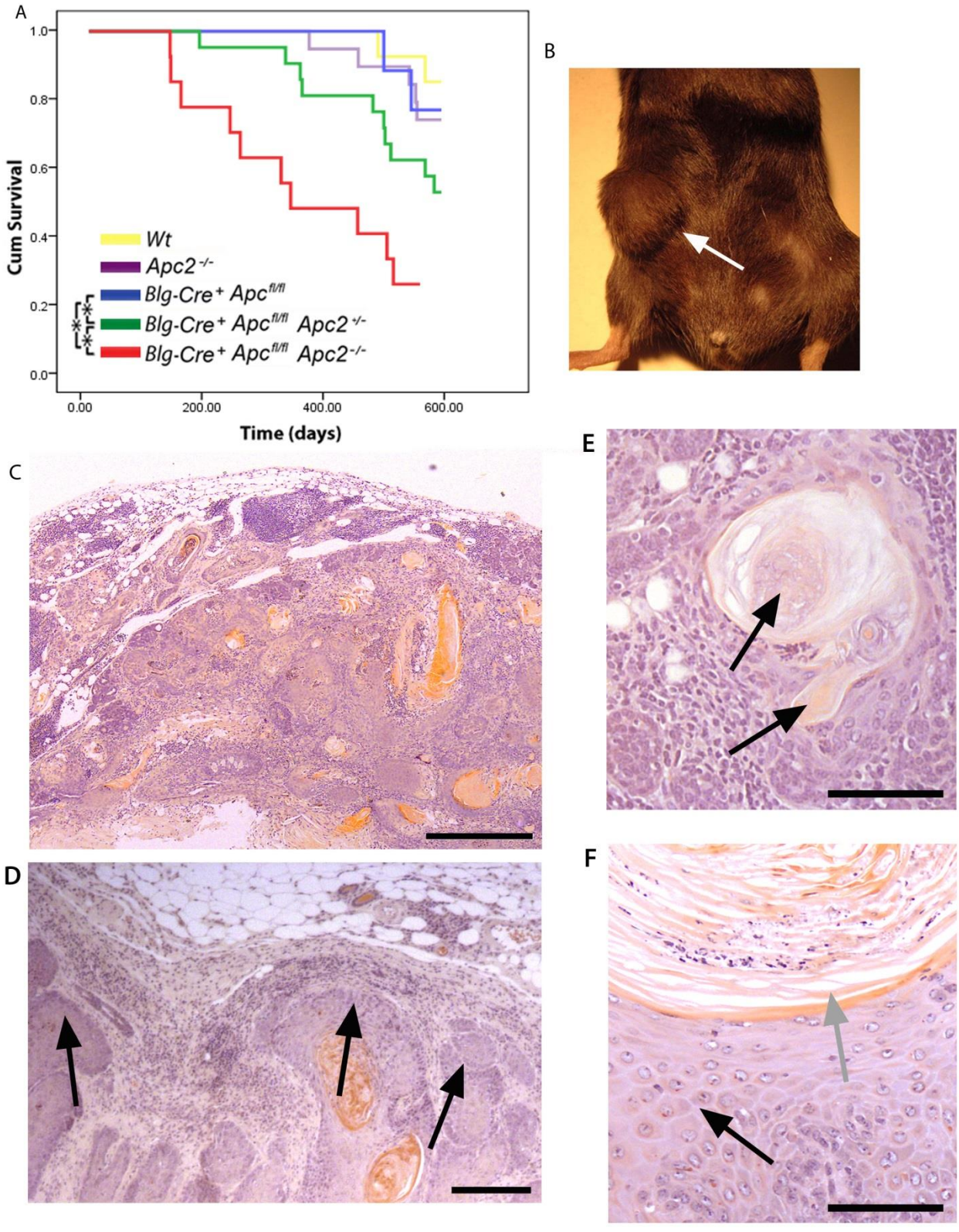

Figure 5.15 Combined deficiency of both Apc proteins in mammary epithelium resulted in mammary tumour formation with age. (**A**) *Wt* (yellow line), *Apc2*$^{-/-}$ (purple line), *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ (Blue line), *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{+/-}$ (green line) and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ (red line) mice were aged out and culled upon signs of ill health (survival presented as Kaplan-meier). *Apc2*$^{-/-}$ and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice displayed no alterations in survival compared to *Wt* mice (Log-rank test, $p>0.32$, $n\geq11$). Both *Blg-Cre*$^{+}$

Apc$^{fl/fl}$ *Apc2*$^{+/-}$ and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice displayed reduced survival compared to other genotypes (asterisks mark statistically different comparisons, p≤0.01, Log-rank test, n≥11). Both *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{+/-}$ and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice displayed reduced health due to mammary tumours. (**B**) Image of a mammary tumour 'in situ' before dissection. (**C**) Representative image of H&E stained section of dissected mammary tumour. Histopathological examination characterised tumours as squamous carcinoma (scale bar represents 2mm). Magnified images show (**D**) 'spiky' tumour periphery invicative of local area tumour invasion (black arrows) (scale bar represents 200µm). (**E**) Ghost cells are still occasionly present (black arrows) (scale bar represents 50µm), (**F**) Squamous differentiation (black arrow) and keratin material/whirls are also present (gray arrow) (scale bar represents 50µm).

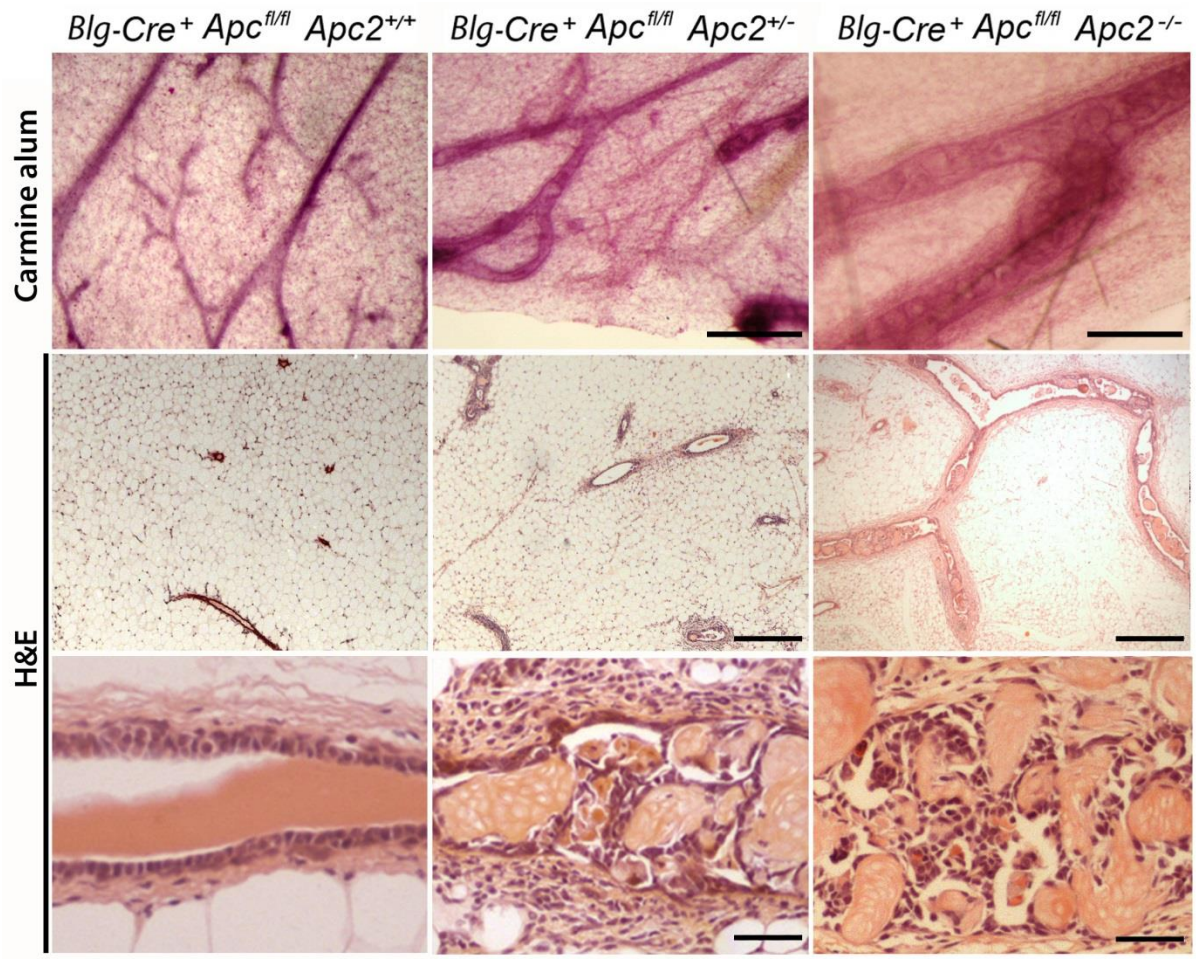

Figure 5.16 Apc2 has a gene dosage effect in the context of homozygous Apc loss in mice at 10wks of age. Top image displays comparative whole mount sections stained with carmine alum and bottom images display corresponding H&E stained sections. *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{+/-}$ mammary tissue displayed similar defects to *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ although to a lesser extent (top images= scale bar represents 2mm, middle images=scale bar represents 1mm, bottom images=scale bar represents 50µm).

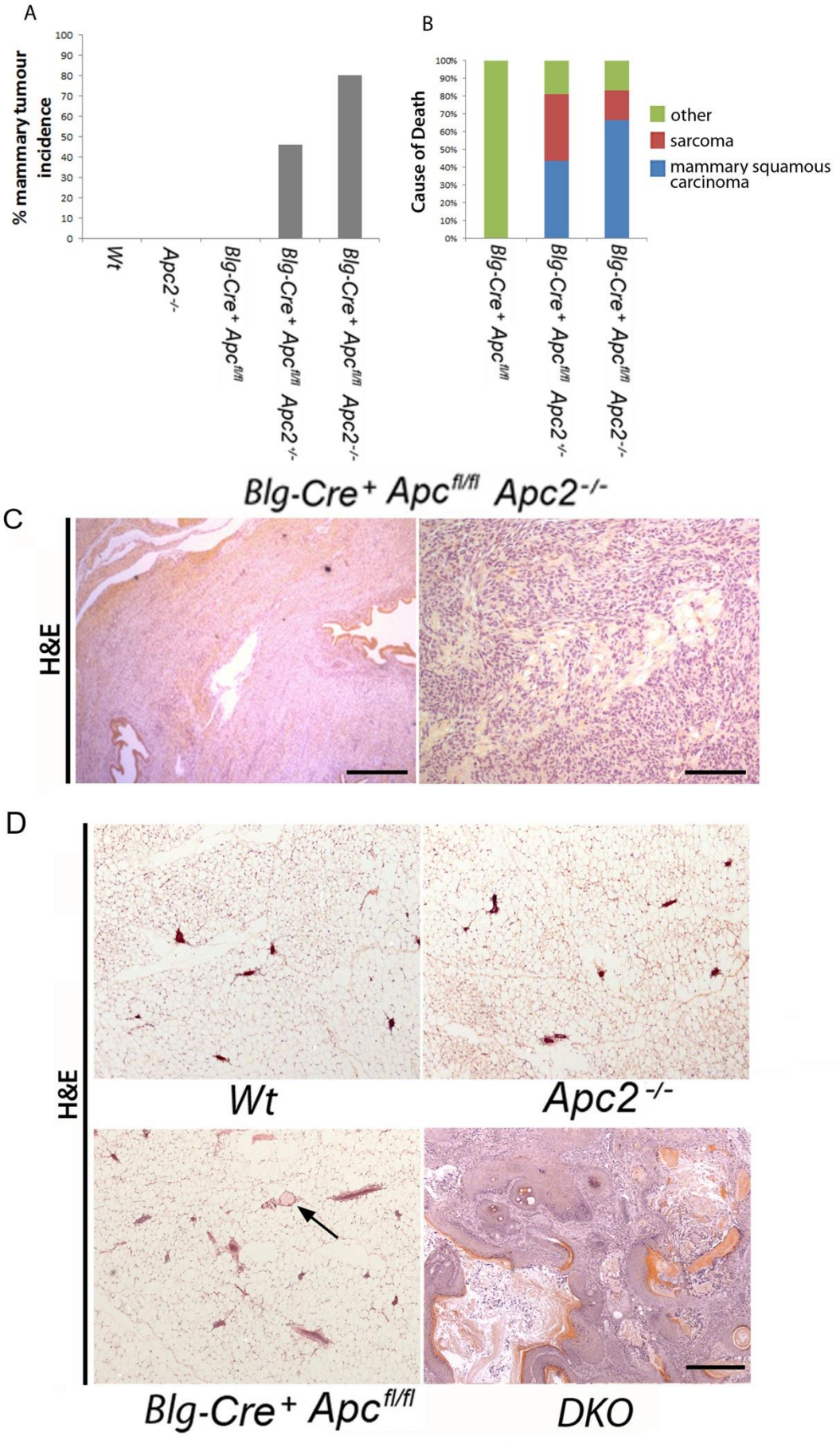
A
% mammary tumour incidence
100
90
80
70
60
50
40
30
20
10
0
Wt
Apc2-/-
Blg-Cre+ Apcfl/fl
Blg-Cre+ Apcfl/fl Apc2+/-
Blg-Cre+ Apcfl/fl Apc2-/-
B
Cause of Death
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
other
sarcoma
mammary squamous carcinoma
Blg-Cre+ Apcfl/fl
Blg-Cre+ Apcfl/fl Apc2+/-
Blg-Cre+ Apcfl/fl Apc2-/-
Blg-Cre+ Apcfl/fl Apc2-/-
C
H&E
D
H&E
Wt
Apc2-/-
Blg-Cre+ Apcfl/fl
DKO

Figure 5.17 Mammary tumours are only apparent in mice with combined Apc and Apc2 loss. (**A**) Mice were examined for presence of mammary tumours at death. Whist *Wt, Apc2*$^{-/-}$ and *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice display no signs of mammary tumorigenesis, 46% of *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{+/-}$ and 80% of *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice exhibited lesions. This suggests a gene dose dependent effect of Apc2 in the context of Apc deletion. (**B**) Cause of death was determined and confirmed by a pathologist. Three main pathologies were identified; mammary squamous carcinoma, sarcoma and other (comprising mainly of HCC, lymphoma and unknown). Whilst *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice all fall into the 'other' category, additional *Apc2*$^{+/-}$ or *Apc2*$^{-/-}$ mutation not only induced an increase in mammary squamous carcinoma, but also sarcoma. (**C**) Example of Sarcoma found in uterine (scale bar represents 1mm & higher maginification 100μm). (**D**) Representative images of mammary gland sections taken from aged mice. Lesions are only present in *DKO* glands. However, small infrequent patches of ghost cells can be found in *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mammary glands (arrow indicates a small patch of ghost cells, scale bar represents 1mm).

Mice from aging cohorts were checked regularly and killed either if general health visually deteriorated or if mammary tumours arose and surpassed a set size, blistered or restricted movement. Cause of death was determined by internal inspection at time of death and/or histopatological examination of harvested organs (performed in conjunction with Prof. Geraint T. Williams). Three categories for the cause of death were comprised; 'mammary squamous carcinoma', 'sarcoma' or 'other' (mostly consisting of lymphoma, HCC and undetermined). *Wt* and *Apc*$^{-/-}$ fall into the 'other' category (discussed in Chapter 3). All *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice also fall into the 'other' category (Figure 5.17B). However, although no differences were noticed in their survival, death was almost certainly attributed to HCC (Discussed in next chapter). Interestingly, in cohorts with deficiency for both Apc proteins, along with a cause of death due to mammary squamous carcinoma, there was an increase in the occurrence of sarcoma. Sarcomas were mostly detected in the uterine and abdomen, in most cases they were poorly differentiated deeming origin hard to determine (Figure 5.17C). Histological analysis of mammary tissue taken at time of death revealed lesions only in glands deficient for both Apc proteins (Figure 5.17D). Although no lesions were present in *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mammary glands, occasional small clusters of ghost cells were observed.

Activation of the Wnt signaling pathway is a common event in a range of tumours (Polakis, 2000; Giles et al., 2003; Reya & Clevers, 2006) including those of the breast (Khalil et al., 2012). As both Apc proteins share the ability to influence Wnt signaling (Nakagawa et al., 1998; Van Es et al., 1999) and combined loss of both in the mammary epithelium leads to

Wnt perturbations, pathway status was addressed in harvested tumours (Figure 5.18). Serial sections from tumours were stained with H&E, β-catenin using florescent IHC and cMyc and CD44 using normal IHC. β-catenin displayed a heterogeneous pattern of nuclear expression. Tumours stained positive for both cMyc and CD44. Together, these results indicate activation of the Wnt signaling pathway in tumours that arise from mammary epithelium deficient for both Apc proteins.

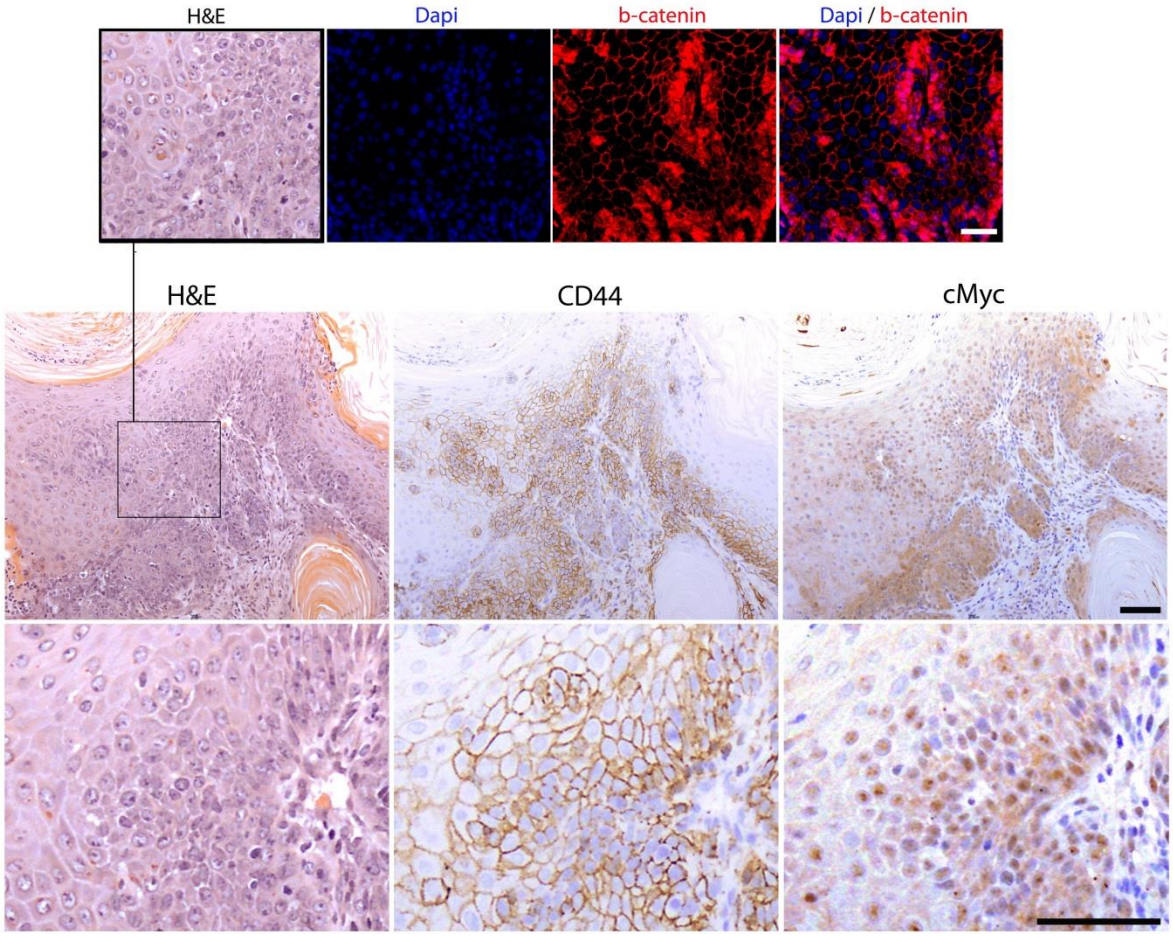

Figure 5.18 Mammary tumours arising in glands with epithelium deficient for both Apc proteins display Wnt activation. Serial sections of tumour tissue were stained with H&E, β-catenin, cMyc and CD44. β-catenin displayed a heterogeneous pattern of nuclear expression. Both cMyc and CD44 were expressed in the tumours demonstrating Wnt activation (top + bottom images scale bar represents 50μm, middle images scale bar represents 100μm).

Axillary lymph nodes represent the first 'port of call' for mammary tumour metastases and incidence of these metastases correlates with reduced patient survival (McGuckin et al., 1996; Weigelt et al., 2005). As combined epithelial loss of both Apc proteins caused local

tissue invasion in young mouse glands and also invasive properties in subsequently developing tumours, lymph nodes were analysed for signs of invasion and metastases. Sections of mammary glands containing lymph nodes from aged *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ and *DKO* mice with tumours were stained with H&E (Figure 5.19). Whilst lymph nodes from *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ displayed a true periphery with epithelial tissue remaining on the outside (all glands

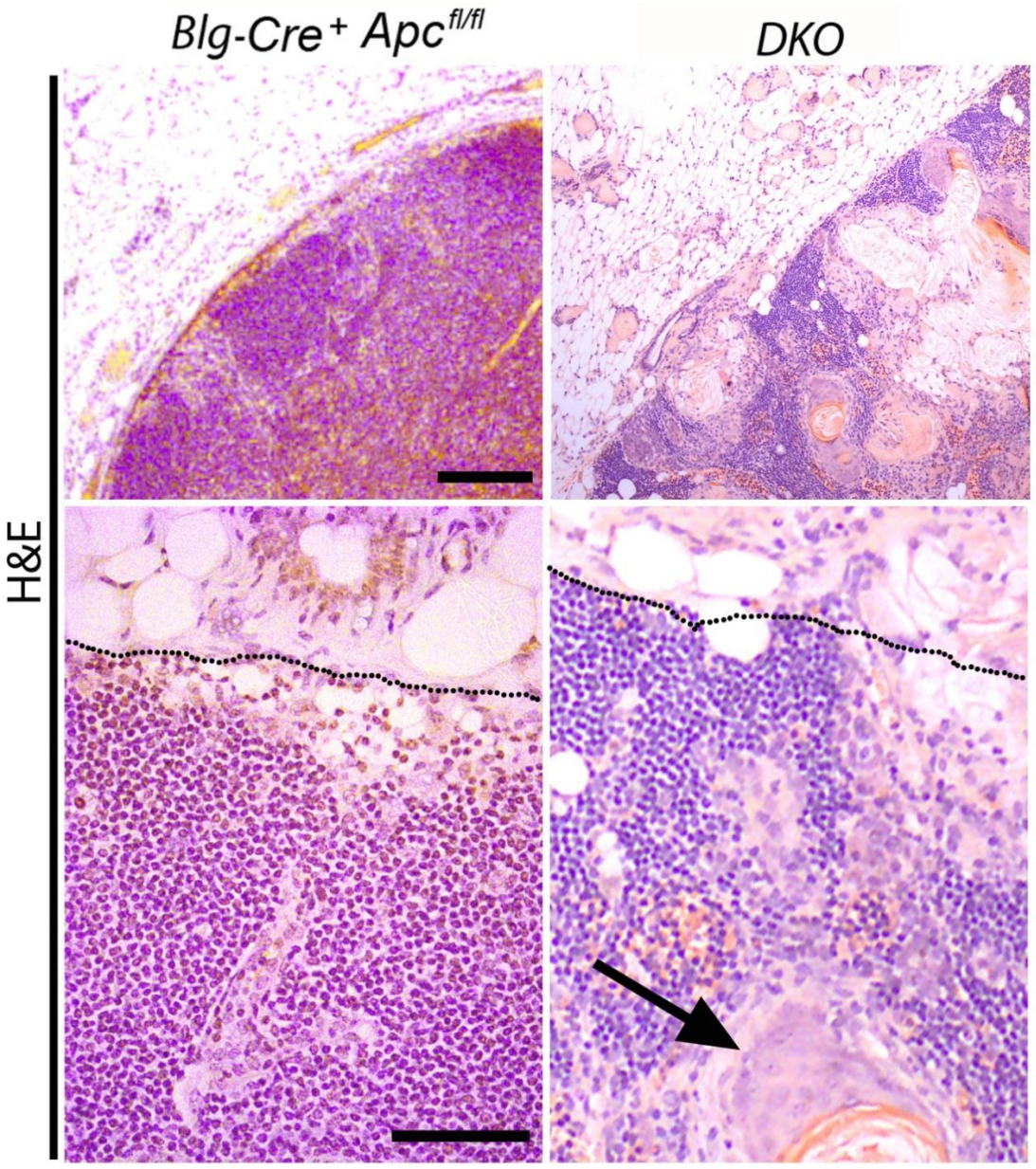

Figure 5.19 Tumours arising from mammary epithelium deficient for both Apc proteins display invasive properties. Image shows a lymph node from a *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ mammary gland on left and a lymph node from *DKO* on right, both from aged animals. Note invasion of squamous carcinoma cells, characterised by production of keratinous material, into the *DKO* node (arrow indicates a clump of cancer cells producing keratinous material within the lymph node periphery, top image scale bar represents 100µm, higher magnification image scale bar represents 50µm).

analysed), glands from *DKO* mice displayed invasion of epithelial tissue through the periphery and into the node (in 2 out of 3 nodes analysed from glands containing tumours). Invading cells within the node still express keratin reminiscent of the squamous carcinoma. This occurrence of lymph node metastasis highlights the invasive nature of tumours arising from epithelium deficient for both Apc proteins.

5.3 Discussion

Breast cancer is one of the most common malignancies in the western world and is accountable for a fifth of all women who die from cancer. Despite increasing advancement in the understanding of this disease, the roles of the APC proteins remain undefined. Evidence has implicated both APC loss (Kashiwaba et al., 1994; Thompson et al., 1993; Jin et al., 2001; Ho et al., 1999; Schlosshauer et al., 2000; Sarrio et al., 2003; Furuuchi et al., 2000) and APC2 loss (Sobottka *et al*., 2000; Yang et al., 2004; Wang et al., 1999; Jarrett et al., 2001; Chan et al., 2008) in breast cancer, however, parallel assessment has never been conducted. It is known both APC and APC2 can regulate the β-catenin/Wnt signaling pathway with indications of functional redundancy in the intestines (Van Es et al., 1999; Roberts et al., 2012). Beguilingly, both are expressed in human mammary epithelium (Kashiwaba et al*.,* 1994; Schlosshauer et al., 2000; Jarrett et al., 2001). In this chapter I utilised mouse transgenics to test the significance of Apc proteins in the mammary epithelium. Apc loss, Apc2 loss and concomitant loss of both were analysed to test their importance in mammary epithelial homeostasis and elucidate possible functional redundancies. Results indicate that both Apc and Apc2 alone are dispensable for mammary epithelial homeostasis. However, combined loss led to a range of defects including; atypical ductal hyperplasia, ghost cell formation, loss of polarity and cohesion, local tissue invasion and subsequent formation of invasive tumours. The role these proteins play in tumour suppression may extend beyond that of Wnt signaling.

Consistent with human tissue, both Apc proteins are expressed in murine mammary epithelium. Apc2 loss in this tissue was achieved through use of the *Apc2*$^{-/-}$ mouse (described in Chapter 3). Loss of Apc2 alone did not lead to any overt mammary phenotype, epithelial tissue retained its ductal architecture and remained polarised. Aged mice

displayed no alterations in lifespan nor developed any lesions within their mammary glands. This, in part, could be attributed to a lack of change in β-catenin intensity, localisation or detectible signs of Wnt pathway alteration. Interestingly, *Apc2*$^{-/-}$ mice did display a statistically significant reduction in mammary epithelial proliferation compared to *Wt.* The reasons behind this decrease remain elusive, but one could speculate that as the mouse model is a constitutive knock out, developmental compensation may have occurred prenatally and perhaps subtle increases in Wnt repression could implement reductions in proliferation. Importantly, this is the first study to analyse Apc2 loss in the context of the mammary gland and together these findings indicate Apc2 alone is dispensable for mammary homeostasis.

Constitutive Apc loss is embryonic lethal (Moser et al., 1995), so a Cre-lox system was employed utilising the *Apc*$^{fl/fl}$ allele (Shibata et al., 1997) and the *Blg-Cre*$^{+}$ promoter (Selbert et al., 1998) to delete Apc from the mammary epithelium. Previous studies using the *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ model have reported a delay in ductal growth although no overall alterations in virgin mammary epithelial physiology. Tumour formation only resulted when combined with an additional Tcf-1 or p53 mutation (Gallagher et al., 2002; Meniel et al., 2005). In another recent study targeted mammary epithelial Apc loss using the WAP-Cre promoter also failed to induce tumorigenesis (Kuraguchi et al., 2009). Results in this chapter confer with these studies where Apc was dispensable for mammary epithelial integrity and tumour suppressor function. Loss of Apc neither affected the ductal architecture, polarisation of the epithelium or proliferative state. Although loss of Apc in the intestinal epithelium has profound effects on the Wnt signaling pathway (Sansom et al., 2004; Andreu et al., 2005), analysis of this pathway following mammary epithelial Apc loss failed to display any detectable differences. This, in part, could explain the lack of phenotype in Apc deficient mammary epithelium in our study.

Interestingly, along with the fact that mice with a heterozygous *Apc* mutation have a higher incidence of mammary tumours (Moser et al., 1993), two recent animal studies have also implicated altered Apc in mammary tumorigenesis (Kuraguchi et al., 2009; Gaspar et al., 2009). In the first study, a *K14-Cre* promoter was used to target mammary epithelial *Apc* loss, which induced tumour formation. Although this promoter does induce recombination

in the mammary epithelium, it is not tissue specific (Kuraguchi et al., 2006). Contributions of other recombined cells in tumour formation cannot be delineated. In the second study, a constitutive Apc hypomorph was generated, permitting intermediate levels of Wnt signaling which pre-disposed mice to mammary tumours as appose to intestinal. This is in line with the consensus that varying levels of aberrant Wnt signaling effect different tissues (Buchert et al., 2010). However again, contribution of other cell types deficient for Apc hinder true consequences of specific epithelial Apc loss. Apc2 status was not addressed in any of these studies.

Strikingly, combined loss of both Apc and Apc2 in these experiments led to a range of extensive epithelial disruptions and subsequently tumour formation. This is the first study to date to demonstrate mammalian functional redundancies between the evolutionarily conserved Apc proteins '*in vivo*'. The implications of this finding are of great importance in the understanding of both of Wnt signaling transduction and cancer biology. The following discussion of results will focus on the dual roles Apc proteins must play.

Upon histopathological examination of presented lesions in young mammary glands with epithelium deficient for both Apc proteins, phenotype was reminiscent of atypical ductal hyperplasia (ADH). ADH is one of the most common premalignant lesions in humans and is a precursor of ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (Arpino et al., 2005). Overall, patients with ADH have a 4-5 times higher risk of developing invasive breast carcinoma (Page et al., 1985). ADH is classified by an increase in ductal cell number and an unusual patterning of these cells (Rosen et al., 2008), both of which were seen in young glands with epithelium deficient for both Apc proteins. The effects of aberrant Wnt signaling on cell division are well documented (reviewed in Reya & Clevers, 2005) and increases in cell division and thus hyperplasia can be explained by the noticed increase in Wnt signaling. Although nuclear β-catenin is the indicative sign of active Wnt in the intestines, in the mammary, aberrant Wnt can be exhibited by both nuclear β-catenin and an increase in β-catenin expression (Schlosshauer et al., 2000). The mechanisms behind an increase in β-catenin expression are unknown. However, in mammary epithelium deficient for both Apc proteins there were regions which display both an increase and nuclear translocation of β-

catenin concomitantly. These areas also displayed an increase in cMyc expression indicating aberrant activation of the Wnt signaling pathway.

The other requisite for classification of ADH is aberrant positioning and patterning of ductal epithelial cells (Rosen et al., 2008). In mammary epithelium deficient for both Apc proteins, polarity and cell positioning was vastly perturbed. The luminal and myoepithelial layers were chaotically organised and grew into the lumen, and markers of polarity such as Zo-1 and E-cadherin were lost. It is known Zo-1 and E-cadherin co-localize with β-catenin at the plasma membrane and co-immunoprecipitate (Rajasekaran et al., 1996). Furthermore, β-catenin has been shown to aid these proteins in the development of tight junctions, structural markers of polarised epithelial cells (Tsukita et al., 1999). Studies have suggested that activation of the Wnt signal pathway and nuclear translocation of β-catenin can suppress Zo-1 activity, diminishing polarity (Mann et al., 1999; Reichert et al., 2000). As concurrent loss of Apc and Apc2 caused nuclear translocation of β-catenin in our model, this is one proposed mechanism behind the aberrant cell positioning.

Interestingly, mouse models with activating β-*catenin* mutations also display ductal hyperplasia, but not ADH (Imbert et al., 2001; Miyoshi et al., 2002; Michaelson et al., 2001; Teulière et al., 2005). This suggests that defects regarding cell positioning, the other requisite for ADH, cannot solely be explained through canonical Wnt signaling. '*In vitro'* and '*in vivo'* work suggest important roles of Apc proteins, independent of β-catenin transcriptional activity (reviewed in Aoki and Taketo, 2007; McCartney and Nathke, 2008) and evidence is emerging implicating a more direct role of Apc proteins in controlling epithelial polarity. Both Apc and Apc2 have been shown to localise to the apical membrane of polarised epithelial cells (Reinacher-Schick & Gumbiner, 2001; McCartney et al., 1999) and both are thought to play a role in polarity through direct interaction with the actin and microtubule skeleton (Wen et al., 2004; Collin et al., 2008; Etienne-Manneville et al., 2005; Inaba et al., 2010). Cytoskeletal defects were noticed in this study in mammary epithelium deficient for both Apc proteins, consistent with a redundant role of Apc proteins beyond Wnt signaling. Regardless of exact mechanism, the evidence suggests that Apc proteins have dual roles in both controlling cell division and maintaining polarity, and combined defects in these functions converge to cause ADH. Although there is a high frequency of ADH in human

patients (Arpino et al., 2005) and an associated increased risk in development of invasive breast cancer (Page et al., 1985), animal models of ADH are seldom. Young *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$glands could present an admirable model of ADH for a whole range of benefits ranging from identification of biomarkers through to screening of therapeutics.

Mammary epithelium deficient for both Apc proteins also led to the appearance of ghost cells. These are characterised as anucleate 'cells' which stain positive for hard keratins and are most likely necrotic (Tanaka et al., 2007). They are extensively reported in a variety of human cancers, including those of the breast (Raman et al., 2000; Rosner et al., 2002; Hernanz et al., 2012). However, their highest occurrence is within tumours of the head and neck region (Hong et al., 1991; Marrogi et al, 1992; Kumaran et al., 2006). Studies of Pilomatricoma (type of Skin tumour), Craniopharyngioma (type of brain tumour) and Odontoma (type of Dental tumour), all of which contain ghost cells, all reveal aberrant β-catenin localisation in surrounding cells (Hassanein et al., 2003; Tanaka et el., 2007). This implies Wnt signaling plays some role in the development of these lesions. However, the status of other Wnt pathway genes, such as *APC* or *APC2* was never addressed. In our model, ghost cells occurred in almost every gland analysed and areas could be found were cells showed transition from normal appearing epithelium to ghost cell like. Upon further analysis it was revealed that in the early stages of transition, cells displayed nuclear/up-regulation of β-catenin and were cycling. As we move furthered through this zone of transition, cells become increasingly anucleate with observable apoptotic bodies and production of a pink material reminiscent of keratin.

A number of mechanisms could be responsible for epithelial transition to ghost cells; Wnt induced cell death, lack of vascularity and supportive cells or genetic re-programed differentiation. It is known cells require a 'just right' level of Wnt signaling for growth incurred advantage (Lamlum et al., 1999; Albuquerque et al., 2002; Lucero et al., 2010) and levels too high induce cell death (Lazarova et al., 2004; Bordonaro et al., 2008). Concurrent Apc and Apc2 loss causing high levels of Wnt may initially induce proliferation which could then be followed by cell death and subsequent keratinisation of cells which remain '*in situ*'. Indeed, concomitant loss of Apc proteins in the intestines induced cell death (discussed in Chapter 4) and in the mammary epithelium where there was an increase in caspase-3

staining. However, this increase in cell death could occur due to other reasons in addition to Wnt hyper-activation, one example being increased DNA damage. Another mechanism behind this transition may lie with reduced vascularity. As these Wnt activated cells proliferate, nutrients or cell signals may not be able to penetrate this increased mass, inducing necrosis and/or differentiation. A further mechanism may lie with re-programed differentiation. It is known keratin genes are Wnt targets (St-Jacques et al., 1998) and the Wnt signaling pathway is imperative in terminal hair shaft differentiation (Tsuji et al., 2001; Zhou et al., 1995; Dunn et al., 1998), a tissue that requires cell keratinisation. Loss of both Apc proteins in the mammary epithelium may activate transcription of these keratin genes through increased Wnt signaling and induce trans-differentiation and thus formation of ghost cells. The mechanisms behind formation of ghost cells in our model do warrant further investigation. One could speculate that this switch from a Wnt hyper-active cell to a terminally differentiated ghost/keratinocyte and the mechanisms behind this switch could expose new potential strategies in the treatment of cancer.

In mammary epithelium deficient for both Apc protein, along with areas which displayed miss-localised, up-regulated or nuclear β-catenin, groups of cells appeared to lose detectable expression altogether. From these experiments it cannot be determined if this heterogeneous patterns of β-catenin expression is a progressive sequence of events caused by loss of both Apc proteins or the effect of recombination in different cell types. In any case, complete loss of detectable β-catenin was also affiliated with epithelial defects. Upon inspection of epithelium in our model, cohesion was lost and cells and/or clumps of cells appeared to detach from the epithelial layer and 'invade' surrounding tissue. Occasionally these cells still retained some cytokeratin labelling suggesting an epithelial origin. These cells may result as a consequence of duct rupture caused by a build-up of ghost cell aggregates. However, cell damage was not overtly obvious and nor was death of these cells. As β-catenin is mostly undetectable in these cells, it is possible that this phenotype is not associated with canonical Wnt signaling. As mentioned previously, the role of Apc proteins in tumour suppression is multifunctional (reviewed in Aoki and Taketo, 2007; McCartney and Nathke, 2008) and evidence toward direct cytoskeletal roles in addition to that of β-catenin degradation is mounting (reviewed in Prosperi & Goss, 2011).

The ability of cells to invade and metastasize is paramount in cancer progression. In order for this to occur, cells from epithelial origin must obtain the ability to alter cohesion and adhesion. Loss of both Apc and Apc2 have been implicated independently in adhesion defects (Reichert et al., 2000; Bienz and Hamada, 2004; Faux et al., 2004 Hamada and Bienz, 2002) and it has been reported previously that this role is mutually exclusive from that of β-catenin degradation (Prosperi et al., 2009; Harris & Nelson, 2010). Apc proteins' role in adhesion is most likely linked with the fact that they both localise with adherens and tight junctions (Reichert et al., 2000; Bienz and Hamada, 2004; McCartney et al., 1999). In our model, areas displayed loss of E-cadherin and Zo-1 which mark both adherens junctions and tight junctions respectively. One could speculate that a direct cytoskeletal presence of at least one Apc homolog is a necessity for formation or maintenance of these junctions and loss of both Apc's leads to dissociation. The exact role Apc and Apc2 play in regulating adhesion and/or how loss of both proteins induces loss of β-catenin, E-cadherin and Zo-1 is unknown. However, disturbed tissue organisation through disrupted adherens junctions and tight junction protein integrity is frequently observed in cancer (Schmalhofer et al., 2009) and correlated with a more aggressive and advanced state (Thiery, 2002; Huang & Muthuswamy, 2010). How combined Apc and Apc2 loss influences adherens junctions and tight junctions is an exciting avenue for further research. Together these results imply that Apc proteins are functionally redundant in controlling cohesion and adhesion, and this may be a role independent of canonical Wnt signalling.

A property associated with invasive ability of cells is epithelial-mesenchymal transition (EMT). This is characterised by a loss of adhesion, cell shape changes and increased motility (reviewed in Thiery et al., 2009; Sleeman et al., 2012). Previous studies have associated Wnt signaling with promotion of EMT (Fu et al., 2011; Valenta et al., 2012; Anson et al., 2012; DiMeo et al., 2009). In mammary epithelium deficient for both Apc proteins, there are cells which displayed positive staining for vimentin concomitant with a loss of E-cadherin. Together these characteristics are indicative of EMT (Blanco et al., 2004). However, although there is a noticed increase in Wnt signaling in our model, cells which display invasive properties were usually negative for β-catenin staining. This suggests a different or concurrent mechanism behind the transition. Indeed, there was no correlation between nuclear β-catenin staining, 'invasive' cells or expression of snail (a known inducer of EMT

influenced by Wnt signaling (Cano at al., 2000)). This is another aspect of this model that warrants further research.

Cultured mammary epithelial cells were used to explore the consequences of combined Apc and Apc2 loss. Pure epithelial cultures help delineate influences on epithelial cell from supportive non-epithelia cells, which would also be deficient for Apc2 in *Blg-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice. Mammary epithelial cells deficient for *Apc2* or *Apc* through targeted recombination with an adenovirus, failed to display overt differences from control tissue. However, combined loss of both Apc proteins led to both polarity and adhesion defects, cells also displayed prominent shape changes consistent with EMT. These '*in vitro*' experiments help validate the functional redundancies noticed in epithelial cells '*in vivo*'. Further '*in vitro*' work concurrent with mammary epithelial transplants (discussed in Raijnkels & Rosen, 2001) could help expose true invasive potential of these cells.

To summarise so far, both Apc proteins alone are dispensable for mammary epithelial homeostasis. However, combined loss led to a range of epithelial defects and these defects may be contributed to by a component beyond that of canonical Wnt signaling. Previous reports have shown defects caused by *Apc* loss in the intestines can be rescued through additional *cMyc* mutation (Sansom et al., 2007). *cMyc* is a Wnt target gene (He et al., 1998) and a global regulator of transcription (Dang et al., 2006). As only combined loss of Apc and Apc2 induced detectible increases in cMyc, it would be interesting to see how much of the phenotype can be accredited to its over-expression. Although transcription of some genes upon nuclear translocation of β-catenin are cMyc independent (Sansom et al., 2007), concomitant mammary epithelial loss of both Apc proteins and cMyc may help delineate Wnt dependent and Wnt independent functions Apc proteins may play in mammary homeostasis.

Upon aging mice with mammary epithelial deficiency for Apc or Apc2 alone, neither displayed survival differences from control mice within the timeframe of the experiment. No lesions were present within the mammary glands of either genotype upon time of death, although occasional small aggregates of ghost cells were present in glands deficient for Apc. It would be interesting to test the status of Apc2 in epithelium surrounding these aggregates

given the vast occurrence of ghost cells in glands with epithelial deficiency for both Apc proteins. Some mechanism causing reduction of Apc2 concomitant with Apc loss in these cells could be a causative explanation. Taken together, these results suggest both Apc and Apc2 alone are dispensable for tumour suppression within the mammary epithelium.

Strikingly, combined deletion of both Apc proteins induced mammary tumourigenesis and significantly reduced survival. Although no redundant roles in tumour suppression are eminent between Apc proteins within the intestine (discussed in Chapter 3 & 4), in the mammary epithelium the redundancies are paramount. Functional redundancies in tumour suppression in a range of other tissues may explain why even though APC is ubiquitously expressed, its loss is mostly associated with intestinal tumourigenesis (Kinzler et al., 1991; Groden et al., 1991; Nishisho et al., 1991). In addition to mammary tumorigenesis, mice with epithelial deficiency of both Apc proteins also occasionally developed sarcomas. These are a type of tumour that is thought to arise from connective tissue origin (Helman & Meltzer, 2003). Most were located within the uterine or abdomen in experimental mice, however, origin of these tumours was difficult to determine. As occurrence of both tumour types requires the *Blg-Cre*$^{+}$ transgene in addition to mutations of both *Apc*s, sarcomas either arise as a consequence of metastasis from the mammary tumours or '*de novo*' occurrence due to tissue non-specific recombination. Both require further exploration.

Mice with both homozygous and heterozygous *Apc2* mutation in the context of mammary epithelial Apc loss developed mammary tumours and occasional sarcomas. The incidence of these tumours and reduction in lifespan were not as severe in the heterozygous mice. This along with the fact they also displayed similar epithelial defects at a young time point, although not as intense, suggests a gene dose dependent effect of Apc2 in the context of Apc loss. Interestingly, whilst *APC2* mutation is infrequent, gene silencing through varying degrees of promoter hypermethylation is extremely common in human breast cancers (Chan et al., 2008). Levels of promoter methylation correlate with levels of reduced protein expression (Suzuki & Bird, 2008; Chan et al., 2008). This complies that reduced expression levels of *APC2* are sufficient for tumourigenesis in the right context. *APC* also displays high levels of promoter hypermethylation (Van der Auwera et al., 2008). It would be interesting to quantify levels of both *APC* and *APC2* expression concurrently in human breast cancer. It

can be speculated that combined reductions are needed in human tissue to induce tumorigenesis.

Following histopathological inspection, the majority of collected mammary tumours were classified as squamous carcinoma. This cancer is a relatively rare human breast carcinoma (Cardoso et al., 2000). However, it is aggressive, invasive and associated with a poor patient prognosis (Wargotz & Norris, 1990). Consistent with human diagnosis, tumours in mice were deemed invasive with some areas of squamous differentiation and characteristic areas of keratin production. It is interesting that whilst most human cancers display aberrations in *APC* and/or *APC2* expression, loss of murine Apc proteins led to a rare cancer subtype. Indeed, the majority of mouse models causing aberrant Wnt signaling in the mammary lead to some sort of squamous differentiation (Sasai et al., 2000; Brennan & Brown, 2004; Gaspar et al., 2009; Kuraguchi et al., 2009), which is uncommon in human breast cancer. This discrepancy might highlight evolutionary differences between human and mouse tissue. However, a more plausible explanation may lay with the additional genetic mutations found in human breast cancers. It is known that most breast cancers contain more than 80 non-silent mutations (Wood et al., 2007) and varying patterns of gene expression can define cancer subtype (Sotiriou & Pusztai, 2009). Regardless of dissimilarities, mice with concurrent mammary epithelial Apc and Apc2 loss mimic human cancer progression as lesions develop from ADH through to invasive carcinoma.

Most of the tumour sample collected displayed a heterogeneous pattern of nuclear β-catenin. This was concomitant with activation of the Wnt gene targets cMyc and CD44 in the majority of cells, indicating tumours are Wnt activated. As the Wnt signaling pathway was only activated in a subset of epithelial cells at an early time point, It is unknown if these tumours arise due to Wnt activation in specific cell types, which one could speculate to be mammary stem cells or due to an accumulation of other oncogenic mutations in other cell types. *APC* loss has been shown to induce chromosome instability in other tissues (Fodde et al., 2001; Green & Kaplan et al., 2003) and in another mouse model targeting mammary epithelial Apc loss, additional activating mutations in *H-Ras* or *K-Ras* were present in a subset of arising mammary tumours (Kuraguchi et al., 2009). Further analysis of obtained tumour samples from this study will help elucidate contribution of other genetic factors.

The importance of the Wnt signaling pathway and aberrations of this pathway on adult stem cells is closely tied with cancer initiation in a range of tissues (Reya & Clevers, 2005). Indeed, the origin of intestinal cancer can be attributed to Wnt alterations in these cells (Barker et al., 2009). The contribution mammary stem cells play, as well as the contribution of aberrant Wnt signaling in these cells, is not well defined in breast cancer. Mammary stem cells have been characterised (Shackleton et al., 2006; Stingl et al., 2006; Van Keymeulen et al., 2011; Shehata et al., 2012) and implicated in tumour initiation (Al-Hajj et al., 2003; Dontu et al., 2003; Ginestier et al., 2007). It has also been show that markers of these stem cells increase in Wnt activated mammary tumours (Shackleton et al., 2006). However, influence of Wnt signaling in these cells and their relationship with cancer origin remain inconclusive (reviewed in Lindvall & Williams, 2007). As concurrent loss of mammary epithelial Apc proteins induces both activation of the Wnt signaling pathway and initiation of tumours, it poses an excellent model to study the role Wnt signaling plays on mammary stem cells in tumourigenesis.

Mammary tumours arising from epithelium deficient for both Apc proteins also possessed invasive capabilities. Histopathological analysis of tumours revealed invasive qualities; local tissue invasion was present as well as occurrence of invasion and metastasis into the mammary lymph nodes. In cancer progression, invasion into the vascular or lymphatic system is a key step in tumour cell dissemination (Fidler, 2003; Christine et al., 1989; Gupta & Massague, 2006). Distant metastases were not confirmed although some mice did display occurrence of multiple tumours (classified as sarcomas of unidentifiable origin). The mechanisms behind promotion of invasion may be two-fold, both indirect through aberrant Wnt signaling and/or direct through cytoskeletal and adhesion alterations. It is known nuclear translocation of β-catenin and subsequent Wnt activation can promote both reductions in adhesion and EMT (Yook et al., 2005; Fu et al., 2011; Valenta et al., 2012; Anson et al., 2012; DiMeo et al., 2009). Also, analysis of mammary epithelium deficient for both Apc proteins at an early time point display diminished junctional complexes, adhesion and cohesion which occurred concomitant with complete β-catenin loss. As mentioned previously, from these experiments we cannot confirm if the differing patterns of β-catenin localisation are a sequence of events. Nuclear translocation of β-catenin and activation of

Wnt may induce adhesion changes and EMT which could then be followed by β-catenin loss. Indeed, the majority of primary tumour cells did display Wnt activation. Interestingly, confusion of β-catenin status in invasion extends to human breast samples. Both β-catenin nuclear translocation (Lin et al., 2000; Geyer et al., 2011; Khramtsov et al., 2010) and protein loss (Dolled-Filhard et al., 2006; Park et al., 2007) have been correlated with metastasis and poor patient outcome. Together this evidence increases the probability of a concomitant Wnt independent influence of loss of Apc proteins, in invasion.

Invasion and metastasis of cancer account for more than 90% of cancer-related deaths (Chaffer & Weinberg, 2011). The pathological progression of human breast cancer is well documented and well understood, however, the molecular factors which control this progression are less well characterised. Progression from deregulated epithelial proliferation, into atypical hyperplasia, carcinoma '*in situ*', and invasive carcinoma, which may develop metastasis, is the accepted biological pathway in human breast cancer progression (Rosen et al., 2008). Mammary deficient for both Apc proteins implement epithelial changes which assist in all these stages from increased proliferation through to increased invasive capabilities. Although Apc proteins are redundant in these functions, it is not clear how much can be account for through effects on Wnt signaling.

To summarise, results in this chapter indicate that both Apc and Apc2 alone are dispensable for mammary epithelial homeostasis. Combined loss led to a range of epithelial defects which subsequently led to tumour formation. This exposes functionally redundant functions between Apc proteins within the mammary gland in homeostasis and tumour suppressor function. While Wnt activation was subtle at an early time point and some defects may be attributed to Wnt independent means, combined loss of Apc and Apc2 synergise to drive Wnt activated tumours. The contrasts and parallels between this model and human disease provide important insights into the factors driving human tumours through subtle deregulation of Wnt.

Chapter 6

Investigating the liver phenotypes associated with reduced levels of Apc proteins

6.1 Introduction

Apc proteins play key roles throughout development, homeostasis and induction of pathogenesis in a variety of tissues. The previous chapters have exposed the roles of Apc proteins in the intestines and mammary gland. The Wnt signaling pathway is increasingly implicated in regulating liver homeostasis, thus the roles of the Apc proteins were explored in this tissue.

The liver is one of the largest organs in mammals and the largest gland. It has many functions but some of the most important include detoxification and production of biochemicals for digestion. The liver can be broken down into basic functional units termed hepatic lobules (Figure 6.0).

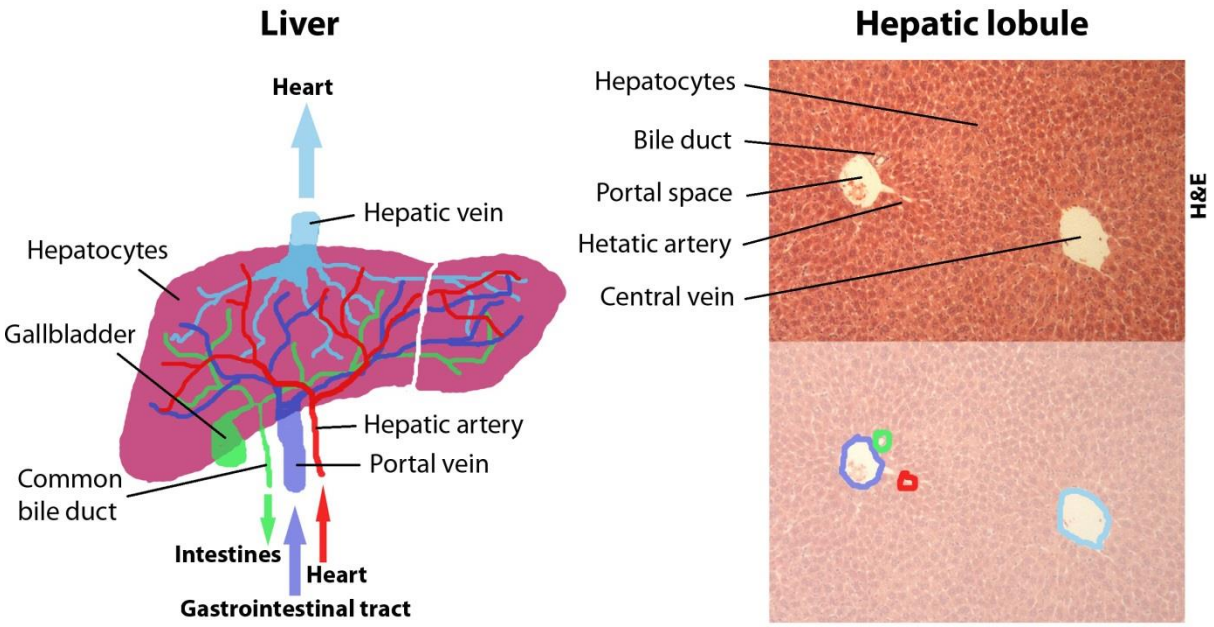

Figure 6.0 The liver and its basic functional unit (hepatic lobule). The liver receives a dual supply of blood from both the portal vein and hepatic artery. Whilst the hepatic artery supplies the liver cells

with oxygenated blood directly from the heart, the portal vein brings nutrient rich blood from the small intestines for processing and metabolising. The liver can be broken down into basic functional units termed hepatic lobules. Blood flows into these lobules through the hepatic artery and portal space, travels through the network of hepatocytes enduring metabolic processes and is collected by the central vein. The central veins of each unit congregate to eventually form the hepatic vein which transports the cleansed blood to the heart. The liver also supplies bile to the small intestine to aid digestion. Bile is produced by the hepatocytes and collected via a network of bile ducts. These ducts congregate into the common bile duct which either directly supplies bile to the small intestine or stores it in the gallbladder.

Analysis of cultured embryonic murine livers and use of knockdown studies '*in vitro*' suggest a role for β-catenin in hepatocyte proliferation and differentiation (Micsenyi, 2004: Monga et al., 2003). Targeted loss of Apc or activating mutations in β-catenin in Hepatoblasts during development resulted in underdeveloped livers and subsequent embryonic lethality (Decaens et al., 2008: Tan et al., 2008). These experiments demonstrate the importance of Wnt signaling during liver embryogenesis however, control of this pathway is also important in adult liver homeostasis. In adult mice targeted disruption of Apc or activating mutations of β-catenin in hepatocytes resulted in abnormal Wnt activation and subsequent hyperproliferation leading to hepatomegaly-associated mortality (Colnot et al., 2004: Reed et al., 2008: Benhamouche et al., 2006). Conversely, loss of β-catenin was dispensable for normal liver function but did result in liver weight reduction (Sekine et al., 2006) and a delay in hepatocyte proliferation after partial hepatectomy (Sekine et al., 2007).

Previous experiments have demonstrated the importance of Wnt signaling in liver cellular patterning, referred to as 'zonation'. Expression of metabolic genes involved in drug metabolism, ammonia detoxification, carbohydrate metabolism, and bile production and secretion are dictated by a cells position along the porto-central axis of the liver lobule. Differential zones of "periportal" (PP) or "perivenous" (PV) hepatocytes are established which express different patterns of metabolic gene expression. Contrasting localization patterns are observed for active β-catenin expressed in PV hepatocytes and Apc, which is expressed in PP hepatocytes (Benhamouche et al., 2006). Various studies inactivating Apc in the murine liver induced a PV genetic program, at a PP expense, supposedly through β-catenin activation (Benhamouche et al., 2006; Reed et al., 2008; Burke et al., 2009). Conversely inactivation of β-*catenin* expression or over expression of *Dickkopf 1* (a Wnt

inhibitor) resulted in an induction of a PP genetic program at the expense of the PV program (Burke et al., 2009; Sekine et al., 2006; Benhamouche et al., 2006). The importance of Apc2 in the regulation of liver zonation has yet to be investigated, but given its known ability to regulate Wnt signaling, it is reasonable to speculate it may also have a role in liver homeostasis.

Aberrations in the Wnt signaling pathway have been noted in a variety of pathological liver conditions ranging from hepatitis to carcinoma (reviewed by Thompson et al., 2007). Patients with mutations in *APC* are at an increased risk of developing hepatoblastoma (Giardiello et al 1996; Hirschman et al 2005) a rare, rapidly progressive, usually fatal childhood malignancy. Although Patients with FAP are usually associated with intestinal malignancies, hepatic tumours have been reported and can include adenoma, fibrolamellar carcinoma, and hepatocellular carcinoma (HCC) (Gruner et al., 1998; Toiyama et al., 2011; Li et al., 2012). A significant fraction of cholangiocarcinomas as well as HCC's exhibit alterations in Wnt signaling (Taniguchi et al., 2002; Tokumoto et al., 2005) suggesting that Wnt can act on several cell types in the liver to induce carcinogenesis. HCC is the most prevalent primary liver cancer, ranking as the fifth most common cancer in the world and being the third most common cause of cancer-related death (Bosch et al., 1999). Studies have shown inappropriate β-catenin signalling in at least 30% and up to 70% of all HCC (de La Coste et al., 1998: Wong et al., 2001: Giles et al., 2003). Hypermethylation of the *APC* promoter in HCC in patients occurs in >80% of cases and results in a reduction of APC protein expression (Sun et al., 2003; Jain et al., 2011; Cspregi et al., 2008). The higher occurrence of *APC* hypermethylation in tumours compared to the level of β-catenin activation suggests, a Wnt independent role, in addition to the Wnt dependent role, for APC in HCC development. Although Wnt signalling may play a part, the major mechanisms at play during the development of HCC remain poorly understood, hindering the development of novel therapeutic approaches (Farazi and DePinho, 2006).

As discussed in the previous chapters, both Apc proteins have the ability to regulate Wnt signaling and harbour overlapping roles in a variety of tissues. Apc2 is highly expressed throughout liver development and in the adult tissue (Shintani et al., 2012) although its role in this organ has never been addressed. One possible way in which Apc2 may play a role is

through its interaction with Proline/serine-rich coiled-coil 1 (*PSRC1*) an oncogene commonly over expressed in human HCC (Yang et al., 2011). *PSRC1* is a micro-tubule-associated protein that promotes cell growth by several different mechanisms, one of which is through binding to *APC2* and enhancing β-catenin dependent transactivation of Wnt target genes (Hsieh et al., 2007). In this chapter I wished to characterise and compare the effects of Apc, Apc2 and combined loss within the liver using a range of transgenic approaches.

6.2 Results

6.2.1 Targeting loss of Apc and Apc2 proteins in the murine liver

In order to investigate the role of Apc proteins within the adult liver, a variety of transgenic approaches were employed*. Foremost, to analyse the consequences of high level Apc loss, a Cre recombinase conditional knock-out system was used to circumvent the embryonic lethality associated with constitutive Apc loss (Moser et al., 1995). The *Tg(Cyp1a1-cre)1Dwi* transgene (abbreviated here as *Ah-Cre*$^{+}$) targets Cre recombination in a variety of tissues, including the liver following intraperitoneal administration of β-napthoflavone (Ireland et al., 2004). To test liver targeted recombination, *Ah-Cre*$^{+}$ mice were crossed with mice containing the *ROSA26*$^{+}$ reporter allele (described in Chapter 5) and harvested 4 days after a β-naphthoflavone induction regime (see methods). Whole mount liver pieces incubated with X-gal revealed high levels of recombination in livers positive for the *Ah-Cre* transgene (Figure 6.1A). β-galactosidase antibody staining of induced *Apc*$^{fl/fl}$ and un-induced *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ liver sections revealed no spontaneous recombination in liver cells. However, more than 95% of induced *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ liver cells displayed recombination events following induction (Figure 6.1B). Un-induced *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers displayed no staining for β-galactosidase activity, which revealed recombination was dependent on induction (data

*Animals involved in the experiments using *Ah-Cre*$^{+}$ were induced and dissected by Dr Karen Reed. X-gal liver whole mount performed by Dr. Reed. The subsequent analysis of the tissue sections was carried out by me.

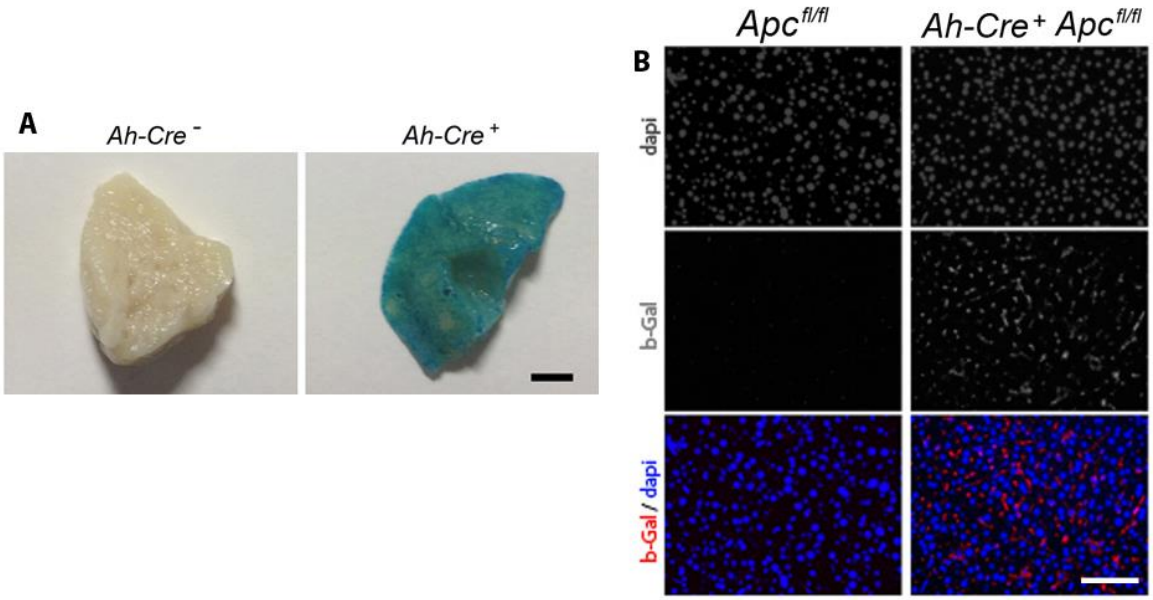

Figure 6.1 Induced *Ah-Cre*$^{+}$ *ROSA26*$^{+}$mice displayed efficient Cre recombination within the liver. (**A**) Whole mount pieces of induced *Ah-Cre*$^{+}$ and *Ah-Cre*$^{-}$ livers, incubated with X-gal, revealed high levels of recombination only in *Ah-Cre*$^{+}$ mice following induction (scale bar 2.5mm). (**B**) β-galactosidase antibody staining of *Apc*$^{fl/fl}$ and *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ liver sections displayed no spontaneous recombination in *Apc*$^{fl/fl}$ cells however, more than 95% of *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ cells displayed recombination events (scale bar 100um).

not shown). In contrast to acute levels of Apc loss within the liver following Cre-recombinase activation, more subtle levels of reduction can be investigated through non-induced *Apc*$^{fl/fl}$ livers. This is due to the hypomorphic nature of the genetically modified *Apc*^{fl} allele which causes reduced levels of *Apc* mRNA expression in mice (Shibata et al., 1997). These reduced Apc levels are sufficient to induce a phenotype within the liver (Buchert et al., 2010). Fluorescent IHC staining using an antibody raised against the C-terminal tail of Apc revealed a slight reduction of Apc protein in un-induced *Apc*$^{fl/fl}$ livers and a vast reduction in induced *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers (Figure 6.2A).

In order to evaluate Apc2's role in the liver, the constitutive *Apc2*$^{-/-}$ mouse was used (described in Chapter 3). Fluorescent IHC using an antibody raised against the C-terminal tail of Apc2 revealed an abundance of Apc2 specific staining in the *Wt* livers, which was lost in *Apc2*$^{-/-}$ livers (Figure 6.2B).

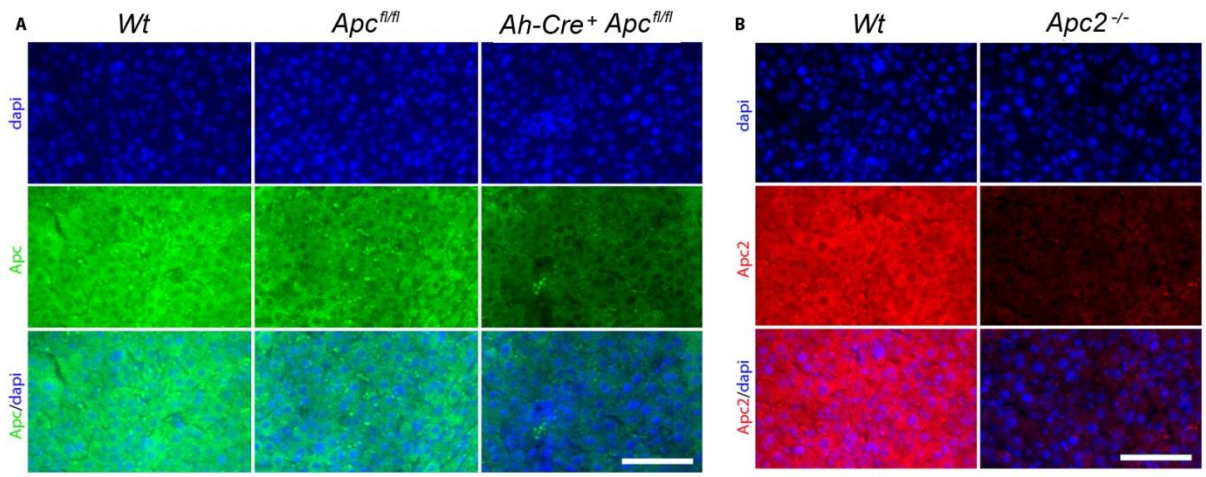

Figure 6.2: Reduction of Apc proteins shown by fluorescent IHC. (**A**)*Wt*, *Apc*$^{fl/fl}$ and induced *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ Liver sections were stained with an anti-Apc antibody raised against the C-terminal tail of Apc. *Ah-Cre*$^{+}$, *Apc*$^{fl/fl}$ liver cells displayed a reduction in Apc protein 4 days post induction compared to *Wt*. Non-induced *Apc*$^{fl/fl}$ (or induced *Apc*$^{fl/fl}$ negative for *Ah-Cre* {not shown}) displayed a slight reduction Apc protein. This was due to the hypomorphic nature of the *Apc*^{fl} allele. (**B**) *Wt* and constitutive *Apc2*$^{-/-}$ liver sections were stained with an Anti-Apc2 antibody raised against the C-terminal tail. No cells in the *Apc2*$^{-/-}$ livers stain for Apc2 (scale bar 100um).

To directly test the functions of Apc proteins in liver homeostasis and elucidate any functional redundancies that may exist, mice were inter-crossed to create six combinations of genotypes (summarised in Figure 6.3). These could allow us to test all possible combinations. However, due to low level embryonic expression of the *Ah-Cre*$^{+}$ transgene, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice die '*in utero*' due to compromised functional redundancies that must exist during development. Full characterisation of liver phenotypes in the other genotypes was carried out.

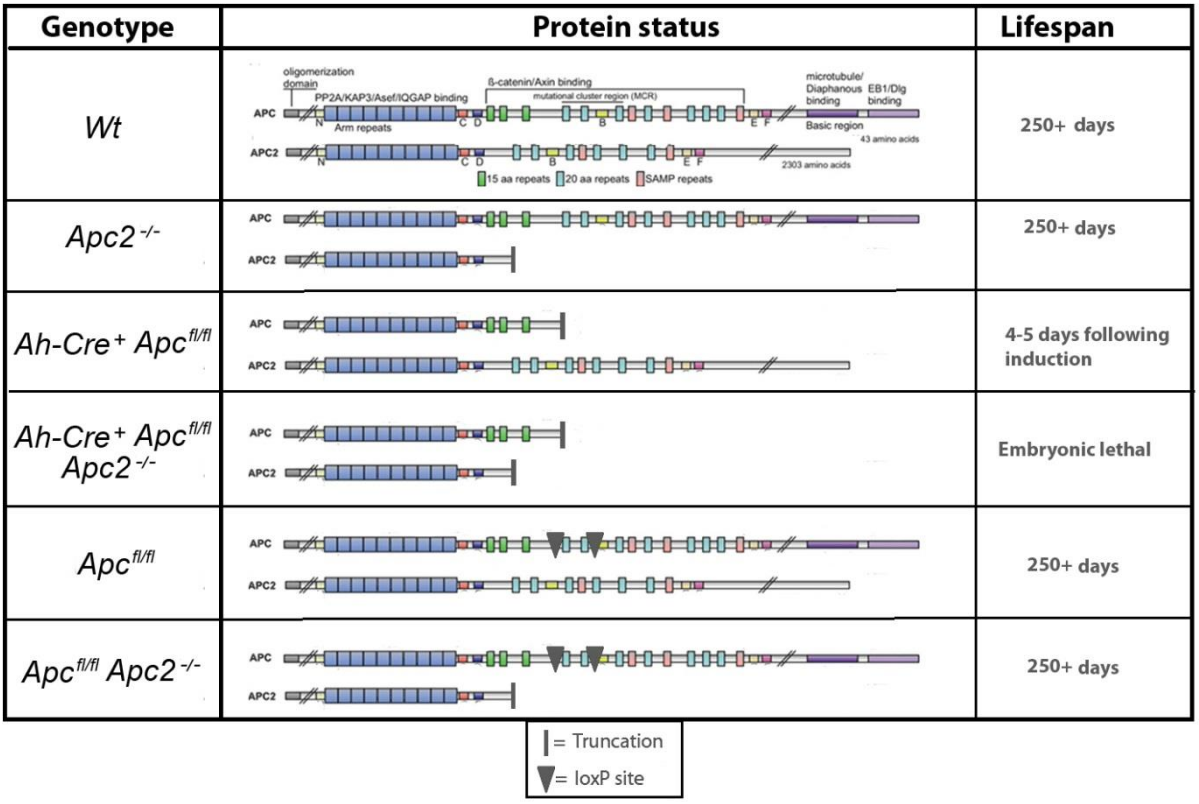

Figure 6.3: Experimental design to test the function of Apc proteins in liver homeostasis. ***Wt* :** livers contains full length Apc & Apc2**.** ***Apc2*$^{-/-}$:** full length Apc protein combined with a constitutive truncating Apc2 mutation. Mice displayed un-altered survival as shown in chapter 3**.** ***Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$:** full length Apc2 but induced Cre mediated Apc truncating mutation in the liver. Mice only survived 4-5 days following induction due to recombination in other tissues, mainly the intestinal track**.** ***Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$** : mice die '*in utero*' due to low level embryonic expression of the Ah-promoter compromising functional redundancies that exist between Apc proteins in embryonic development. ***Apc*$^{fl/fl}$:** consists of full length Apc2 but had reduced levels of Apc due to the genetically inserted loxP sites. These mice are known to develop HCC at around 400 days of age. ***Apc*$^{fl/fl}$ *Apc2*$^{-/-}$** : consists of both the hypomorphic *Apc*$^{fl/fl}$ alleles and constitutive truncating Apc2 mutation. These mice were viable and thus allowed us to directly test the significance of Apc2 on the development of HCC. Heterozygous combinations were not assessed (protein schematics adapted from Webb et al., 2009).

6.2.2 Levels of cell division and liver integrity are only altered following loss of Apc

To compare the effects of acute loss of either Apc or Apc2 on liver homeostasis cohorts of *Wt*, *Apc2*$^{-/-}$ or *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice were established. To induce high level liver loss of Apc, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice were induced with a β-naphthoflavone regime. Although loss of Apc2 alone did not lead to alterations in animal survival (Chapter 3), acute loss of Apc using the

Ah-Cre^{+} transgene led to morbidity 4-5 days following induction. This is due to both manifestations of the intestinal tract (Samson et al., 2004) and liver hepatomegaly (Reed et

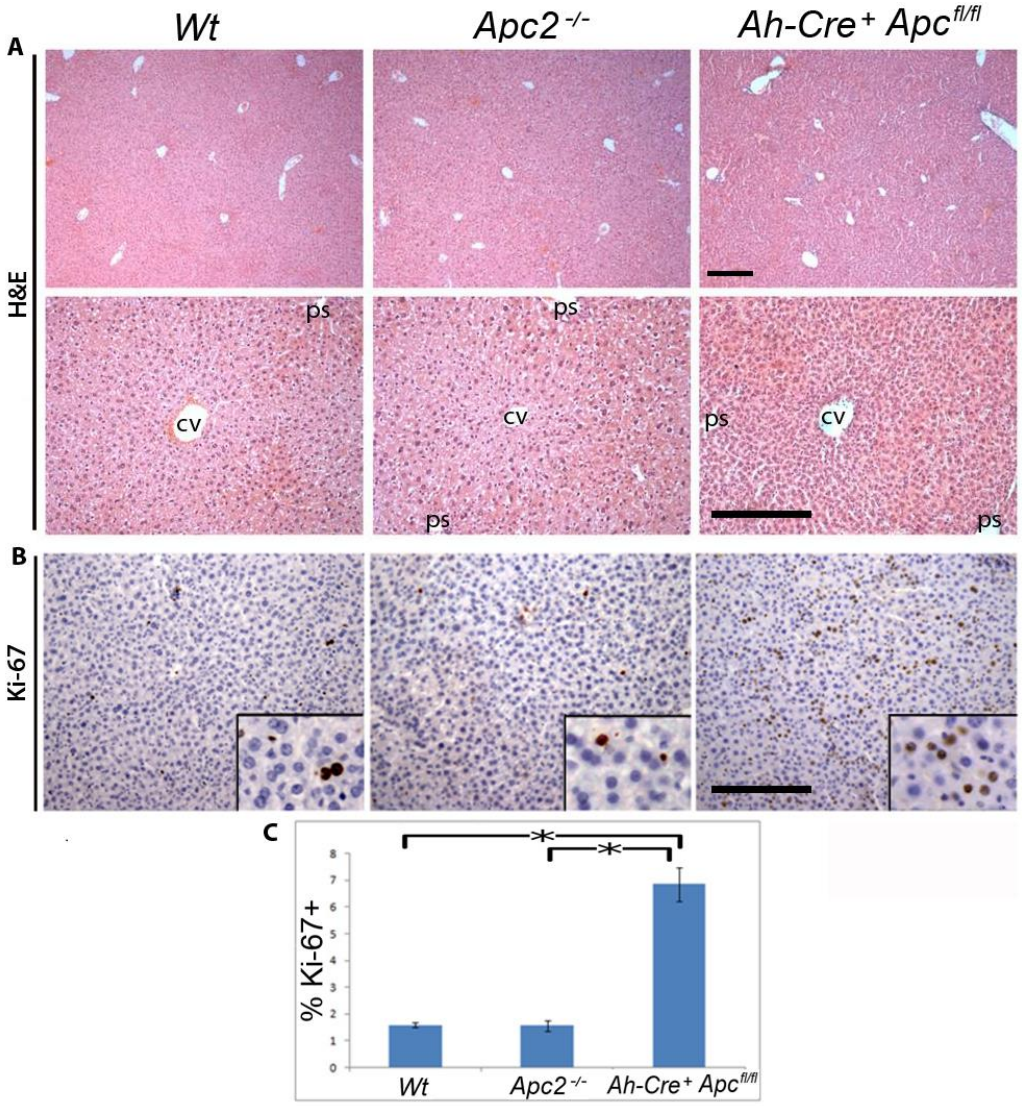

Figure 6.4: loss of Apc2 had no effect on liver histology or proliferation. However, acute loss of Apc caused hepatomegaly and hyper proliferation. (**A**) H&E stained liver sections from *Wt*, *Apc2*$^{-/-}$ and *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice, bottom row higher magnification of top panel (PS= Portal Space, CV= Centrilobular vein). *Apc2*$^{-/-}$ livers displayed normal histology compared to *Wt*, however, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers displayed an increase in cell number and increase in liver to body weight ratio (data not shown) (scale bar represents 200um). (**B**) Liver sections were stained with an anti-Ki-67 antibody to assess levels of cell division. Whilst *Wt* and *Apc2*$^{-/-}$ had a comparably relatively few cells cycling within the liver, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers displayed a high frequency of KI-67 labelled cells. Inlet shows same image at higher magnification. (**C**)Quantitative analysis of Ki-67 positively labelled cells revealed no significant difference between *Wt* and *Apc2*$^{-/-}$ livers however, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers exhibited a statistically significant increase (error bars represent SD, asterisks mark p≤0.01, Mann-Whitney *U*-test, n≥3).

al 2008). This compromised use of these mice for long term study. Cohorts were sacrificed at similar time points between *Wt*, *Apc2*$^{-/-}$ and *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice following a β-naphthoflavone induction regime (n≥3). Only livers from *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice displayed hepatomegaly (Data not shown) as previously reported (Reed et al., 2008). Histological analysis using H&E stained liver sections did not reveal major structural differences between genotypes (Figure 6.4A). All genotypes displayed similar frequencies of centrilobular veins (CV) and portal spaces (PS). However, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers did seem more compacted with a possible increased cell number. Liver sections from each genotype were stained with a Ki-67 antibody to label proliferative cells (Figure 6.4B). Quantitative analysis revealed no statistical difference in Ki-67 positive cells between *Wt* and *Apc2*$^{-/-}$ livers (1.56±0.11 and 1.49±0.21, p=0.865, Mann-Whitney *U*-test, n≥3). However, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers displayed a more than a 3 fold increased in positively labelled cells (1.56±0.11 and 6.89±0.79, p≤0.01, Mann-Whitney *U*-test, n≥3) compared to *Wt* (Figure 6.4C).

6.2.3 Increased cytoplasmic levels of β-catenin occur due to loss of either Apc or Apc2 however Lect2 is only up regulated following Apc loss

Liver sections were co-labelled with β-catenin and Leukocyte cell-derived chemotaxin-2 (Lect2) antibodies, using florescent IHC. This allowed us to assess protein co-localisation (Figure 6.5). Previous studies have implicated Lect2 as a β-catenin target within the liver (Ovejero et al., 2004; Cadoret et al., 2002). In *Wt* liver, β-catenin is exclusively membrane associated. However, as previously reported following loss of Apc, β-catenin displayed an increase in cytoplasmic and nuclear accumulation (Colnot et al., 2004; Reed et al., 2008; Benhamouche et al., 2006). In *Apc2*$^{-/-}$ livers, the majority of cells still displayed membrane associated β-catenin. However, there were occasional cells which displayed an increase in cytoplasmic/nuclear staining. In *Wt* livers, Lect2 expression is limited to a layer a few cells thick surrounding the CV, with proposed activated β-catenin (Benhamouche et al., 2006). Following loss of Apc, Lect2 is expressed in the majority of cells regardless of position. Cells co-labelled with increased cytoplasmic/nuclear β-catenin, although this was not a requisite. Interestingly although *Apc2*$^{-/-}$ livers display alterations in β-catenin staining patterns, Lect2 status is maintained, even cells with clear nuclear β-catenin accumulation fail to stain for Lect2.

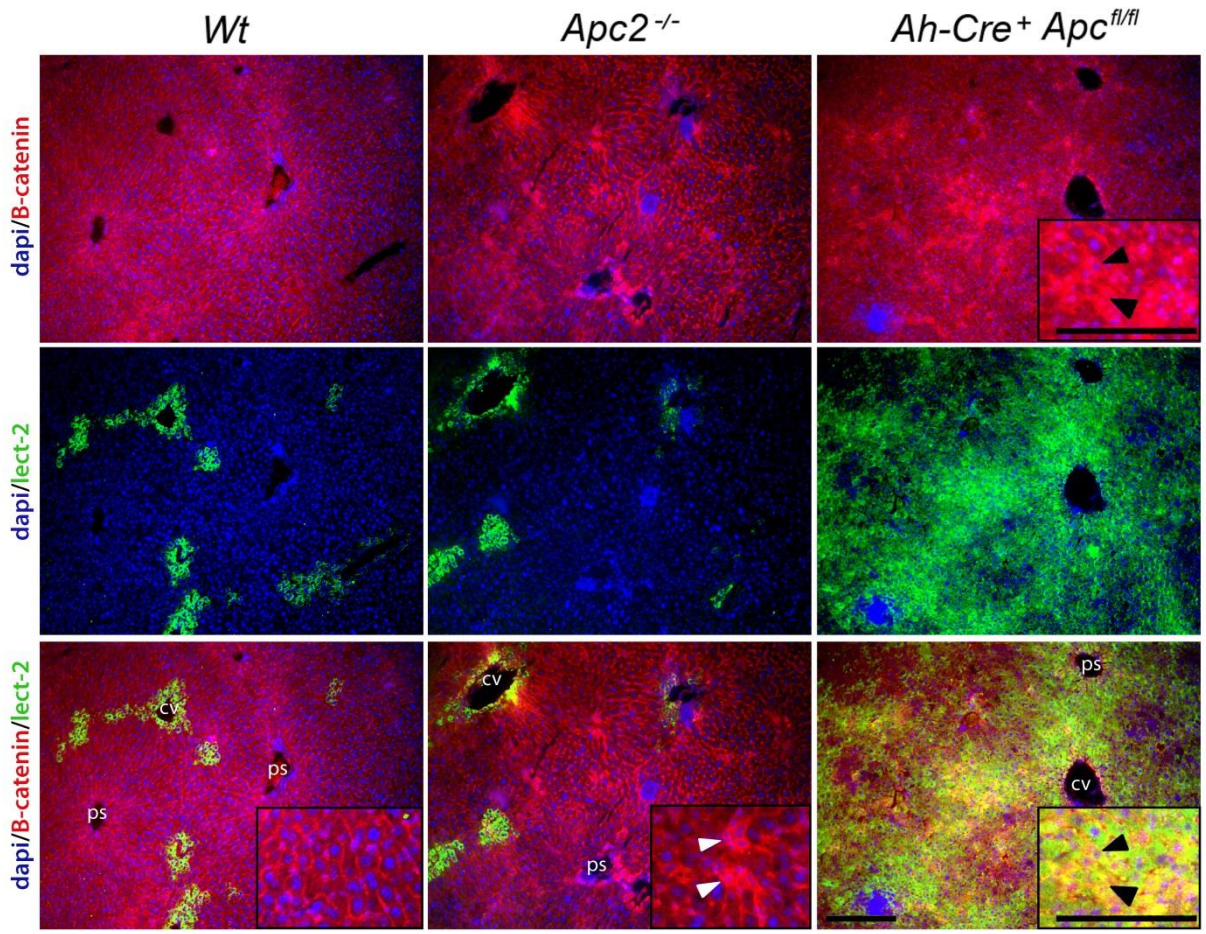

Figure 6.5: Loss of either Apc or Apc2 led to nuclear/cytoplasmic β-catenin accumulation. However, Lect2 was only up regulated following Apc loss. Double label fluorescent IHC was performed on liver sections to assess β-catenin and Lect2 status. In the *Wt* β-catenin staining was membranous and Lect2 expression limited to a few cells around the centrilobular vein. In the *Apc2*$^{-/-}$ livers, although the majority of cells displayed membranous β-catenin, a few cells showed increased nuclear/ cytoplasmic β-catenin. Interestingly, these cell did not display an up regulation of the Wnt target gene Lect2 (white arrowheads) and the pattern of expression remained similar to *Wt*. Following induction, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers almost completely lost membrane associated β-catenin, the majority of cells now show nuclear or cytoplasmic β-catenin staining concurrent with an up-regulation of Lect2 (black arrowheads). The Lect2 pattern of expression seen in *Wt* and *Apc2*$^{-/-}$ is lost in *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers and now the majority of cell displayed expression regardless of position. Inlet shows a higher magnification of bottom panel (scale bar 200um).

6.2.4 Liver zonation is maintained with loss of Apc2 but severely perturbed following loss of Apc

In order to evaluate the effects on liver zonation due to loss of either Apc or Apc2, liver sections from each genotype were co-labelled using fluorescent IHC for glutamine synthetase (GS) and carbamoyl phosphate synthetase (CPS). GS is an enzyme related to ammonia detoxification, part of a gene signature pattern zonally expressed in PV hepatocytes and a direct liver β-catenin target gene (Cadoret et al., 2002; Ovejero et al., 2004). CPS is a urea cycle enzyme, part of a gene signature pattern expressed mostly in PP hepatocytes, intermediates, and the first few layers of the PP zone. Loss of Apc2 did not lead to changes in liver zonation (Figure 6.6). However, loss of Apc led to an increase in GS and a decrease in CPS staining. This is in agreement with previous studies showing an increase in a PV genetic program, at a PP expense (Benhamouche et al., 2006; Reed et al., 2008; Burke et al., 2009). Whilst Lect2 was only expressed in a layer of a few cells around the CV in *Wt* and *Apc2*$^{-/-}$ livers, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers displayed labelled cells regardless of zone. This data indicates that although Apc loss effects liver zonation, Apc2 was dispensable for this function.

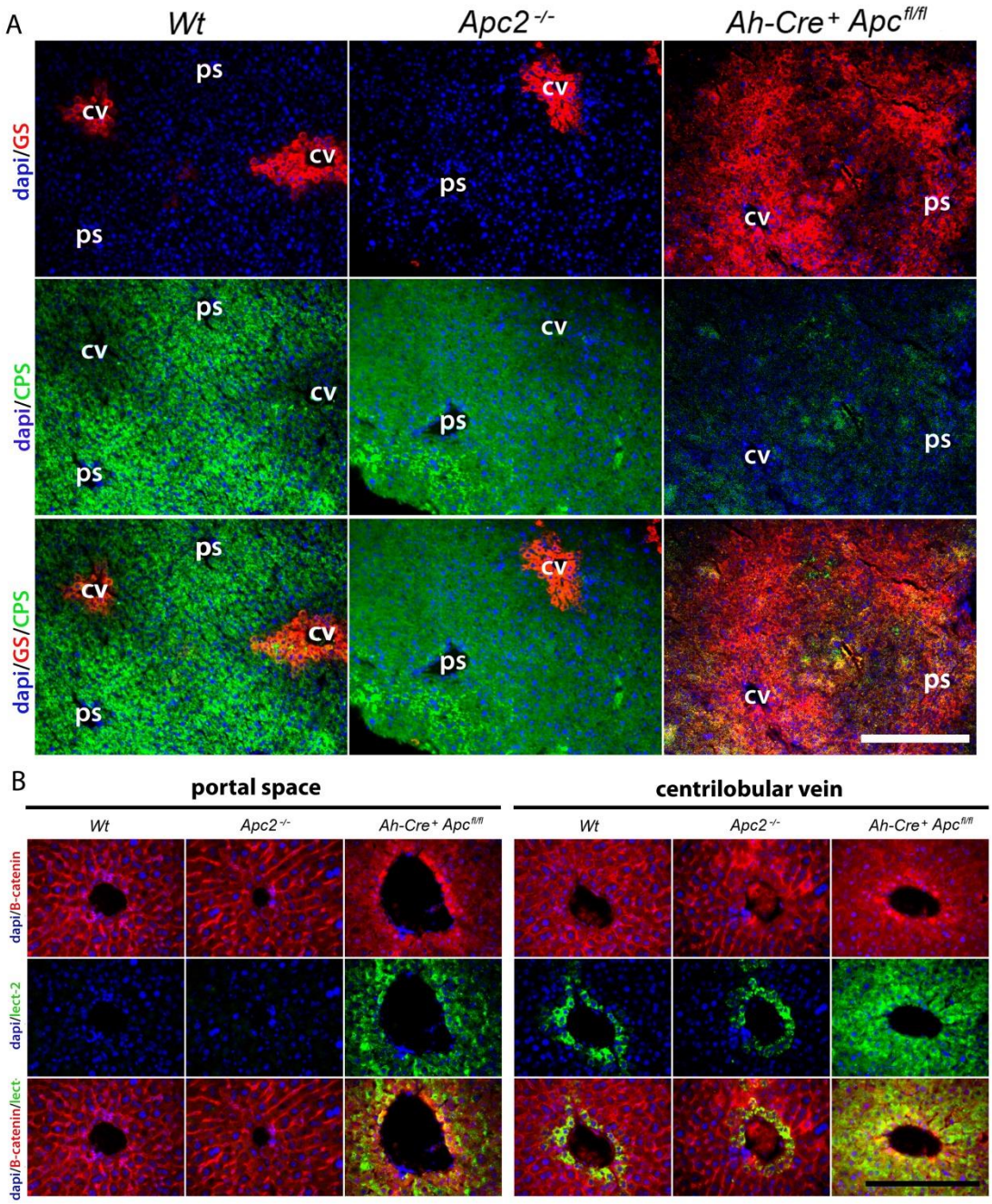

Figure 6.6: Acute loss of Apc alone altered Liver zonation. (**A**) Fluorescent IHC for CPS and GS displayed normal zonation in *Wt* and *Apc2*$^{-/-}$ animals, GS was located in a layer a few cells thick surrounding the central vein and CPS was located in a complementary fashion in periportal, intermediate, and the first few layers of the perivenous zone. However, in *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice, CPS was expressed in a few cells whilst GS was expressed in most cells regardless of position in regards to portal space or centilobular vein position. (**B**) Analysis of expression patterns of Lect2 in cells surrounding the portal space or centilobular vein indicated that *Apc2*$^{-/-}$ livers displayed a similar pattern to *Wt* with Lect2 only expressed in a few cells surrounding the centrilobular vein. However, in *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice, Lect2 was expressed in the majority of cells regardless of position to portal space or centilobular vein position (scale bar 200um).

6.2.5 Aberrant Wnt signaling occurs following loss of either Apc or Apc2 but differs in each situation

To determine if Apc2 loss altered gene expression within the liver and how these changes might compare to those of Apc loss, quantitative analysis of known Wnt target and repressors genes was performed. Total RNA from liver samples was extracted, subjected to reverse transcription and compared using quantitative real-time PCR (qRT-PCR). *Apc2*$^{-/-}$ liver gene expression levels were directly compared to that of aged matched *Wt*s. Induced *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ gene expression levels were directly compared to that of induced age matched *Apc*$^{fl/fl}$ controls. The shortened lifespan following induction of *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice, combined with the hypomorphic nature of the *Apc*^{fl} allele (assessed in 6.2.9) necessitated the requirement of separate controls for the different comparisons. Indirect comparisons were made between Wnt gene expression profiles following Apc2 or Apc loss (Figure 6.7). *β-catenin* and *Dickkopf2* expression levels remained unchanged following either Apc2 or Apc loss. *C-Myc* and *CD44* expression were unchanged in *Apc2*$^{-/-}$ livers compared to control. However, *Ephrin B2* and *Ephrin B3* were significantly increased in *Apc2*$^{-/-}$ livers, whilst *Cyclin D1* and *Groucho* where significantly decreased. *cMyc*, *CD44*, *Cyclin D1* and *Groucho* were all significantly increased following Apc loss, whilst *Ephrin B2* and *Ephrin B3* remained unchanged from controls. Axin2 is part of a Wnt negative feedback loop and both a Wnt target gene and Wnt repressor. It was significantly up-regulated following lack of either Apc2 or Apc in the liver (Figure 6.7, for all statistically significantly differences $p \leq 0.05$, Whitney *U*-test, $n \geq 3$).

6.2.6 Apc2 deficiency attenuates the HCC phenotype associated with reduced levels of Apc expression

Apc2$^{-/-}$ mice did not display altered survival compared to *Wt* (chapter 3) or any associated liver pathology. Conversely, it has been reported that mice with reduced Apc expression through low level Cre mediated deletion develop HCC (Colnot et al., 2004). Indeed, work

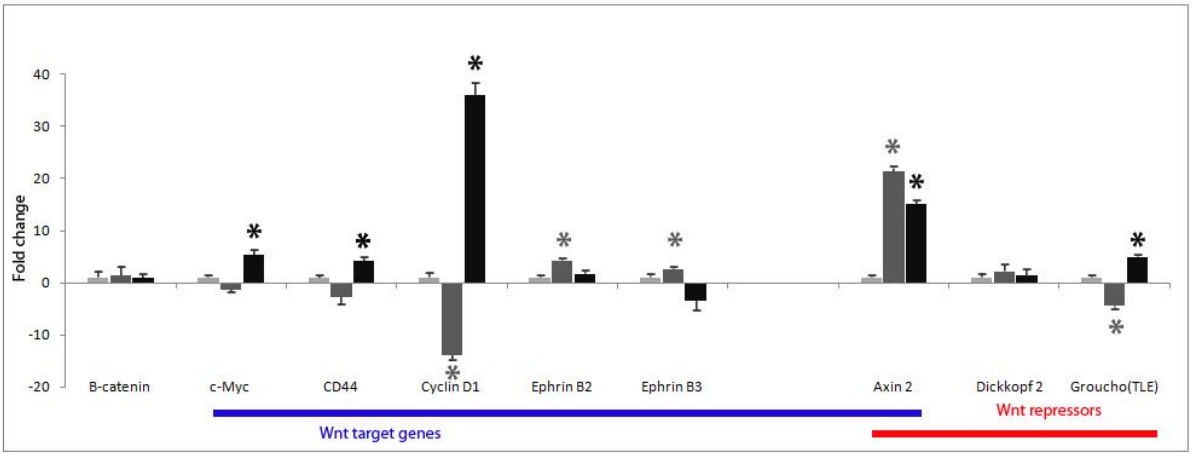

Figure 6.7: Expression analysis of Wnt target genes and Wnt repressors displayed different profiles following loss of either Apc or Apc2 protein. Quantitative RT-PCR was used to compare expression levels between **i**, *Wt* control vs. *Apc2*$^{-/-}$ aged match livers (light gray vs. dark gray bars) and **ii**, *Apc*$^{fl/fl}$ control vs. *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers 4 days following tamoxifen Induction (light gray vs. Black bars*). β-catenin* and *Dickkopf 2* expression levels remained unchanged following either Apc2 or Apc loss. *cMyc* and *CD44* expression was unchanged in *Apc2*$^{-/-}$ livers compared to control. However, *Ephrin B2* and *Ephrin B3* were significantly increased whilst *Cyclin D1* and *Groucho* where significantly decreased. Following Apc loss, *cMyc*, *CD44*, *Cyclin D1* and *Groucho* were significantly increased whilst *Ephrin b2* and *Ephrin b3* remained unchanged compared to control liver. Axin2 is part of a Wnt negative feedback loop and both a Wnt target gene and Wnt repressor, It was significantly up regulated due to lack of either liver Apc2 or Apc (Gray asterisks mark the comparisons that were found to be significantly different between *Wt* and *Apc2*$^{-/-}$ aged matched livers, black asterisks between *Apc*$^{fl/fl}$ and *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ livers 4 days following tamoxifen Induction $p \leq 0.05$, Mann-Whitney *U*-test, $n \geq 3$, error bars represent SEM of C_t values).

from our laboratory and others has shown that the attenuated expression of Apc in un-induced *Apc*$^{fl/fl}$ mouse leads to HCC development with a 100% penetrance by 400 days of age (Buchert et al., 2010). As Cre negative *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice are viable, we were able to directly test the significance of Apc2 on HCC development. Cohorts of *Wt*, *Apc2*$^{-/-}$, *Apc*$^{fl/fl}$, and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice were aged to 400 days, sacrificed and livers harvested for analysis. Upon sacrifice it was noticed that *Apc2*$^{-/-}$ livers appeared normally sized, while *Apc*$^{fl/fl}$, displayed an increase in liver size (Figure 6.8A). This was attenuated in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice. Quantitative analysis of liver to body weight ratio (Figure 6.8B) revealed a significant increase in *Apc*$^{fl/fl}$ mice which was rescued in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice (statistically significant differences $p<0.01$, Whitney *U*-test, $n \geq 3$). Strikingly, macroscopic tumours were

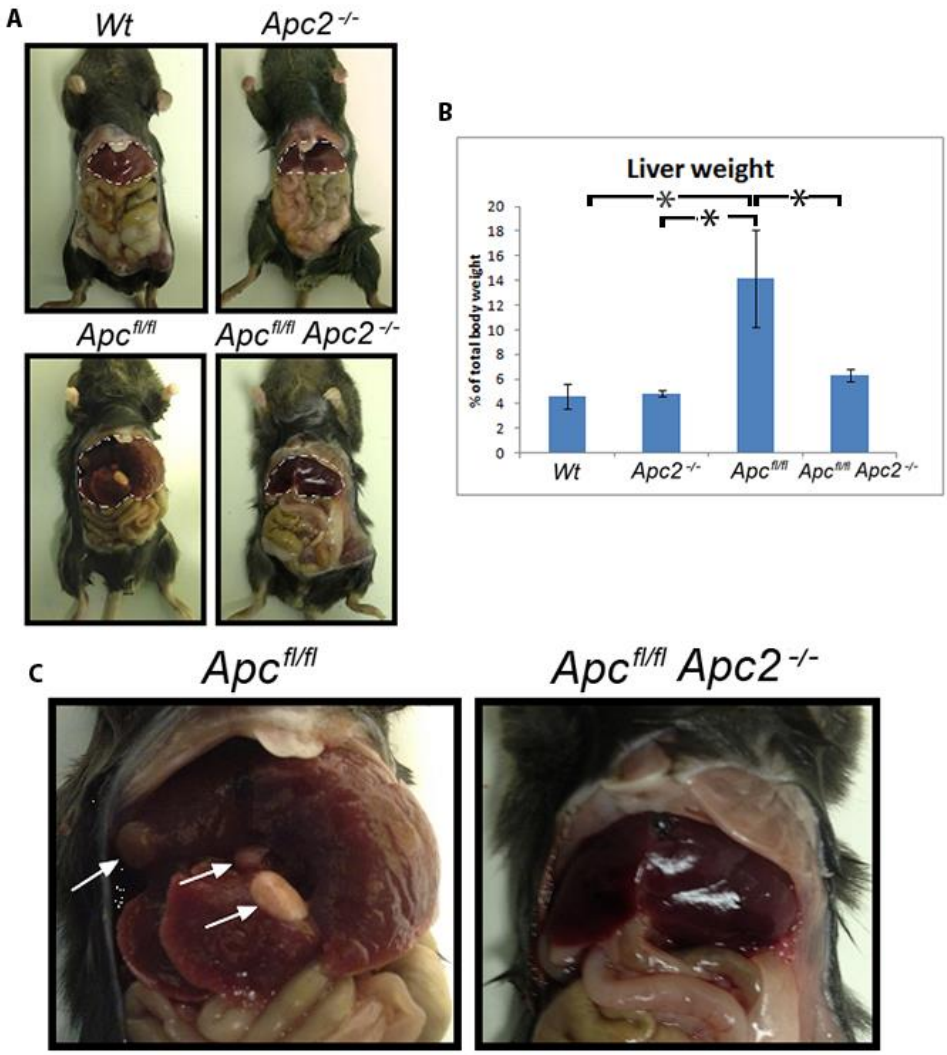

Figure 6.8: loss of Apc2 did not lead to a liver weight increase or macroscopic tumour formation in aged animals. However, it attenuated the phenotype associated with reduced levels of Apc expression. Mice from cohorts of *Wt*, *Apc2*$^{-/-}$, *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ were aged and sacrificed at 400 days. (**A**) Macroscopic examination revealed no difference in *Apc2*$^{-/-}$ livers but an increase in liver size in *Apc*$^{fl/f}$ mice. This increase in liver size was not observed in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers (white dotted line indicates liver outline) suggesting a rescued phenotype. (**B**) Quantitative analysis of liver to body weight ratio revealed a statistical increase in *Apc*$^{fl/fl}$ livers compared to other genotypes. Additional Loss of Apc2 on an *Apc*$^{fl/fl}$ background rescued this phenotype. Asterisks mark the pair-wise comparisons that were found to be significantly different $p \leq 0.05$, for all comparisons $n \geq 3$, error bars represent SD. (**C**) Macroscopic tumours were only visible in the *Apc*$^{fl/fl}$ aged livers. Additional loss of Apc2 rescued the HCC phenotype (white Arrows indicate macroscopic HCC).

only visible in *Apc*$^{fl/fl}$ livers (Figure 6.8C). Liver sections from *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice were stained with H&E to assess histology. Large tumours were present in *Apc*$^{fl/fl}$ mice with 100% penetrance, consistent with previous studies. Astonishingly, no macroscopic tumours

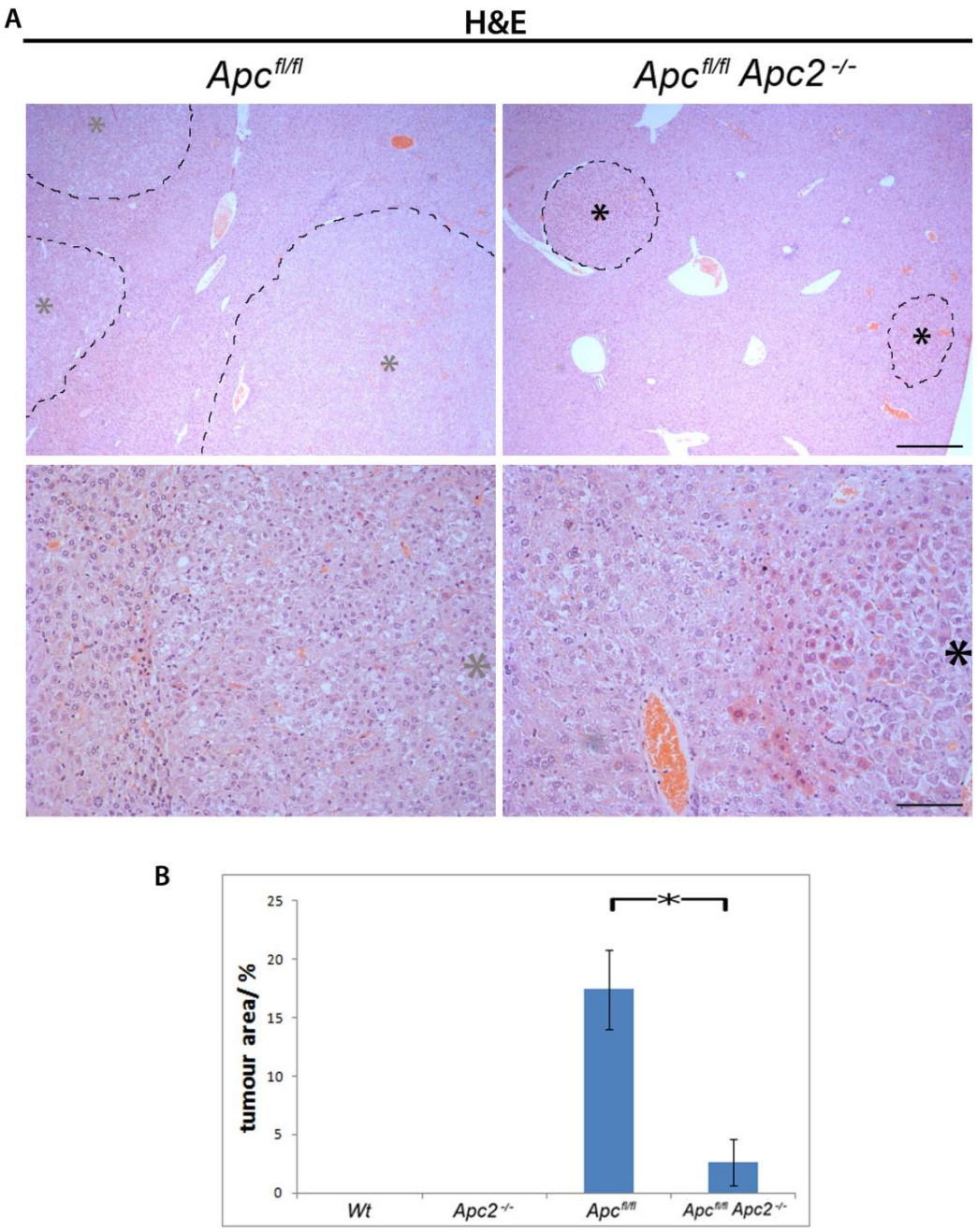

Figure 6.9: Histological analysis revealed the presence of large tumours in *Apc*$^{fl/fl}$ livers attenuated by the loss of Apc2. (**A**) H&E staining of sections from *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers revealed large tumours in livers with reduced levels of Apc (Gray asterisks) and small abnormal foci in livers with reduced levels of Apc and loss of Apc2 (Black asterisks). Lower panel represents higher magnification of top image (150µm and 500µm respective scale bars). Dotted line represents outline of abnormal tissue. (**B**) Scoring of tumour area between liver section from *Wt*, *Apc2*$^{-/-}$, *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers shows no tumour formation following loss of Apc2 and an attenuation of tumour growth in *Apc*$^{fl/fl}$ livers with additional Apc2 loss (error bars represent SD, asterisks mark p≤0.01, Mann-Whitney *U*-test, n≥3).

were visible in *Apc*$^{fl/f}$ *Apc2*$^{-/-}$ livers. Upon histopathological examination of H&E stained liver sections (performed in collaboration with Prof. Geraint T. Williams), *Apc*$^{fl/fl}$ livers all displayed large tumours which were classified as HCC (Figure 6.9A). HCC was not present in any *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers, although small abnormal foci were observed which were classified as microadenomas. In order to provide a quantitative value of tumour area per animal, the area of abnormal tissue was scored and divided by total area of liver (Figure 6.9B). Whilst *Wt* and *Apc2*$^{-/-}$ mice did not develop liver lesions, almost 18% of *Apc*$^{fl/fl}$ liver was tumour tissue. Additional *Apc2*$^{-/-}$ mutation attenuated this to ~ 2.5% ($p \leq 0.01$, Whitney *U*-test, $n \geq 3$). This reduction in tumorigenesis suggests that the presence of Apc2 in a hypomorphic Apc background positively contributes to tumour development. This contribution may be directly though modulating the level of Wnt signalling required for a neoplastic initiation or indirectly though providing an environment that positively contributes to tumour progression/growth or a combination of both.

6.2.7 Additional loss of Apc2 reduced the amount of dividing cells found within abnormal foci of livers with reduced Apc expression

As a means of determining the contribution of Apc2 to tumour initiation/progression, serial sections of abnormal foci from both *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ were stained for β-catenin and Ki-67 (Figure 6.10). Nuclear β-catenin is well documented as a Wnt signal with potential to induce a proliferative program (for review see Reya &Clevers, 2005). Both *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ lesions displayed a heterogeneous pattern of increased cytoplasmic/nuclear β-catenin however, only *Apc*$^{fl/fl}$ lesions displayed an increase in Ki-67 positive cells compared to surrounding tissue. This suggests additional loss of Apc2 has an effect downstream of β-catenin reducing the proliferative signal.

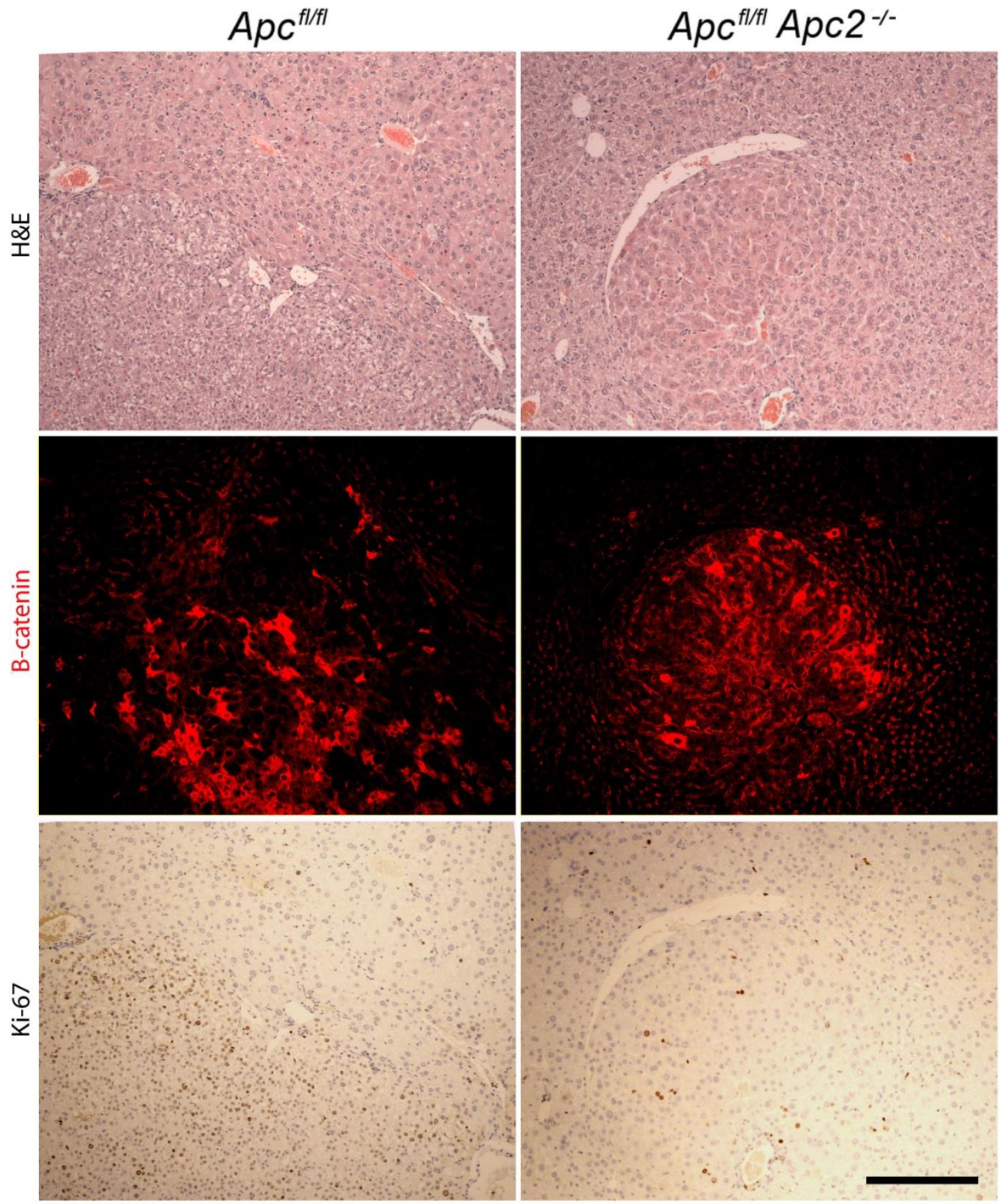

Figure 6.10: Both *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ lesions within the aged livers displayed elevated cytoplasmic levels of β-catenin however, less actively cycling cells were present within lesions of *Apc*$^{fl/fl}$, *Apc2*$^{-/-}$ livers compared to that of *Apc*$^{fl/f}$. IHC carried out on abnormal foci found in both genotypes showed elevated cytoplasmic levels of β-catenin suggesting perturbed Wnt signalling. Ki-67 antibody was used to label actively dividing cells. Less dividing cells were seen in the abnormal foci of *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers compared to that of *Apc*$^{fl/fl}$ (Scale bar 200um).

Lect2 is implicated as a direct target gene of β-catenin in the liver (Ovejero et al., 2004; Cadoret et al., 2002). In order to test the hypothesis that additional Apc2 loss attenuates activation of Wnt target gene expression produced by reduced Apc levels, lesions from each genotype were co-labelled with β-catenin and Lect2 antibodies using florescent IHC (Figure 6.11). Surprisingly, although there was an up regulation of Lect2 in both genotypes, the Lect2 staining pattern was mosaic and failed to correlate with nuclear β-catenin in either genotype.

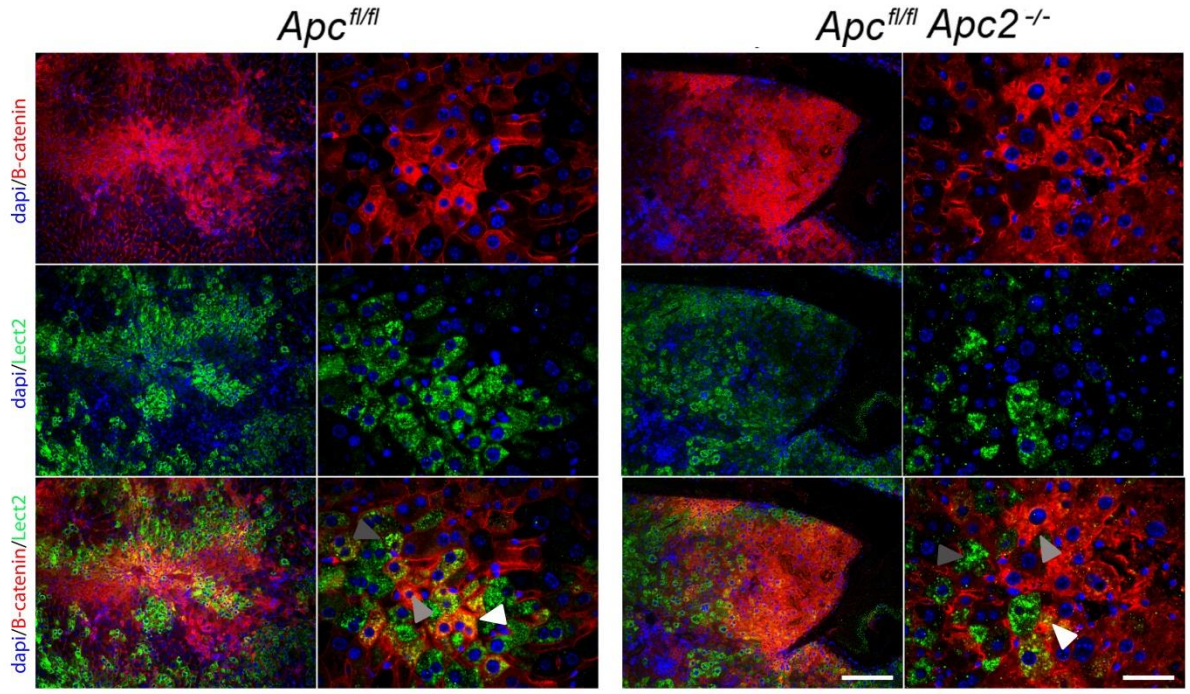

Figure 6.11: Abnormal foci from *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ both display increased nuclear/cytoplasmic β-catenin. Lect2 status is not associated with β-catenin location or intensity in either genotype and displayed a mosaic expression pattern. Fluorescent IHC was performed on lesions from *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers staining for β-catenin and Lect2. Right hand panels show higher magnification of the left hand panels, white arrow heads indicate cells with elevated nuclear/cytoplasmic β-catenin, dark gray arrow heads indicate cells with only Lect2 expression, and light gray arrow heads indicate cells with combined elevated nuclear/cytoplasmic β-catenin and Lect2 expression (scale 200µm in left hand panel, 50µm in right).

6.2.8 Loss of liver zonation associated with reduced Apc expression can be partially rescued in double mutant mice

In order to address if Apc2 contributes to a pro-tumour environment in an *Apc*$^{fl/fl}$ mouse liver, non-tumour tissue between *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ was analysed and compared to *Wt*. Sections were co-labelled with β-catenin and Lect2 antibodies using florescent IHC to assess protein status (Figure 6.12). In *Wt* liver β-catenin was exclusively membrane associated however in *Apc*$^{fl/fl}$ liver there were cells displaying increased cytoplasmic and nuclear β-catenin. This was also apparent in the *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers also but to a lesser extent. In *Apc*$^{fl/fl}$ liver, Lect2 was up regulated and displayed a mosaic pattern. No zonation was apparent and cells displayed Lect2 positivity regardless of PV or PP position. Interestingly in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers, Lect2 was also up regulated but there was clear zonation. A Lect2 free zone was maintained around the PS albeit it reduced compared to *Wt* liver. Lect2 status did not correlate with β-catenin location or intensity in either genotype.

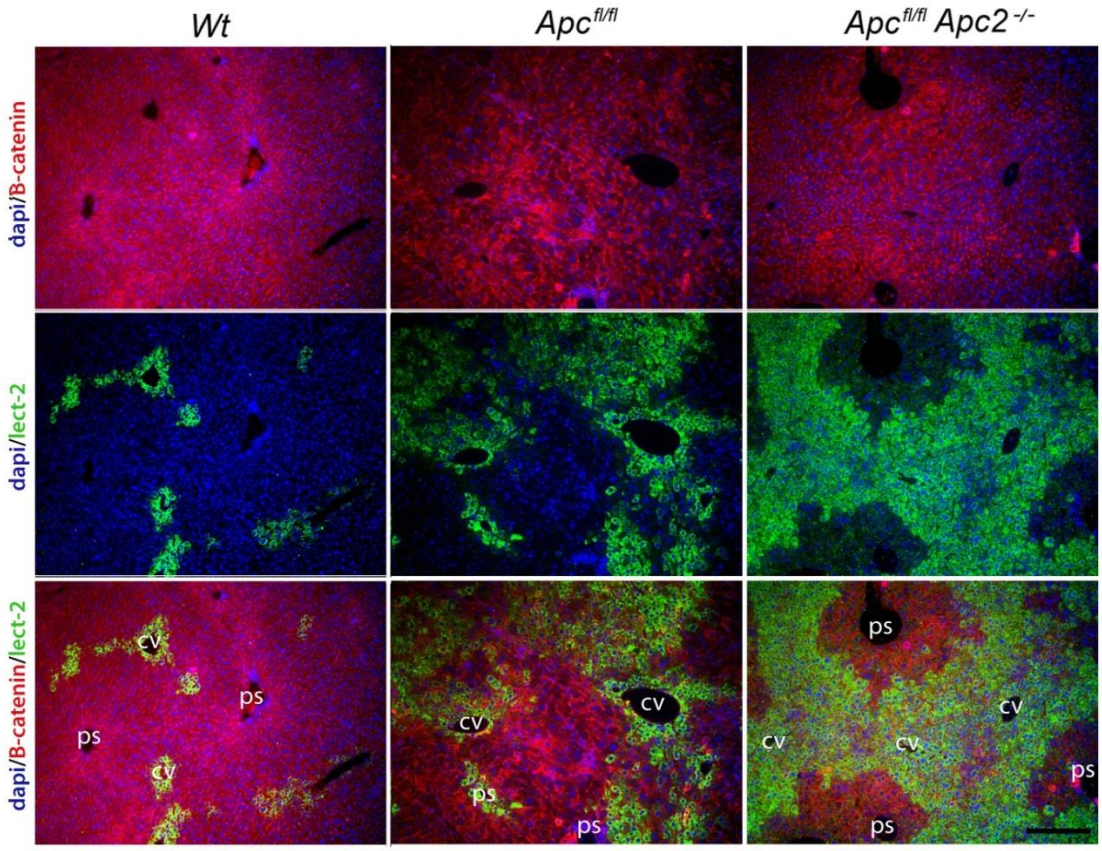

Figure 6.12: Lect2 expression was increased in both *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers however, in *Apc*$^{fl/fl}$, *Apc2*$^{-/-}$ livers a Lect2 clear zone remained around the portal space. Fluorescent IHC was carried out on livers from each genotype to assess β-catenin and Lect2 expression patterns. β-

catenin was membrane associated in the majority of all cells, in non-tumour tissue, regardless of genotype. However, in the *Apc*$^{fl/fl}$ livers there were more cells with increased cytoplasmic/nuclear β-catenin compared to *Wt* controls, this was not so apparent in the *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers. Lect2 expression was increased in *Apc*$^{fl/fl}$ livers compared to *Wt;* cells stained Lect2 positive regardless of perivenous or periportal position with a mosaic appearance. *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers also displayed an increase in number of cell stained positive for Lect2 compared to *Wt* however, a clear lect2 free zone was maintained, albeit a lot smaller than in the *Wt* livers. Lect2 status did not correlate with β-catenin location or intensity in any genotype (scale bar 200μm).

To properly assess zonation, liver sections from *Wt*, *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice were co-labelled with GS and CPS using florescent IHC (Figure 6.13). *Apc*$^{fl/fl}$ livers show an increase in expression of the PV gene marker GS in comparison to *Wt*, GS is expressed in the majority of cells regardless of position in comparison to PS or CV position. Also, in the *Apc*$^{fl/fl}$ livers the PS marker CPS is down regulated and only a few sporadic cells show CPS expression. In contrast, although *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers also displayed an increase in GS and decrease in CPS expression, a clear pattern of zonation is still apparent. CPS expression is not as reduced as in *Apc*$^{fl/fl}$ livers and clear zonation is maintained in PP hepatocytes surrounding the portal space.

6.2.9 Additional loss of Apc2 altered the Wnt target gene profile associated with reduced Apc expression

To address how additional Apc2 loss effected Wnt pathway changes invoked by reduced Apc levels, quantitative analysis of levels of known Wnt pathway genes was performed on aged liver tissue from *Wt*, *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ animals (n≥3). Total RNA from liver tissue was extracted and subjected to reverse transcription. Gene expression levels of Wnt pathway genes were compared using quantitative real-time PCR (qRT-PCR) (Figure 6.14). Interestingly, *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ liver samples displayed reduced expression of *β-catenin* in comparison to *Wt*. *cMyc* and *Axin2* expression levels were significantly higher in both *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ liver samples compared to *Wt* however, no statistically significant differences were observed between these two genotypes. *CD44* expression was reduced in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ liver samples in comparison to both *Wt* and *Apc*$^{fl/fl}$ samples. *Cyclin D1*

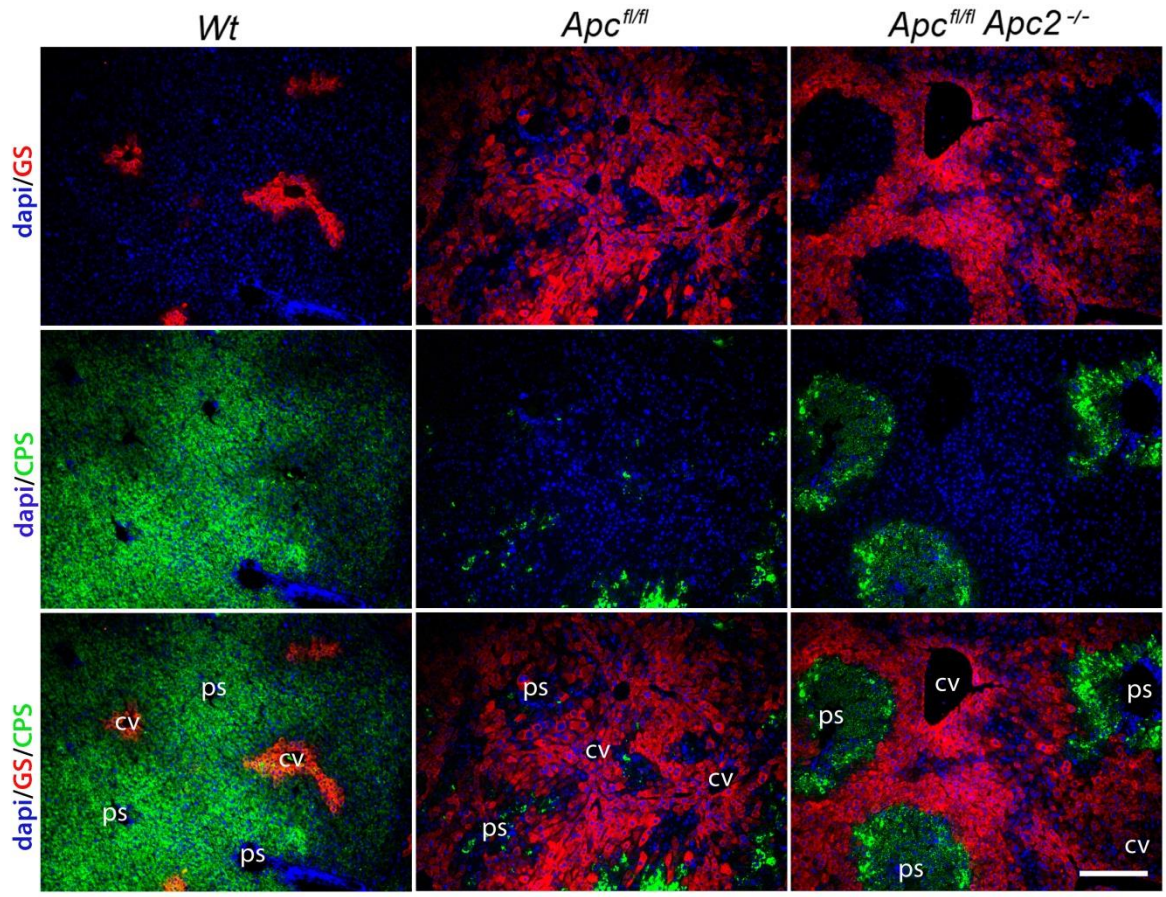

Figure 6.13: Additional loss of Apc2 on an *Apc*$^{fl/fl}$ background helps maintain some liver zonation after aging, albeit zone sizes differ from *Wt*. Fluorescent IHC was carried out on livers from each genotype to assess perivenous (GS) and periportal (CPS) gene expression patterns. At 400 days *Apc*$^{fl/fl}$ livers show an increase in expression of the PV gene marker GS in comparison to Age matched *Wt* controls. GS is expressed in the majority of cells regardless of position in comparison to portal space or centilobular vein position. Also in the *Apc*$^{fl/fl}$ livers the PP marker CPS is down regulated and only a few sporadic cells show CPS expression in no organised manner. In contrast, although *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers also show an increase in GS and decrease in CPS expression in comparison to age matched *Wt* controls, a clear pattern of zonation is still eminent. CPS expression does not seem as reduced as in *Apc*$^{fl/fl}$ and it is well 'zoned' in periportal cells surrounding the portal space (scale bar 200µm).

expression levels were reduced in *Apc*$^{fl/fl}$ liver samples compared to *Wt*. Additional loss of Apc2 attenuated this reduced expression. *Dickkopf2* displayed no statistically significant differences between any genotype. *Apc*$^{fl/fl}$ liver samples showed an increase in expression of the Wnt repressor *Groucho* compared to *Wt* controls. Additional loss of Apc2 significantly increased *Groucho* expression even more (for all statistically significantly differences $p \leq 0.05$, Whitney *U*-test, $n \geq 3$).

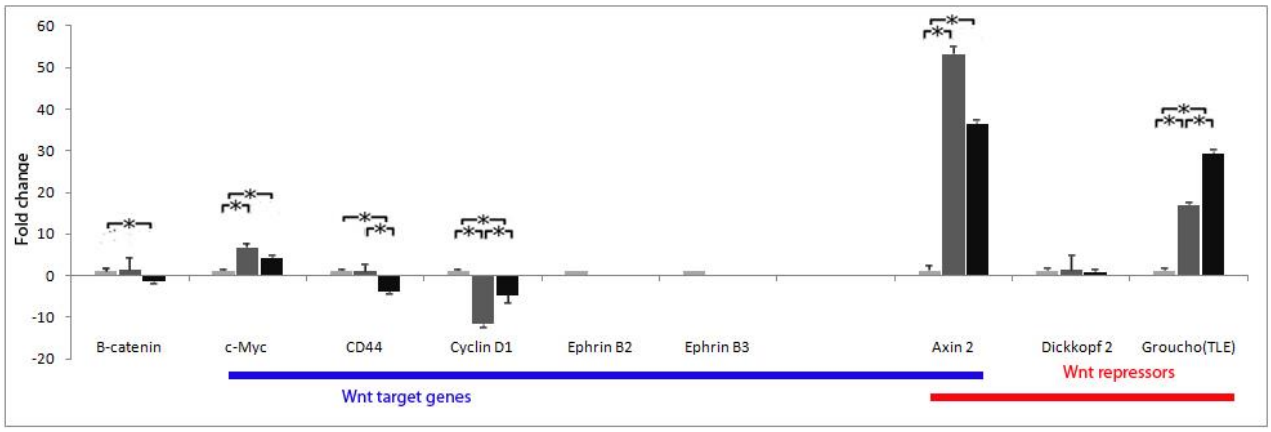

Figure 6.14: Additional *Apc2*$^{-/-}$ mutation on an *Apc*$^{fl/fl}$ background altered the expression pattern of some Wnt target genes and repressors. Quantitative RT-PCR was used to compare expression levels between *Wt* (light gray bars), *Apc*$^{fl/fl}$ (dark gray bars) and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ (black bars) liver samples taken from animals sacrificed at 400 days of age. *β-catenin* expression was shown to be down regulated only in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ compared to *Wt*. *cMyc* and *Axin 2* expression levels were significantly higher in both *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers compared to *Wt*. *CD44* was down regulated in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ in comparison to both *Wt* and *Apc*$^{fl/fl}$ livers. *Cyclin D1* was down regulated in *Apc*$^{fl/fl}$ liver samples in comparison to *Wt*. Additional *Apc2*$^{-/-}$ mutation attenuated this reduced expression. *Apc*$^{fl/fl}$ livers displayed an increase in expression of *Groucho* compared to *Wt* controls. *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers exhibited an even greater increase in expression of expression of *Groucho* (asterisks mark the pairwise comparisons that were found to be significantly different, p≤0.05, Mann-Whitney *U*-test, n≥3, error bars represent SEM of C_t values).

6.3 Discussion

A transgenic approach was used to study the '*in vivo*' consequences of loss of Apc proteins within the liver. As previously reported, acute loss of Apc resulted in liver hyperproliferation causing hepatomegaly, increased nuclear/cytoplasmic β-catenin, loss of zonation and an increase in Wnt target gene expression (Colnot et al., 2004; Reed et al., 2008; Benhamouche et al., 2006). Livers deficient for Apc2 however, failed to show changes in liver homeostasis and/or any liver associated pathology. *Apc2*$^{-/-}$ Liver size remained comparable to *Wt* even after 400 days, levels of cell division and zonation also remained indistinguishable from *Wt*. Interestingly livers deficient for Apc2 do have cells with increased nuclear/ cytoplasmic β-catenin however, these cells fail to display abnormal histology or express Lect2 a known liver Wnt target gene.

Although both Apc proteins have the ability to mediate Wnt signaling through β-catenin, and loss of either within the liver can lead to changes in β-catenins' sub-cellular intensity/ localisation, Wnt gene profiles differ between genotypes and only Apc loss induces a phenotype. This may be due to different affinities of β-catenin destruction between the two Apc homologs (Nakagawa et al., 1998) that extend to the liver. Loss of Apc or Apc2 may lead to different threshold levels of Wnt, indeed fewer cells did show aberrant β-catenin intensity/localisation in *Apc2*$^{-/-}$ livers compared to that of Apc deficient livers. However, *Axin2,* which is a commonly used readout of Wnt (Lustig et al., 2002; Jho et al., 2002) was up-regulated in both genotypes, suggesting high levels of Wnt activation in both genotypes. Another hypothesis is that, as the Apc2 mutation is constitutive, levels of Wnt may have been readjusted due to developmental compensation. However, *Dickkopf 2* a known Wnt repressor (Mao et al., 2001; Kawano & Kypta, 2003) which antagonises Wnt in early hepatic development (Bi et al., 2009), did not display any difference in levels of expression between *Apc2*$^{-/-}$ and control livers. *Groucho*, another known Wnt repressor (Cavallo et al., 1998) which directly competes with β-catenin in Tcf/Lef binding (Daniels & Weis, 2005) surprisingly displayed reduced levels of expression in *Apc2*$^{-/-}$ livers compared to controls. The reasons behind this reduction remain unclear.

A third explanation for why only Apc loss induces a phenotype may lay with unique functions of Apc either downstream or even independent of β-Catenin phosphorylation. Non-canonical functions of Apc have been reported in other tissues (Fodde et al., 2001; Kaplan et al., 2001; Näthke, 2006; Prosperi et al., 2009; Qian et al., 2008; Harris & Nelson, 2010). β-catenin itself is dispensable for normal liver function (Skekine et al., 2006) and activation of β-catenin in hepatocytes (Cre mediated, low level) can lead to an increase in nuclear/cytoplasmic β-catenin in cells that still appear healthy and quiescent (Harada et al., 2002). However, high levels of β-catenin activation have caused hepatomegally in some studies (Harada et al., 2002; Cadoret et al., 2001; Stein et al., 2011) although not all (Miyoshi et al., 2009; Nejak-Bowen et al., 2009). Together the evidence raises the possibility of a β-catenin phosphorylation independent role of Apc in liver homeostasis. However, immediate phenotypes of Apc loss can be rescued upon additional knockout of β-Catenin (Reed et al., 2008) suggesting some function of β-catenin must be present to allow the effects of Apc loss to become manifest. β-catenin's role in the liver, either independently or through

interaction with Apc or Apc2 is complex. Further studies are needed to elucidate their roles in liver homeostasis.

Unfortunately due to low level embryonic expression of the *Ah-Cre*$^{+}$ transgene, *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice died '*in utero*' due to compromised functional redundancies that must exist between the Apc proteins during development. This hindered our ability to study the immediate consequences of acute loss of both proteins within the liver. This could be circumvented by the use of a more tissue specific Cre promoter, for example, the *Albumin-Cre* (Postic et al., 1999) or *Transthyretin-Cre* (Tannour-Louet et al., 2002) or Cre mediated deletion could be achieved through the use of an injectable adenoviral Cre (Colnot et al., 2004). Although acute loss of both Apc proteins within the liver was not achievable through our experimental design, this is the first study to analyse liver specific consequences of Apc2 loss and compare them to that of Apc loss.

The Hypomophic nature of the *Apc*^{fl} allele (Shibata et al., 1997) allowed us analyse liver phenotypes associated with attenuated Apc expression. Furthermore, as *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice were viable it permitted us to directly test the significance of Apc2 in an attenuated Apc background. Consistent with other studies (Burke et al., 2009; Buchert et al., 2010), aged *Apc*$^{fl/fl}$ livers possessed cells with eminent nuclear/cytoplasmic β-catenin, aberrant zonation and mice developed HCC with 100% penetrance. Strikingly, although livers from *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice still displayed cells with elevated nuclear/cytoplasmic β-catenin and some zonation perturbation, HCC was prevented. This reduction in tumorigenesis clearly demonstrates that the presence of Apc2 in the hypomorphic *Apc*$^{fl/fl}$ environment, positively contributes to liver tumour formation. Small microscopic areas of abnormal foci were noticed in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers which failed to progress from microadenomas. On comparing β-catenin status between abnormal foci from each genotype, it was revealed that both displayed a heterogeneous pattern of increased cytoplasmic/nuclear staining. However, in the lesions in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers, proliferation was attenuated in comparison to those in *Apc*$^{fl/fl}$. There are various possible explanations behind why additional Apc2 loss reduces proliferation and prevents tumour progression.

'Just right' levels of Wnt signaling have been suggested to be required for oncogenesis in a variety of tissues (Lamlum et al., 1999; Albuquerque et al., 2002; Lucero et al., 2010). As

both Apc proteins have the ability to alter Wnt signaling, whilst attenuated Apc may lead to appropriate levels of Wnt for HCC formation and proliferation, addition of an *Apc2* mutation may alter these levels outside of that permissive for tumour progression. In an attempt to estimate levels of Wnt, Lect2 status was examined. Unfortunately, in contrast to published data (Ovejero et al., 2004; Cadoret et al., 2002), Lect2 did not correlate with β-catenin expression. Interestingly, despite inappropriate β-catenin signalling in up to 70% of human HCC tissue (de La Coste et al., 1998: Wong et al., 2001: Giles et al., 2003), most mouse models with liver specific β-catenin activating mutations fail to develop tumours (reviewed in Nejak-Bowen & Monga, 2011). Lack of oncogenesis in mice with liver targeted activating β-catenin mutations and the fact that mutations in *β-catenin* are only found in HCC and not adenomas in diethylnitrosamine (DEN)-induced mice (Ogawa et al., 1999), suggest that activated β-catenin may be a secondary rather than causative event in liver tumourigenesis. How Apc loss induces tumourigenesis may lie with functions independent of Wnt signaling. However, it is important to note that β-catenin activation does define a genetically distinct subset of human HCC's (Llovet and Bruix, 2008) clinically relevant to treatment strategies.

The mechanisms at work during the development of HCC remain poorly understood (Farazi and DePinho, 2006). However, it is known the development of HCC is likely to result from a multistep process beginning with the accumulation of genetic and epigenetic alterations in regulatory genes (Tischoff et al., 2008). Along with Wnt alterations, the genetic changes accumulated during oncogenic progression can include mutations in p53, PTEN, pRB, CDKN2A, PIK3CA and RAS family members (Zender et al., 2010). How attenuated Apc expression in our mice leads to the accumulation of genetic and epigenetic alterations causing HCC remains unresolved. On one hand, reduced non-canonical functions of Apc can lead to chromosome instability (Fodde et al., 2001; Kaplan et al., 2001; Näthke, 2006) which may invoke genetic changes needed for HCC progression. On the other hand, HCC can be rescued by limiting expression of β-catenin (Buchert et al., 2010). This suggests β-catenin is needed and involved for HCC development in $Apc^{fl/fl}$ livers. Interestingly $Apc^{fl/fl}$ $Apc2^{-/-}$ livers displayed reduced *β-catenin* expression in comparison to both *Wt* and $Apc^{fl/fl}$ livers. Although the mechanisms behind reduced β-catenin expression are unknown, it is possible that it contributes to attenuation of tumour progression seen in $Apc^{fl/fl}$ $Apc2^{-/-}$ mice.

An alternative explanation of how attenuated Apc expression may lead to HCC through accumulation of genetic changes might lie with its effects on zonation. In *Apc*$^{fl/fl}$ mice, a PV genetic program was induced at the expense of a PP program, 'zones' were diminished and hepatocytes of either fate displayed a mosaic pattern. Whilst hepatocytes which express genes like GS, ornithine aminotransferase (OAT) and glutamate transporter (GLT1) were increased and mis-localized, hepatocytes which express genes like CPS, Arginase (ARG1) and succinate dehydrogenase (SDH) were reduced and also mis-positioned. Perturbation of these hepatocytes will undoubtedly have detrimental effects on metabolism. Metabolic changes have proven paramount in cancer progression (reviewed in Marín-Hernández et al., 2009). CPS is a liver-specific, intramitochondrial enzyme which has a focal role in converting ammonia to less toxic urea (Haussinger et al., 1992). Various studies have demonstrated reduced to non-existent expression of CPS in HCC compared to normal tissue (Siddiqui et al., 2002; Butler et al., 2008; Liu et al., 2011). It is unknown whether CPS loss is a consequence or cause of HCC progression however, it is known that exposure to its substrate ammonia is implicated in tumour initiation in rodents (Uzvölgyi & Boján, 1980; Tsujii et al., 1995) and humans (Garland et al., 2004). SDH a krebs cycle gene, is mutated both germinally and somatically in a large subset of human cancers (reviewed Bardella et al., 2011) leading to an accumulation of its substrate succinate. Succinate accumulation leads to alterations in genome-wide histone and DNA methylation that can initiate and contribute to tumorigenesis (Xiao et al., 2012). It would be of interest to directly test the significance of these substrates on HCC development in our mice.

Interestingly, zonation was partially rescued in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers, adding support to the premise that zonation alterations can provide a permissive environment for tumour progression. This rescue of zonation by additional loss of Apc2 in an attenuated Apc background could partially be explained by differences in Wnt signaling. It is unknown whether Wnt expression changes precede, cause or are consequences of zonation changes. A sub-set of genes involved in the Wnt signaling pathway were profiled and compared between aged non-neoplastic tissue from *Wt*, *Apc*$^{fl/fl}$ and *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers. Whilst *cMyc* and *Cyclin D1* did show changes in expression between genotypes, phenotypes of liver Apc loss do not require cMyc (Reed et al., 2008), and neither are targets of β-catenin activation within the liver (Cadoret et al., 2001; Nejak-Bowen et al., 2009). *CD44* was down regulated

in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers and previous studies have indeed shown reduction of CD44 can reduce tumorigenesis in HCC cell lines (Xie et al., 2008). This reduction in *CD44* expression may contribute towards the HCC prevention. *Groucho* was significantly up-regulated in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers compared to both other genotypes. This suggests that increased Wnt repression may also contribute to the observed attenuation of HCC development. A genome wide array of expression changes could prove useful in delineating the molecular mechanisms behind the prevention of HCC in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ mice.

Surgical resection remains the treatment of choice for HCC patients with well-preserved liver function. However, it is associated with a high risk of post-operative complications and tumour recurrence (Nguyen et al., 2009). Liver transplantation is another favoured treatment but is hindered by the shortage of suitable liver grafts (Mazzaferro et al., 2008). Chemotherapy could provide a feasible option however, most drugs to date have proved ineffective in patients (Cao et al., 2012). Therapeutic targeting of Wnt/β-catenin in chemoprevention or as a possible treatment of cancers has been previously suggested (Luu et al., 2004; Kundu et al., 2006). Indeed, targeting of this pathway has shown some promise in animal models of HCC (Liu et al., 2006; Behari et al., 2007). As Apc2 is dispensable for normal liver function and has the ability to attenuate tumour progression poses it as an exciting therapeutic target. In this reported, it would be interesting to see how additional Apc2 loss may affect other models of HCC.

To conclude, varied expression of Apc alone has the ability to alter liver homeostasis and induce tumorigenesis. Evidence from these experiments suggests a role in addition to that of β-catenin phosphorylation. Whilst Apc2 is dispensable for liver homeostasis and tumour suppression, additional loss on an *Apc*$^{fl/fl}$ background prevented HCC.

Chapter 7

General discussion

Cancer is the largest primary cause of death in the UK and one of the largest in the world. Although incidence is on the rise, increased understanding of the disease has led to earlier detection and better treatment strategies thus increasing patient survival. It is known that cancer is the endpoint of a transformation of cells from normalcy into malignant tumours. For any cells to become cancerous they must acquire a full collection of necessary physiological adaptions or 'hallmarks' (Hanahan & Weinberg, 2011). Cells accumulate these traits through the multi-step process of tumour pathogenesis. It is known that genetic alterations enforce this multi-step process, so an immense effort has been placed into understanding how these alterations implement the necessary hallmarks on a molecular level.

Studying the genetics of cancer inducing syndromes and the genomic composition of sporadic tumours, gives us an insight into the genetic abnormalities that contribute to tumorigenesis. In colorectal cancer, the *APC* gene was mutated in the vast majority of cases (Kinzler et al., 1991; Groden et al., 1991; Powell et al., 1992; Nagase & Nakamura, 1993; Rowen et al., 1999, Ilyas et al., 2000). It was also mutated in a range of other cancers (Damjanov et al*.,* 1996; Virmani et al., 2001; Furuuchi et al., 2000; Kashiwaba et al., 1994; Giardiello et al 1996; Hirschman et al 2005; Gruner et al., 1998; Toiyama et al., 2011; Li et al., 2012). This strongly suggests APC is important in tumour suppression. Through the use of mouse transgenics, alterations in *Apc* were proven sufficient to induce tumourigenesis (Moser et al., 1993; Kuraguchi et al., 2009; Gaspar et al., 2009).

The *APC* gene has a highly evolutionally conserved homolog. APC2 displays structural similarities to APC (Van Es et al., 1999), so may have comparable functions. Loss of its gene

product has also been implicated in a range of human cancers (Jarrett et al., 2001; Sobottka et al., 2000; Yang et al., 2004; Wang et al., 1999; Nakagawa et al., 1999; Chan et al., 2008; Schuebel et al., 2007; Hsieh et al., 2007; Mokarram et al., 2009; Kumar et al., 2009). Both proteins are expressed in a wide range of human tissues (Van Es et al., 1999; Jarrett et al., 2001) and work from *Drosophila* demonstrates that these proteins display functional redundancies (Ahmed et al., 2002). It has also been shown that APC2 can complement APC loss in human colorectal cancer cell lines (Nakagawa et al., 1998; Van Es et al., 1999; Roberts et al., 2012). Together, these studies suggest that APC2 is also important in tumourigenesis and may have overlapping roles with APC.

Both APC proteins are known to mediate the Wnt signaling pathway, a pathway dysregulated in the majority of human cancers. Both have other roles beyond Wnt signaling regulation, which when disrupted may contribute to tumourigenesis. Although APC has been extensively studied, APC2 remains relatively uncharacterised. Due to the availability of a mouse model with mutated *Apc2* we could now study the relevance of Apc2 in a mammalian setting. The Aims of this thesis were: to elucidate Apc2's functions in a range of tissues, expose any roles of Apc2 in tumour suppression, to test these factors in the context of tissue specific Apc loss and elucidate any functional redundancies between the two mammalian Apc proteins.

Mice with homozygous *Apc2* mutation did not die '*in utero*' and were viable, in contrast to the situation with homozygous *Apc* mutations (Moser et al., 1995). Interestingly, whilst *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice were also viable, addition of a homozygous *Apc2* mutation induced embryonic lethality. Given that *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ are viable, low level embryonic expression of the *Ah-Cre*$^{+}$ promoter and subsequent *Apc* loss in conjunction with *Apc2* deficiency must account for this lethality. Together this highlights that functional redundancies exist between mammalian Apc homologs during embryonic development.

Although *Apc2*$^{-/-}$ mice were viable, on a purer C57BL/6 background, a proportion developed hydrocephalus. Apc2 is known to be expressed at its highest levels within the developing brain in both mice (Yamanaka et al., 2002) and humans (Van Es et al., 1999), so it is unsurprising that its loss affects this organ. This agrees with two recent studies whereby brain defects were apparent following Apc2 loss in both chick (Shintani et al., 2009) and mouse (Shintani et al., 2012) brains. Interestingly, in both studies Wnt signaling displayed no detectable differences and the phenotypes were attributed to direct cytoskeletal defects.

Upon aging mice that displayed no residing brain defects, loss of Apc2 did not induce tumour formation in any tissue nor did it alter overall lifespan. This suggests that Apc2 is dispensable for tumour suppression and that the loss of APC2 observed in human tumours is most likely associated with tumour progression rather than initiation or is just a passive event. Analysis of APC2 status alongside tumour grade and/or other genetic alterations may help elucidate APC2's true role in human cancers.

Within the intestines, Apc2 did display a role in regulation of the Wnt signaling pathway and this occurred in a gene dose dependent manor, suggesting that the level of Apc2 is important. Although no differences were noticed in macro- or microscopic intestinal morphology or composition of terminally differentiated cell types, cell division and markers of ISC's were increased. This occurs concurrent with an increase in crypt cells displaying nuclear localised β-catenin and an increase in Wnt target gene expression. However, tumour formation was not induced in this tissue. It is possible that Apc2 loss alone is insufficient to induce a 'just right' level of Wnt signaling need for intestinal tumourigenesis. Indeed, APC2 does display a reduced regulatory ability of Wnt signaling in comparison to APC (Nakagawa et al., 1998).

Another possible explanation as to why Apc2 loss does not lead to intestinal tumourigenesis may lie with a lack of disruption of the cytoskeleton. Migration and polarity defects have been associated with tumourigenesis in both mice and humans with altered *APC,* and this most likely lays with APC's ability to interact with microtubules. It is predicted that APC2

lacks domains that directly or indirectly interact with microtubules (reviewed in Allan & Nathke, 2001) so unlike APC, may have no influence on polarity or migration. However, as mentioned previously, it was shown by Shintani et al. (2009, 2012) that Apc2 does interact with the cytoskeleton within neural tissue, but this may be tissue specific. Also, Apc2 deficient mice displayed increased intestinal epithelial migration. However, this is most likely compensatory for the noted increase in cell division as opposed to direct effects on the cytoskeleton. Together these findings might highlight disruption of polarity and migration as necessities for tumourigenesis within the intestine which are only disrupted following Apc loss.

Apc2 loss was analysed in conjunction with targeted intestinal *Apc* deletion. This was done in an effort to elucidate any functional redundancies or co-operation between Apc proteins in intestinal epithelial homeostasis and delineate any role Apc2 may have in Wnt activated tumour progression. Additional Apc2 loss in the context of immediate intestinal homozygous *Apc* deletion surprisingly, reduced overall Wnt signaling and attenuated the phenotype in comparison to *Apc* deletion alone. The mechanisms behind this may be more effective Wnt repression and/or feedback loops which govern this repression. Indeed, the Wnt repressors Groucho and Dickkopf 2 were higher in intestines deficient for both Apc proteins.

A further explanation may again fall with 'just right' levels of Wnt signaling. Whereas moderate levels of Wnt activation incur a growth advantage (Lamlum et al., 1999; Albuquerque et al., 2002; Lucero et al., 2010; Chandra et al., 2012), higher levels induce cell death (Lazarova et al., 2004; Bordonaro et al., 2008). Whilst immediate intestinal Apc loss permits a suitable level of Wnt activation for manifestation of its phenotype, additional Apc2 loss may cause Wnt hyper-activation and selective death or terminal differentiation of some cells, such as ISC's, and attenuate this phenotype. Given these cells usually display high levels of Wnt activation in both wild type and Apc deficient intestines, selective loss of these cells in intestines deficient for both Apc protein would account for a reduced overall Wnt signature in comparison to that of Apc loss alone. Indeed, additional Apc2 loss

concomitant with intestinal Apc loss, increased cell death and reduced markers of intestinal stemness in comparison to Apc loss alone.

From these findings, one would postulate that additional Apc2 loss would have an effect on intestinal tumourigenesis caused by heterozygous *Apc* mutation. However, this was found not to be the case. Additional Apc2 mutation did not alter tumour number, tumour grade or overall survival in the context of heterozygous *Apc* mutation. A likely explanation for this may lie with the nature of the 'second hit' (mutation of the normal remaining *Apc* allele) that initiates these tumours. For a 'just right' level of Wnt signaling needed for tumourigenesis, one would predict that the cells that form tumours in the intestines with additional *Apc2* mutation will probably contain a second *Apc* mutation whereby Apc retains slightly more Wnt regulatory ability than in intestines heterozygous for Apc alone.

A further explanation for a lack of difference in tumourigenesis may lie with Wnt independent effects. For example, additional *Apc2* mutation may increase Wnt activation however, cytoskeletal disruptions may be detrimental for tumourigenesis. A balance between these effects may be why no differences in tumourigenesis are apparent.
How *APC2* influences human cancer and how it affects tumours with other oncogenic mutations remains to be elucidated.

Apc2 loss or targeted mammary epithelial Apc loss alone, failed to induce any overt phenotypes in the mammary gland. No detectable differences were noticed in β-catenin subcellular location or Wnt target gene expression, no lesions were present in the glands after aging and overall lifespan displayed no differences from wild type mice. Together this indicates that either Apc or Apc2 alone is dispensable for mammary epithelial homeostasis and tumour suppression function.

Beguilingly, combined loss of both Apc proteins led to severe mammary epithelial disruption, tumour formation and a reduction in survival. This again occurred in an *Apc2*

gene dose dependent manor, reiterating the importance of levels of Apc2. Together, these results imply that mammalian Apc proteins are functionally redundant in mammary gland homeostasis and tumour suppressor function. Given only subtle changes were noticed in Wnt signaling at an early time point in mammary epithelium deficient for both Apc proteins, whilst tumour cells were mostly Wnt activated, it is unclear if tumours arise from Wnt activation in certain cell types or cells acquire further genetic aberrations assisting in progression. Cytoskeletal defects were noticed in epithelium deficient for both Apc proteins and defects in spindle mechanics and mitosis may implement genomic instability which can assist this transformation. Also, it is important to note whilst disrupted functional redundancies between Apc proteins in Wnt regulation and/or cytoskeletal organisation may account for tumourigenesis, one cannot rule out the possibility that each protein plays a separate role, which combined loss of each is needed to induce tumourigenesis.

At an early time point, mammary epithelium deficient for both Apc proteins displayed a phenotype reminiscent of human atypical ductal hyperplasia, a common premalignant lesion and precursor of invasive carcinoma (Arpino et al., 2005; Page et al., 1985). Given mice deficient for both Apc proteins also developed invasive carcinoma, this suggests that these mice show some parallels to progression of human disease and may pose as a useful animal model of breast cancer. This model could be used for a range of potential benefits ranging from detection of biomarkers through to screening of therapeutics.

Within the liver, although Apc2 loss altered the subcellular location of β-catenin in some cells and altered overall Wnt target gene expression, it was dispensable for homeostasis and tumour suppression. This is in contrast to effects of acute loss of liver Apc, where cell division was increased and subsequent hepatomegaly became manifest. The differences noticed between loss of Apc or Apc2 in the liver, may again be due to the different affinities of β-catenin destruction between Apc proteins (Nakagawa et al., 1998). Whilst Apc loss activates Wnt signaling at a level that surpasses a threshold required to induce a liver phenotype, loss of Apc2 may not. However, it is not possible to rule out other reasons for differences in phenotype following loss of either Apc protein, such as different effects on the cytoskeleton.

Acute liver loss of Apc was achieved through the use of induced *Ah-Cre*$^{+}$ *Apc*$^{fl/fl}$ mice. Although efficient liver recombination is apparent, mice died within 4-5 days due to recombination in other tissues. This hindered the used of these mice for long term studies. However, un-induced *Apc*$^{fl/fl}$ mice display reduced levels of Apc expression (Munemitsu et al., 1995) and these develop perturbations in liver physiology and HCC (Buchurt et al., 2010).

As *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ were viable, it allowed us to directly test the significance of Apc2 in HCC development in mice with attenuated Apc expression. Surprisingly Additional loss of Apc prevented development of HCC. Although micro-adenomas were apparent in *Apc*$^{fl/fl}$ *Apc2*$^{-/-}$ livers at 400 days of age, these had failed to progress to HCC as in *Apc*$^{fl/fl}$ livers at the same time point. Given both Apc proteins have the ability to alter Wnt signaling, one could postulate that whilst attenuated Apc expression may lead to appropriate levels of Wnt for HCC formation, addition of an *Apc2* mutation may alter these levels outside of that permissive for tumour progression. This again would be consistent with a 'just right' level of Wnt needed for tumourigenesis. Given Apc2 is dispensable for normal liver function and its loss can attenuate HCC, it may be a potential therapeutic target. How Apc2 loss affects other models of HCC and how this may translate to human disease warrants further investigation.

Upon combining the data, it is clear that the effects on tumourigenesis of Apc, Apc2 or combined loss are tissue specific (Figure 7.1). Although the extent of the contribution of Wnt independent effects is difficult to delineate, these results are consistent with a 'just right' level of Wnt activation that is optimal for tumour initiation and progression, and this optimal level may differ between tissues.

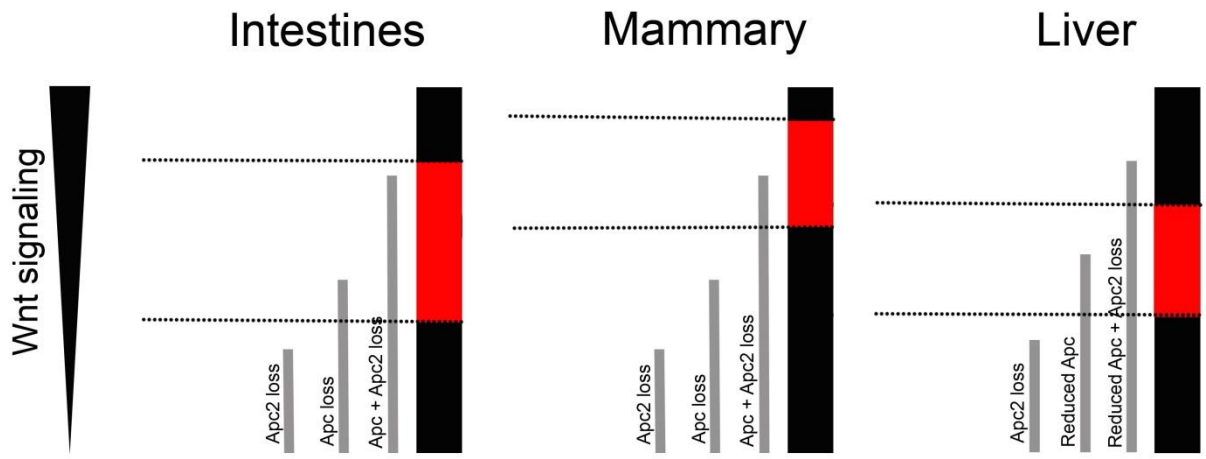

Figure 7.1 Effects of Additional Apc2 loss on tumourigenesis induced by altered levels of Apc are tissue specific. Within the intestines additional Apc2 loss has no overall effect. In the mammary gland, loss of both Apc proteins are required to induce tumourigenesis. However, within the liver, additional loss of Apc reduces tumourigenesis caused by reduced levels of Apc. This model proposes the 'just right' hypotheses (marked in red). Different tissues require different levels of Wnt activation to achieve this 'just right' level.

To summarise, results from this thesis reveal that both mammalian Apc proteins are important in homeostasis and tumourigenesis. Although Apc2 is mostly dispensable, when combined with Apc loss, its effect is dependent upon the tissue. Additional Apc2 loss has no overall effect on tumourigenesis in the context of a heterozygous intestinal *Apc* mutation. In the mammary, loss of both Apc proteins synergise to promote tumourigenesis, whilst in the liver additional Apc2 loss attenuates tumour progression caused by reduced levels of Apc expression.

The data presented here suggest that Apc proteins are functionally redundant in some contexts. Their roles include both Wnt dependent and Independent functions, which when disrupted can lead to perturbed tissue homeostasis and induction of tumourigenesis. This is the first study to demonstrate mammalian functional redundancies between the evolutionarily conserved Apc proteins '*in vivo*'. The implications of this finding are of importance in the understanding of both of Wnt signaling transduction and cancer biology.

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