Investigating the functional significance of the upregulation of Cyclin D2 and p21 following *Apc* loss *in vivo*

Alicia M Cole

PhD

Beatson Institute for Cancer Research
Glasgow University Faculty of Medicine
Graduate School

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Declaration

I declare that all of the work in this thesis was performed personally. No part of this work has been submitted for consideration as part of any other degree or award.

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Abstract

The *Apc* gene encodes the Adenomatous polyposis coli tumour suppressor protein, the germ line mutation of which characterizes Familial Adenomatous Polyposis (FAP), an autosomal syndrome characterized by multiple colorectal lesions. Inactivation of the *Apc* gene is recognized as a key early event in the development of colorectal cancers and leads to the deregulation of the Wnt pathway and the activation of TCF/LEF target genes. This project focuses on the proto-oncogene *c-Myc* as it is a key Wnt target gene which is activated following loss of Apc *in vivo*. This upregulation is noteworthy as c-Myc is implicated in stem cell survival, proliferation, apoptosis and tumourigenesis. Previous studies have shown c-Myc dependency for both apoptosis and proliferation following activation of the Wnt pathway, however little is known about the role c-Myc plays in inducing apoptosis following DNA damage *in vivo*. To study this I have conditionally deleted c-Myc from the intestinal epithelium and examined the response of intestinal enterocytes following DNA damage. Remarkably, following DNA damage, c-Myc deficient enterocytes were unable to upregulate p53 and induce apoptosis, which was mechanistically due to an upregulation of MDM2. Taken together, results from this study showed for the first time *in vivo,* a key role for c-Myc in inducing apoptosis following DNA damage through control of p53.

Previous studies from this lab have shown that within the intestinal epithelium, c-Myc is absolutely required for the hyper-proliferative phenotype that is observed following loss of Apc. Therefore one of the key aims of this thesis is to look downstream of c-Myc in order to delineate how c-Myc induces and controls this proliferation. Given that one of the key postulated functions of c-Myc is the

transcriptional repression of p21, this thesis examines this hypothesis by investigating the significance of the upregulation of p21 following c-Myc deletion in Apc deficient intestinal enterocytes. To do this, I have generated triple knockout (TKO) intestines by intercrossing p21 knockout mice to mice where we can conditionally delete both Apc and c-Myc within the murine intestinal epithelium. Surprisingly, the levels of proliferation were the same between double knockout Apc Myc and TKO intestines, which had markedly less proliferation than Apc deficient intestines. However, unlike double knockout enterocytes, TKO intestinal enterocytes no longer moved up the crypt-villus axis and failed to generate villus. To examine which of these phenomena were key to tumourigenesis (differentiation or proliferation), we investigated whether TKO intestines could form intestinal adenomas and found that even in the absence of p21, c-Myc deficient cells were unable to form tumours. Taken together we have identified a novel role for p21 in driving differentiation following Apc and Myc deletion. This is consistent with the expression of p21 in the normal crypt at the crypt villus junction. Remarkably this function of p21 is independent of its key role as a cell cycle inhibitor.

Moreover, this study also examined the importance of the upregulation of the Cyclin D/CDK4 complexes following Apc loss and their role in c-Myc dependent proliferation. Results from these studies showed that Cyclin D2 is required for efficient proliferation immediately following loss of Apc as well as for tumourigeneis in the *Apc* $^{Min/+}$ mouse. Taken together, results from these studies showed that the upregulation of Cyclin D2 and CDK4 are c-Myc dependent and that the upregulation of these complexes are key for Wnt driven proliferation and tumourigenesis.

Lastly, in this study I have examined whether Apc loss within the intestinal epithelium, where it is a *bona fide* tumour suppressor gene, can provoke senescence, and compared this to the ability of Apc gene deletion to trigger senescence in the renal epithelium, where it is not mutated in human cancer. This study showed that deletion of Apc within the renal epithelium invoked a p21 dependent senescence response, and Apc deficient renal epithelial cells were cleared and very rarely initiated tumourigenesis. However, combined *Apc* and *p21* gene deletion rapidly initiated tumourigenesis, with all mice developing renal carcinoma by 2 months of age. In contrast to Apc deficient intestinal epithelium, this process was unaffected by loss of c-Myc. However within the intestinal epithelium, deletion of Apc did not invoke senescence, but lead to a highly proliferative, p21 independent response. Combined *Apc* and *p21* gene loss had no impact on either the short term phenotypes of *Apc* loss or upon tumourigenesis.

Taken together these results show for the first time that *Apc* loss *in vivo* can invoke a senescence program but in a context dependent fashion. This implies escape from senescence is not a crucial pathway in colorectal cancers that are initiated by *Apc* loss, and goes to explain why renal carcinoma is not observed in FAP patients who are germline heterozygous for *APC*. Therefore the aims for this thesis are:

- To investigate the role of c-Myc in inducing apoptosis within the intestinal crypt, and whether this is p21 dependent?

- To investigate the role of p21 in causing senescence of Apc deficient cells, and whether this is c-Myc dependent?

- To determine the functional importance of repression of p21 by c-Myc in Apc deficient cells.

- To determine the significance of Cyclin D2 upregulation within Apc deficient cells.

1. Introduction to Colon Cancer

1.1 Colon Cancer Epidemiology

Colorectal cancer (CRC) is the second most common form of cancer in women after breast, and the third most common form of cancer in men, after lung and prostate cancer, with approximately 60% occurring in more westernised areas. CRC mostly affects older people, with more than 80% of cases occurring in people over 65 and 40% over the age of 80 (Cancer Research UK- http://info.cancerresearchuk.org/cancerstats) . The incident rate of CRC in men and women under the age of 50 is similar; however the incidence of CRC becomes significantly higher in men after the age of 50. Worldwide, CRC cancer kills on average 500,000 people a year, and various studies have shown that CRC is more prevalent in more westernized areas such as the United Kingdom and the United States. Within Europe, mortality rates are the highest in Hungary and the Czech Republic, and the lowest worldwide in Africa and Asian countries [IARC. GLOBOCAN 2002. Cancer incidence, Mortality and Prevalence Worldwide (2002 estimates) 2005]. One exception is Japan, due to it recently adapting a more western diet (Marchand, 1999, Koyama and Kotake, 1997).

In 2007 in the UK, approximately 16,000 people died from CRC. Although the incident rate of CRC has remained relatively stable for the past ten years, mortality rates from CRC have been on the decline since the 1990's (Figure 1.1.)

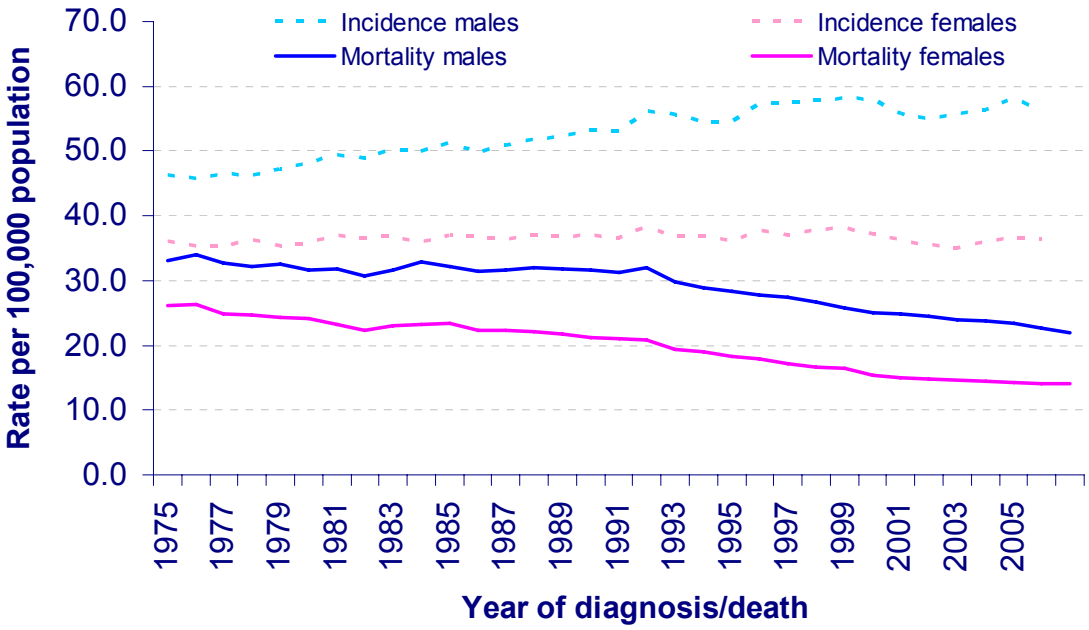

Figure 1.1: Age-standardized incidence and mortality rates by sex, colorectal cancer in Great Britan

(Source Cancer Research UK
http://info.cancerresearchuk.org/cancerstats/types/bowel/incidence/?a=5441)

Within the past 30 years, the 5 year survival rates for CRC have increased from approximately 20% to 50% in both males and females. This is due to a more highly effective screening process, as studies have shown that cases that are caught early have a highly significant increase in 5 year survival. For example, patients classified with Dukes stage A have a 93% chance of 5 year survival versus only 7% for those classified with Dukes stage D. [NICE. Improving Outcome in Colorectal Cancers: Manual update 2004 (Figure 1.2 (Dukes, 1932, Sobin and Fleming, 1997)]

TMN	Description	Dukes Stage
Stage 0	Carcinoma in situ	A
Stage 1	No nodal involvement, no distant metastasis Tumour invades submucosa (T1, N0, M0) Tumour invades muscularis propria (T2, N0, M0)	A
Stage 2	No nodal involvement, no distant metastasis Tumour invades into subserosa (T3, N0, M0) Tumour invades into other organs (T4, N0, M0)	B
Stage 3	Nodal involvement, no distant metastasis 1 to 3 regional lymph nodes involved (any T, N1, M0) 4 or more regional lymph nodes involved (Any T, N2, M0)	C
Stage 4	Distant metastasis (any T, any N, M1)	D

Figure 1.2: Dukes stage classification of colorectal cancer

Adapted from Cancer Research UK http://www.cancerhelp.org.uk/type/bowel-cancer/treatment/dukes-stages-of-bowel-cancer

CRC can be regarded as an environmental disease, as a number of lifestyle choices are known to affect its onset. CRC is known to predominately affect areas that have adapted a 'western' diet and lifestyle. For example, a lack of exercise and increase in obesity has also been shown to increase the onset of this disease (Moghaddam et al., 2007). Similarly alcohol intake increases colorectal cancer risk (Cho et al., 2004). On the other hand, studies have shown a high uptake of dietary fibre decreases the risk of colon cancer (Bingham et al., 2005). Similarly so does a diet high in fruit and vegetables, vitamin D (Feskanich et al., 2004, Peters et al., 2001, Peters et al., 2004, Braun et al., 1995), B6 (Wei et al., 2005) and folate (Sanjoaquin et al., 2005). Given these studies, one of the primary strategies for CRC prevention has been awareness of good dietary and lifestyle habits.

Currently a national screening program called The Scottish Bowel Screening Program will encourage people aged 50-74 years to use the faecal occult blood testing (FOBT) kits, which in other randomized trials have been shown to reduce colorectal mortality by 15% (Hardcastle et al., 1996, Kronborg et al., 1996, Mandel et al., 1993, Atkin, 1999)

1.1.1 Biology of the Intestinal Epithelium

The intestinal tract can be described as a tube that is composed of three tissue layers. The outer layer consists of several sheets of innervated smooth muscle that executes peristalsis. The middle layer consists of stromal tissue, whilst the inner surface is termed the mucosa which consists of sheets of cuboidal epithelial cells and serves to absorb nutrients and compact stool (Sancho et al., 2004). The intestinal epithelium consists of numerous luminal protrusions, termed villi, and invaginations into the submucosa, termed crypts of Lierberkühn which increase its absorptive surface. In the case of the large intestine, the mucosa contains only crypts and villi are replaced with a flat surface epithelium (Sancho et al., 2004).

The intestinal crypt contains columnar and paneth cells that occupy the base of the crypt. Paneth cells secrete antimicrobial peptides and enzymes such as cryptidins, defensins and lysozyme (Porter et al., 2002). Intestinal stem cells reside immediately above the paneth cells and give rise to daughter progenitor cells. Daughter progenitor cells cease proliferation at the crypt villus axis and differentiate into three distinct cell lineages that populate the villus. These include enterocytes, which secrete hyrdrolases and absorb nutrients; goblet cells which produce a protective mucous lining. Enteroendocrine cells secrete hormones including serotonine, substance P and secretin (Hocker and Wiedenmann, 1998)(Figure 1. 3)

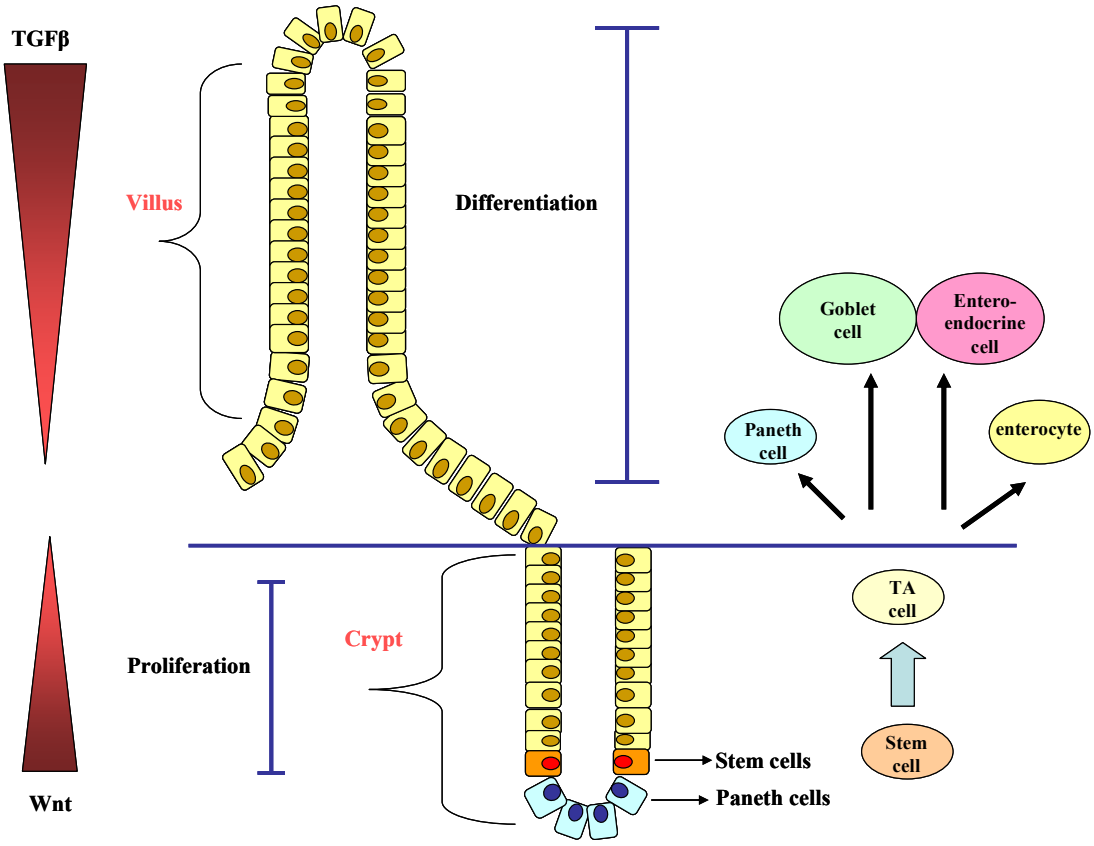

Figure 1.3: Architecture of the Intestinal Crypt

The small intestine which is divided in to 2 compartments. The crypt and the villus. Epithelial stem cells at approximately position 4 in the crypt base, proliferate in to transit amplifying cells which migrate up the crypt-villus axis in to the villus where they differentiate in to 3 lineages, Goblet cells which produce mucins, hormone producing enteroendocrine cells and absorptive enterocytes. A fourth cell type the antimicrobial Paneth cell differentiates and travels down to the base of the crypt. The key regulatory pathway for the proliferation process in the crypt is the Wnt pathway, which is highest at the base of the crypt

1.1.2 Cell Renewal of the Intestinal Epithelium

In order to maintain the cellular intestinal crypt architecture, crypt progenitor cells divide every 12-16 hours, generating 200 cells per crypt every day [reviewed in (Sancho et al., 2004)]. In order to maintain epithelial homeostasis, the epithelial sheet is in a continuous upward 'escalator' type motion, whereby cells continually move up the crypt-villus axis and eventually shed off once they have reached the top of the villus (Heath, 1996). This process takes approximately 5 days. Proliferation in the intestine is not a cell autonomous feature, rather it is dictated by the crypt niche (Hermiston et al., 1996). Therefore, proliferative and differentiated compartments are maintained as cells transit up the crypt-villus axis (Potten and Loeffler, 1990).

Studies have gone on to define intestinal stem cells by four features. These include: retention of an undifferentiated phenotype, continuous production of all cell lineages, retention of self-maintenance capabilities throughout life, and the ability to regenerate upon injury (Sancho et al., 2004). Recent studies have identified leucin-rich repeat-containing G-protein coupled receptor 5 (LGR5) as the first definitive marker of both colonic and intestinal stem cells (Barker et al., 2007). This was confirmed by studies that showed that LGR5 positive cells were capable of repopulating the entire crypt-villus axis will all the correct cell lineages following irradiation (Barker et al., 2007).

1.1.3 Using the Intestinal Crypt as a model system for examining apoptosis in vivo

One of the most tractable systems for studying the DNA damage response *in vivo* is the intestinal crypt. This is due to the fact that the small intestine provides a well characterized system to study apoptosis (Potten et al., 1997). Four to six intestinal stem cells are proposed to reside at the base of the crypt, and have been found to be very susceptible to apoptosis; undergoing apoptosis following low levels of irradiation (1Gy) (Potten, 1998). Studies showed that once the original stem cell has been destroyed they can be replaced by an additional six clonogenic cells, following up to 9Gy irradiation. Following higher doses of irradiation, it is believed that up to 1/3 of the crypt (16-24 cells) can act as clonogenic cells (Roberts et al., 1995). Therefore this allows clonogenicity to be investigated *in vivo*, by the analysis of crypt survival, which can be related to the ability to induce apoptosis (Roberts and Potten, 1994).

In this study, the tractability of this system in conjunction with our previous data showing that C-Myc deletion is not immediately deleterious to intestinal enterocytes, is used to determine whether C-Myc is important for signalling apoptosis in normal cells following DNA damage (discussed in Chapter 3).

1.1.4 Somatic and germline mutations in familial cancer

CRC can be divided into two classes; sporadic and familial (hereditary) cancer, with sporadic cancers accounting for approximatley 80% (Houlston 1992). The remaining 20% of familial cases are further divided into two categories based on the presence of polyposis; **F**amilial **A**denomatous **P**olyposis (FAP) and **H**ereditary **N**on-**P**olyposis **C**olorectal **C**ancer (HNPCC).

1.1.5 FAP (Familial adenomatous polyposis)

FAP is an autosomal dominant disease that affects approximately 1/7000 people and is characterized by the development of hundreds to thousands of colorectal adenomas, usually arising by the second decade in life (Kinzler and Vogelstein, 1996). Due to the sheer volume of adenomas, inevitably one or several adenomas progress to carcinomas, with 100% of FAP patients developing colorectal cancer by the average age of forty (Sancho et al., 2004). FAP patients can also develop other intestinal lesions such as gastric fundic gland polyposis, duodenal adenomas, and gastric, pancreatic, bilary or distal small intestinal lesions. At much lower frequencies, FAP patients can also develop lesions out with the GI tract such as desmoids of the skin, retinal lesions, osteomas and brain tumours (Kinzler and Vogelstein, 1996).

The first genetic elucidation of FAP was the discovery of an interstitial deletion of chromosome 5q in patients with polyposis (Herrera et al., 1986, Bodmer et al., 1987). Further evidence emerged suggesting that mutations within the same gene may be responsible for both somatic and inherited forms of colon cancer. These studies analyzed

sporadic colorectal carcinomas for loss of alleles on chromosome 5q, revealing that over 20% had loss of one allele compared to neighboring normal tissue (Solomon et al., 1987). Comparing germline mutations in FAP patients and somatic mutations in sporadic colorectal tumours, the *Adenomatous Polyposis Coli (APC*) gene was identified and proven to cause FAP (Eldeiry et al., 1993, Groden et al., 1991, Nishisho et al., 1991). In accordance with these findings, studies went on to show that the rate limiting step for tumour initiation in both cases of sporadic and hereditary CRC, was the somatic mutation of the other wild type allele (Ichii et al., 1992, Levy et al., 1994, Luongo et al., 1994). This was demonstrated in FAP patients and animal models with an analogous mutation of the murine homolog of *Apc* (Ichii et al., 1992, Levy et al., 1994, Luongo et al., 1994), as well as in human sporadic colorectal tumours (Solomon et al., 1987). Therefore illustrating that in order for cancer to develop, both copies of Apc must be lost; very much in accordance with Knudson's 'two hit' model for tumour initiation (Knudson, 1971, Knudson, 1986).

Truncations of the Apc protein account for the majority of mutations observed in both FAP patients as well as sporadic adenomas and cancers. Apc mutations in colorectal neoplasia have been well characterized and are summarized in the table below (Table 1.4)

	FAP	Sporadic adenomas	Sporadic Cancers
Population incidence	1 in 7000	1 in 2	1 in 20
APC mutation prevalence	> 85%[b] (germline mutation)	> 80%[c] (somatic mutation)	> 80%[d] (somatic mutation)
Nature of Mutations[a]			
Truncating	96%[e]	89%[f]	98%[g]
Missense	4%[e]	11%[f]	2%[g]

[a] Based on APC mutations that could be precisely defined at the nucleotide level. For the purposes of this table, framshift, nonsense and splice site mutations were considered "truncating"

[b] Based on 62 kindreds (Powell et al., 1993)

[c] Based on analysis of 12 colorectal polyps (Jen et al., 1994)

[d] Based on analysis of 23 colorectal cancer cell lines (Smith et al., 1993)

[e] Based on 174 mutations(summarized in Nagase and Nakamura, 1993)

[f] Based on 19 mutations (Miyoshi et al., 1992; Powell et al., 1992)

[g] Based on 56 mutations (Miyoshi et al., 1992; Powell et al., 1992)

Table 1.4: APC mutations in colorectal neoplasia

Adapted from (Kinzler and Vogelstein., 1996)

1.1.6 Hereditary Nonpolyposis Colorectal Cancer (HNPCC)

Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is an autosomal - dominant cancer syndrome that accounts for approximately 5% of all colorectal cancers (de la Chapelle, 2004). This syndrome not only predisposes to colorectal cancer, but also to a wide range of cancers that occur out with the intestinal tract. These include cancers that arise from the endometrial, gastric, ovarian, and urinary tract origins (Lynch and de la Chapelle, 2003). Unlike FAP patients that present with disease usually in the second decade of life, the average age of onset of HNPCC is approximately 40-45 years of age. Patients are diagnosed with HNPCC if they conform to a set of criteria known as the "Amsterdam criteria". These criteria include; having a first degree relative with colorectal cancer, at least 2 successive affected generations, the exclusion of FAP and one or more of the cancers must have developed before the age of 50 (Vasen et al., 1992). Studies have now identified a group of genes, in which germline mutations are known to cause HNPCC. These are within mismatch repair (MMR) genes, MSH2, MLH1, MSH6 and PMS2, which are key componets of the mismatch repair system, which recognise the mismatch and recruit repair machinery to it (Bronner et al., 1994, Leach et al., 1993, Miyaki et al., 1997, Nicolaides et al., 1994, Papadopoulos et al., 1994). Mismatch repair has a conserved fundamental role to correct mispairs produced by DNA during DNA replication (Modrich and Lahue, 1996). Because of these deficiencies in the DNA mismatch repair pathway, patients with HNPCC generate mutations at 2 to 3 orders of magnitudes higher than in normal cells, which is believed to account for the rapid progression to malignancy (Lynch and Smyrk, 1996). Mutations in MLH1 and MSH2 account for approximately 90% of all mutations. As MMR genes are important in

repairing microsatellite instability, this explains why the hallmark of HNPCC tumours is microsatellite instability (Thibodeau et al., 1993). Germline mutations in these genes have a 80% penetrance for colorectal cancer, 60% for endometrial cancers and less than 20% for gasrtic, ovarian and urinary cancers (Lynch and de la Chapelle, 2003).

1.1.7 The Min mouse

The *Apc* $^{Min+}$ (**M**ultiple **I**ntestinal **N**eoplasia) mouse was the first intestinal mouse model of carcinogenesis to phenotypically recapitulate FAP. The *Apc* $^{Min+}$ mouse was generated using a random mutagenesis screen with the carcinogen ethylnitrosourea (ENU), which resulted in random germline mutagenesis (Moser et al., 1990). Following exposure to ENU, mice showed signs of anaemia and were moribund by 120 days. Upon dissection, multiple adenomas were observed in the small intestine, which occasionally progressed to adenocarcinomas in older mice. Each mouse presented with on average 30 intestinal adenomas, hence the name **M**ultiple **I**ntestinal **N**eoplasia (Moser et al., 1990). The mutation was mapped to codon 850 of the murine homologue of the Apc protein, and was similar to those observed in FAP patients (Su et al., 1992). Similar to FAP patients this mutation was characterized as a fully penetrate autosomal dominant disorder (Moser et al., 1990). Despite similar germline mutations, FAP patients have predominately colorectal adenomas which routinely progress to invasive adenocarcinoms, in contrast to the small intestinal adenomas observed in the *Apc* $^{Min+}$ mouse. This is due to the fact that *Apc* $^{Min+}$ mice present with a high tumour burden, and as a consequence mice need to be sacrificed before tumours are able to progress to adenocarcinomas.

Differences in phenotypes and colonic burden have been observed in FAP patients which harbor identical mutant *APC* alleles. These differences are thought to be due to differences in diet, environment and genetic modifiers or genetic backgrounds.

Similar differences have been observed in the *Apc* $^{Min+}$ mouse. However these discrepancies are attributed to genetic modifiers since diet, environment and genetic backgrounds are controlled within the laboratory setting. Genetic modifiers are genes that alter the disease severity by interacting either directly or indirectly with the primary genetic mutation causing the disease. For example the genetic background of *Apc* $^{Min+}$ mice has been shown to significantly affect tumour burden, as mice with C57BL/6J backgrounds develop significantly more tumours than those on the AKR background. Studies using linkage analysis demonstrated that a single locus on mouse chromosome 4 was responsible for the majority of differences between strains, namely modifier of MIN (MOM1) (Dietrich et al., 1993). The MOM1 gene has been identified as the *phospholipase* A2 (*Pla2g2a)* gene, however as of yet, no associations between polymorphisms in this gene and human disease have been reported (Tomlinson et al., 1996).

A homozygous mutation for the *Min* allele results in embryonic lethality by day 6.5 dpc (days post coitum), as Apc $^{Min/Min}$ embryos are unable to maintain continued development of the primitive ectoderm of the early egg cylinder (Moser et al., 1995).

Analysis of polyps from both mice (on a C57BL/6J background) and human FAP patients have shown all tumours acquire an additional somatic mutation in the other wild type copy of *Apc* (Luongo et al., 1994) (Levy et al., 1994, Albuquerque et al., 2002),

illustrating the importance of loss of *Apc* for tumour initiation. This notion is further observed as mice with different genetic backgrounds exhibit different frequencies of loss of heterozygosity (LOH). For example, *Apc* $^{Min/+}$ mice on the AKR background showed only 63% LOH, compared to 100% LOH in *Apc* $^{Min/+}$ mice on a C57BL/6J background, which may explain why *Apc* $^{Min/+}$ mice on the AKR background develop significantly less tumours (Shoemaker et al., 1998). Therefore mouse models can serve as invaluable tools to investigate the effect of genetic modifiers in different tissues.

1.1.8 Genetics of colorectal cancer initiation and progression

Colon cancer is believed to arise from a combination of the activation of oncogenes and the inactivation of tumour suppressors. This confers a selective growth advantage to mutated cells leading to the formation of benign adenomas, and eventually upon the acquisition of further mutations, to invasive carcinomas (Foulds, 1958).

As previously stated, germline mutations of the Apc gene cause FAP, and it has been discovered that mutations in Apc occur in the majority of all sporadic colorectal tumours (Miyoshi et al., 1992, Powell et al., 1992). Given the high frequency of mutations in the Apc gene, it has been proposed that Apc acts as a key 'gatekeeper' gene of colonic homeostasis, importantly controlling colonic epithelial cell proliferation. Therefore a mutation in this 'gatekeeper' gene results in uncontrolled cellular renewal and proliferation (Kinzler and Vogelstein, 1996).

Although it is now widely accepted that mutations in Apc initiate the neoplastic process, it is also known that a sequential series of mutations are necessary for tumour

progression, specifically including Apc, KRAS, SMAD2/4 and TP53 (Figure 1.5). This is in accordance with the notion proposed by Hanahan and Weinberg, where each genetic change confers a selective advantage to cells, allowing them to aquire the six hallmarks of cancer, namely; self sufficiency in growth signal, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potencial, sustained angiogenesis as well as tissue invasion and metastasis (Hanahan and Weinberg, 2000)

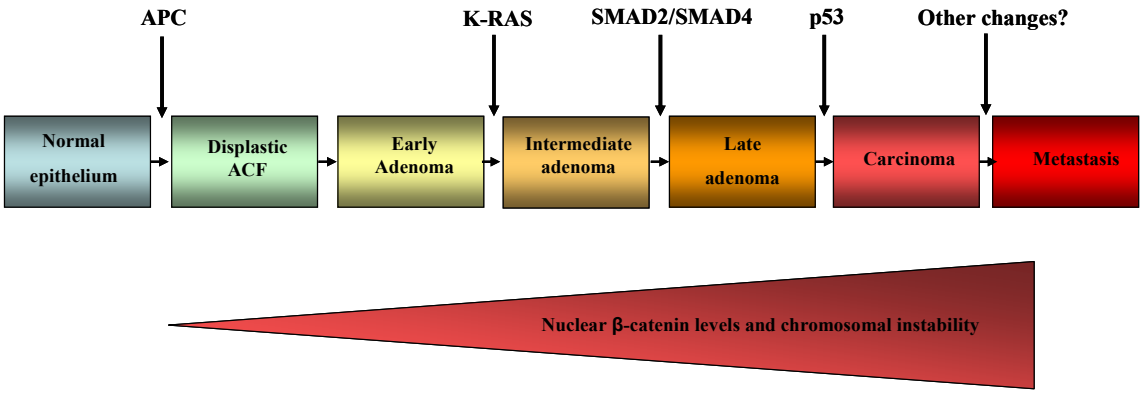

Figure 1.5: Genetic changes associated with colorectal tumourigenesis

Adapted from (Kinzler and Vogelstein., 1996)

Apc mutations initiate the neoplastic process. FAP patients acquire a germline mutation in Apc and form displastic Aberrant Crypt foci (ACF). These continue to progress to adenomas and carcinomas through the acquisition of the above mutations. In the case of K-Ras, activation of only one allele is required. Loss of both alleles are required for the tumour suppressors Smad 2, Smad4 (due to deletion of chromosome 18) and p53. Other genetic alterations occur in advanced colorectal cancers and may account for the differences in biological and clinical features between cases.

Further studies have shown that the order of these mutations are as important as their combined accumulation. For example although p53 mutations occur in approximately 80% of colorectal cancer (Baker et al., 1990), patients which solely harbour a germline mutation of p53 do not develop multiple adenomas, like FAP patients, and are even not at great risk to developing colorectal cancer (Garber et al., 1991). Similarly, a high frequency of KRAS mutations are observed throughout the progression of colonic tumourigenesis, however cells harbouring KRAS mutations have normal intracellular and intercellular organization, and display no predisposition to tumourigenesis (Jen et al., 1994, Shpitz et al., 1996). Taken together, these studies show that although mutations in KRAS and p53 are required for effective tumour progression, on their own they are unable to initiate tumour formation in the absence of a mutation in *APC*. Taken together, these results highlight the importance of an initial mutation in the 'gate-keeper' gene *APC* for the initiation of colorectal cancer tumourigenesis.

1.2 Apc and it's role in the canonical Wnt signalling pathway

The Wnt pathway is widely conserved throughout many species including *C.elegans*, *Drosophila*, *Xenopus* and mammalians. The Wnt pathway was originally discovered in *Drosophila* where the gene *wingless (wg)* was shown to control segment polarity during larval development (Nussleinvolhard and Wieschaus, 1980). Studies in mice, identified a gene called *Wnt1* (originally named *Int*-1) as a proto-oncogene in breast tumours virally induced by the mouse mammary tumour virus (Nusse and Varmus, 1982). Further studies went on to show that *wingless (wg)* was a fly homologue of the mouse *Wnt1* gene

(Rijsewijk et al., 1987). Hence the name Wnt, was coined from combination of *wg*, from the *Drosophilia* wingless gene and *Int*, the mouse homologue.

It is widely accepted that the Wnt pathway controls many events during embryonic development, and regulates homeostatic self-renewal in a number of adult tissues. As a consequence, mutations in this pathway are associated with the onset of various cancers, due to the control the Wnt pathway has on various factors such as proliferation, morphology, motility and cell fate at the cellular level (Oving and Clevers, 2002, Polakis, 2000). Wnt factors (ligands) are cell signalling glycoproteins which interact with a seven-transmembrane Frizzled cell surface receptor, leading to Wnt pathway activation. Three different pathways are believed to be activated upon receptor activation and these include the Wnt/Ca^{2+} pathway, the noncanonical planar cell polarity pathway and the canonical pathway (Clevers, 2006).

1.2.1. The canonical Wnt signalling pathway

The canonical Wnt pathway is a ligand dependent system whereby Wnt proteins bind to Frizzled and LRP family member receptors at the cell surface. This in turn activates Dishevelled family proteins and ultimately results in an increase in the amount of β-catenin that reaches the nucleus (Reya and Clevers, 2005). Consequently β-catenin now complexes with T-cell Factor (TCF) resulting in the transcriptonal activation of Wnt target genes. In the presence of a Wnt ligand, Frizzled (Fz) proteins cooperate with a single pass transmembrane molecule called LRP6 to bind the ligand. Upon this binding, the scaffold protein Axin translocates to the membrane where it interacts with the

intracellular tail of LRP5. This results in the disassociation of the destruction complex comprising Axin, GSK3β (glycogen synthase kinase) , CK1 (casein kinase), Apc, and β-catenin [reviewed in (Reya and Clevers, 2005, Clevers, 2006)]. Also, by an unknown mechanism, the binding of Wnt to Frizzled results in the hyperphosphorylation of Dishevelled (Dsh), which inhibits the activity of GSK3β making it unable to phosphorylate β-catenin (Mao et al., 2001, Yanagawa et al., 1995). As a result of being unphosphorylated, β-catenin is now not recognized by the F-box β-TRCP protein, a component of the E3 ubiquitin ligase and therefore is not degraded. Subsequently, β-catenin is now free to translocate to the nucleus, where it binds to the N terminus of LEF/TCF (lymphoid enhancer factor/T cell factor), and activates transcription of a number of Wnt target genes (Behrens et al., 1996, Molenaar et al., 1996, vandeWetering et al., 1997, Polakis, 2000), including c-Myc (He et al., 1998), CD44 (Wielenga et al., 1999), TCF-1 (Roose et al., 1999), LEF-1 (Hovanes et al., 2001). (A full list of all Wnt target genes can be found at : http://www.stanford.edu/~rnusse/pathways/targets.html)

In the absence of a Wnt signal, CK1 and GSK3β phosphorylate β-catenin at a series of highly conserved Ser/Thr residues near the end terminus. Consequently, β-catenin is now recognized by the F-box β-TRCP protein, ubiquitinated and targeted for degradation via the proteosome (Aberle et al., 1997, Polakis, 2000). (Figure 1.6)

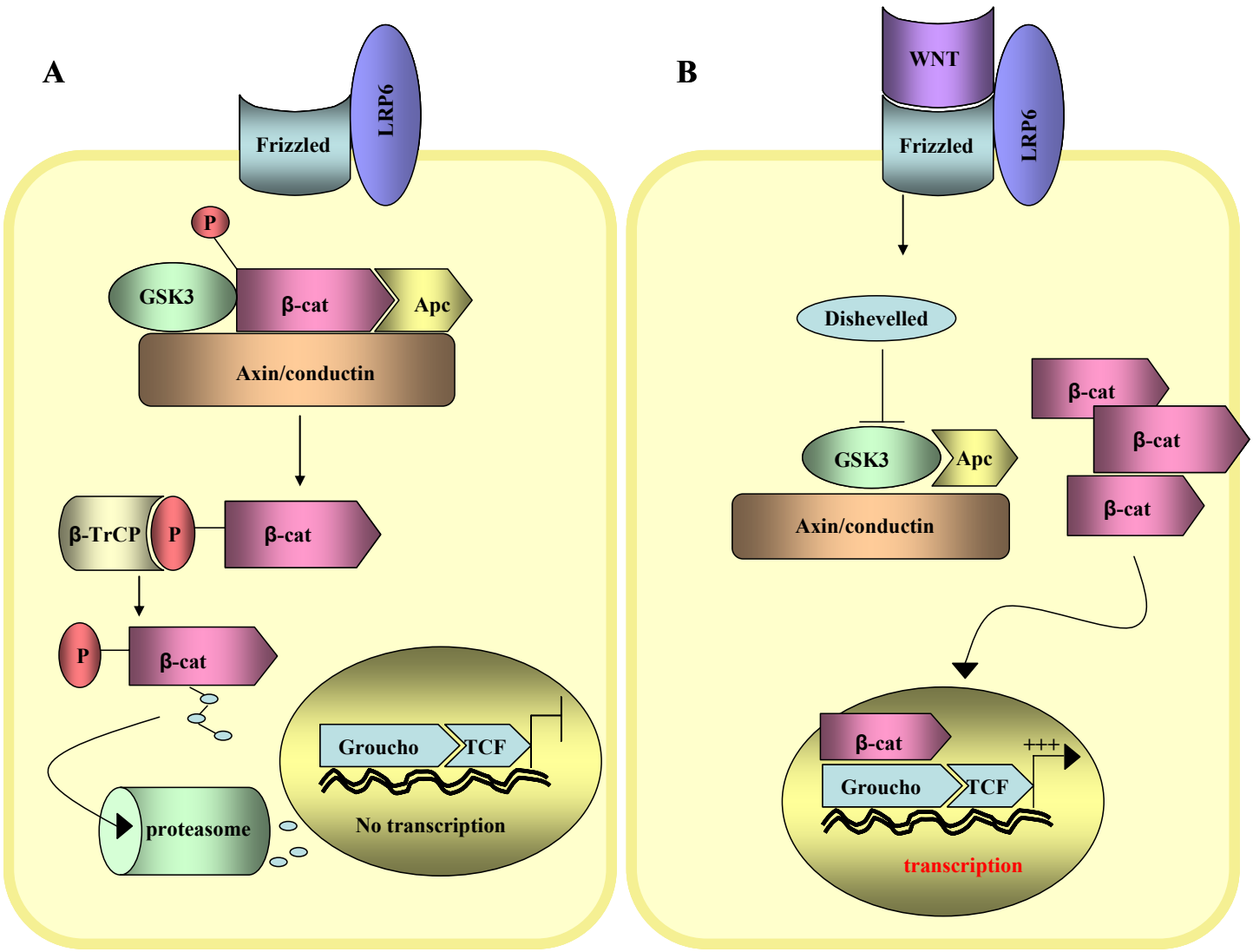

Figure 1.6: The Canonical Wnt signalling pathway

Adapted from (Fodde et al., 2001b)

In the absence of Wnt signalling **(A)**, GSK3 in the Apc -β-catenin-axin complex is active and phosphorylates β-catenin. Phosphorylation of β-catenin creates a recognition site for ubiquitin ligase and leads to its destruction by the proteasome. As β-catenin is now degraded, it is unable to translocate to the nucleus and act as a transcription factor; therefore Wnt target genes are not activated.

In the presence of the Wnt signal **(B)**, the protein dishevelled (Dsh) inactivates GSK3 , and consequently GSK3 is unable to phosphorylate β-catenin. This results in a decrease in the amount of β-catenin targeted for degradation which increases the amount available to activate LEF/TCF transcription factors, and thereby activating a series of Wnt target genes.

Amongst the vast array of target genes that are upregulated following Wnt activation, the upregulation of the proto-oncogene c-Myc has been shown to be pivotal within the intestinal epithelium (van de Wetering et al., 2002, Sansom et al., 2007) (discussed in Chapter 3). Therefore during inappropriate Wnt signalling activation, caused by an Apc mutation, activation of key Wnt target genes such as c-Myc leads to an uncontrolled burst in proliferation. This once again, serves to highlight the important role that Apc plays as a key 'gate keeper' in controlling cell proliferation (Kinzler and Vogelstein, 1996).

1.2.2 The structure of the Apc protein and its role in the β-catenin destruction complex

The human *APC* gene encodes a large 312kda protein (Groden et al., 1991). However despite Apc mutations being identified in both germline mutations in FAP patients, as well as in sporadic colorectal tumours, the sequence of the Apc protein did not elude to any information about its intracellular function. It was only when β-catenin was shown to be a binding partner of Apc did the link between Apc and the canonical Wnt signalling pathway become clear (Su et al., 1993, Rubinfeld et al., 1993). The structure of the Apc protein is depicted in figure 1.7

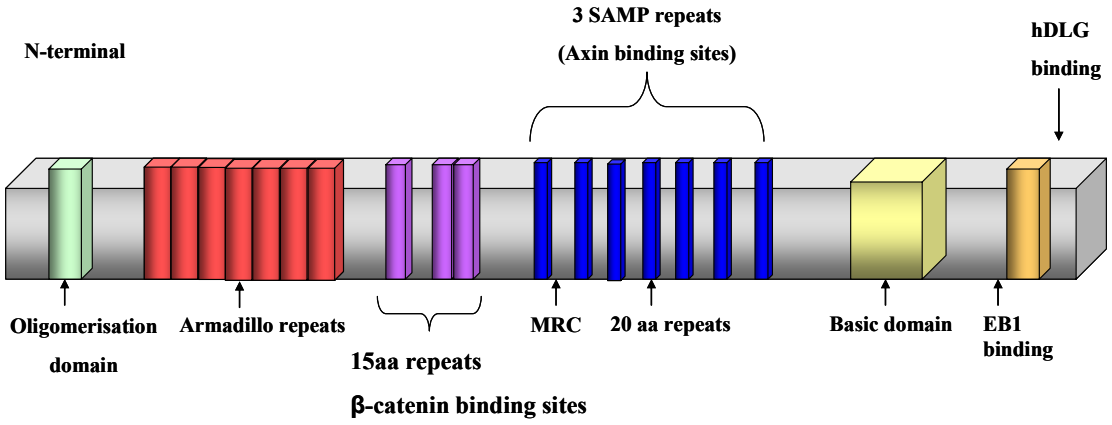

Figure 1.7: Functional domains of the APC (Adenomatous Polyposis Coli) and its binding partners

Adapted from (Fodde, 2001)

Schematic showing the structure and binding domains of the Apc protein:

Armadillo repeats bind to the regulatory unit of phosphatase 2a(PP2A), Apc-stimulated guanine nucleotide exchange factor for Rho family proteins (Asef) and Kap3 a linker proteins for kinesins. MRC denotes mutation cluster region. The 15 aa repeats and the 20 aa repeats are both involved in binding β-catenin. SAMP repeats are involved in binding axin and thought to be the most important repeat in the Apc molecule through the regulation of β-catenin. Apc binds to microtubules through its EB1 binding domain and therefore is believed to contribute to chromosomal instability.

Apc is a large protein of approximately 2800 amino acids (aa). There are three domains. The N-terminal consists of a series of armadillo repeats which bind to the regulatory unit of phosphatase 2a (PP2A), Apc-stimulated guanine nucleotide exchange factor for Rho family proteins (Asef) and Kap3 a linker proteins for kinesins. The Apc protein contains three 15 aa repeats and seven 20 aa repeats, both of which are known to bind β-catenin (Su et al., 1993, Rubinfeld et al., 1993). In both cases of hereditary and sporadic cancers, mutations result in a protein that is truncated at its C-terminus, importantly

eliminating five or more of the seven 20-aa repeats as well as all the axin/conductin binding motifs, both of which are important for the binding and downregulation of β-catenin. In FAP patients, the majority of mutations occur at the 5' end of the Apc gene, however in sporadic tumours, the majority of mutations occur in what is known as the mutation cluster region (MCR), which is between codons 1285 and 1513 and disrupts its binding to axin (Ichii et al., 1993, Miyoshi et al., 1992). Previous studies have suggested that in FAP patients, that specific somatic mutation of the wild type allele are preferentially selected during tumour formation. These include mutations within the Apc gene that still allow for partial binding and downregulation of β-catenin, rather than mutations that have the β-catenin regulatory function completely inactivated. These studies have proposed a 'just right signalling' model, where the mutation needs to result in enough accumulation of β-catenin to activate Wnt target genes, but not enough to result in excessive levels which have been shown to result in programmed cell death (Fodde et al., 2001b). This notion was further investigated by *Pollard et al.* by generating a mouse model harbouring a truncating Apc mutation at codon 1322 (now referred to as Apc^{1322T}). Mutation at this codon resulted in a truncated protein which contained one β-catenin binding 20 Amino Acid Repeat (AAR) sequence. Tumourigenic studies were then performed comparing Apc^{1322T} mice to $Apc^{Min/+}$ mice, which carry no 20 AAR sequences. Results from this study showed that Apc^{1322T} mice developed adenomas much more rapidly, and adenomas were larger, more dysplastic and more numerous than those observed in $Apc^{Min/+}$ mice. Moreover, adenomas from Apc^{1322T} mice displayed significantly lower levels of β-catenin than in $Apc^{Min/+}$ mice. The authors from this study go on to suggest that high levels of β-catenin may be suboptimal for tumourigenesis,

possibly due to; inhibition of the expression of target messenger RNAs, unbalanced target expression, hyperactivation of specific negative feedback inhibition loops and effects on the noncanonical Wnt pathway (Pollard et al., 2009). Taken together these results illustrate that mutant Apc proteins that continue to harbor at least one 20AAR sequence, are able to maintain lower levels of nuclear β-catenin and consequently exert more severe tumourigenic effects.

The C-terminal region of Apc binds to a wide variety of different proteins important for growth control and cycle progression, including EB1, hDLG, Bub1 and PTP-BL (Oving and Clevers, 2002). Previous studies have suggested a role for Apc in chromosomal instability due to its abilty to bind to the kinetochore protein Bub1 at its C-terminus (Kaplan et al., 2001). Similarly, the C-terminal region of Apc also binds to EB1, a protein, that in combination with Apc, mediates the attachment of microtubules to kinetochores (Su et al., 1995, Tirnauer and Bierer, 2000). A role for Apc in chromosomal instability was further elucidated in a study involving ES cells, which showed that cells with mutant Apc proteins lacking the EB1 binding site resulted in the failure of mircotubules to properly connect to kinetochores, resulting in chromosomal missegregation (Fodde et al., 2001a). As mutations are in the MCR (mutation cluster region), all mutations that occur in CRC, as well as driving the activation of Wnt signalling will lack the carboxy-terminal domains that bind to DLG, EB1 and microtubules, creating N-terminal fragments. Given that all CRC mutations lack the C-terminal domain of Apc, it is difficult to interpret if these functions of Apc are significant for its role as a tumour suppressor. This is due to the fact that all these mutations will also

have activated Wnt signalling. To test this hypothesis, *Smits et al.* created the *Apc*^{1638T} mouse model which carried a targeted mutation at codon 1638 of the *Apc* gene. This resulted in a truncated Apc protein encompassing three of the seven 20 amino acid repeats and only one SAMP motif, but lacks all the carboxy-terminal domains thought to be associated with tumourigenesis. Unlike homozygous mutations for mouse Apc which result in early embryonic lethality, homozygosity for the Apc 1638 mutation was viable. However these animals were characterized by growth retardation. Most importantly however, Apc $^{1638T/1638T}$ animals that did survive to adulthood were tumour free. Through this study the SAMP motif was shown to be important for proper β-catenin signalling and regulation, as its targeted deletion results in a further reduction in the ability to properly control β-catenin/Tcf signalling. Therefore these results suggest that the C-terminal domain that associates with DLG, EB1 and microtubules is not critical for maintenance of homeostasis by Apc. Thus illustrating that while C-terminal domain functions of Apc are important for development, it is Apc's role in β-catenin regulation that is crucial for normal embryonic development and tumour suppression (Smits et al., 1999).

Taken together, these studies have shown that the major tumour suppressive function of Apc in colorectal cancer is to control the levels of Wnt signalling. Therefore mutations that result in the truncation of Apc results in the stabilization of β-catenin and the activation of TCF/LEF target genes, and activation of the Wnt pathway. However these truncating mutations also result in abnormal chromosomal segregation, resulting in

genomic and chromosomal instability which may also drive tumour progression (Nathke, 2006)

1.3 Murine models of Colorectal Cancer

Murine models of cancer have become invaluable tools to study the molecular pathways involved in the initiation and progression of tumourigenesis . Many genetically modified mouse strains have been generated in order to recapitulate the mutations that are observed in both familial and sporadic colorectal cancers. Given that mutations in Apc are responsible for the vast majority of tumours in both types of colorectal cancer, many mouse models have been engineered with different mutations in Apc which result in a different onset, severity and location of tumours. However despite these differences, the tumour histology amongst these different mutations are very similar. Table 1.8 contains a summary of various Apc genetically engineered mice.

GEM	Mutation	Polyp burden	Histology/Pathology	Reference
Apc $^{Min/+}$	Truncating mutation at codon 850 (ENU induced)	~ 30 → 100	Polypoid, papillary and sessile adenomas. Colonic polyps	*Moser et al.* 1990
Apc $^{\blacktriangle 716/+}$	Neomycin inserted into exon 15; protein truncated at codon 716	~ 300	Polypoid, papillary and sessile adenomas. No colonic ACF	*Oshima et al.* 1995
Apc $^{1638N/+}$	Neomycin inserted in antisense orientation into exon 15; protein truncated at codon 1638	< 10	Polypoid and hyperplastic polyps. Moderate to highly differentiated adenocarcinoma with infiltration into mucosa and submucosa. Gastric lesions and a single liver metastasis. Desmoids, cutaneous cysts and spontaneous colonic ACF's	*Fodde et al.* 1994
Apc ^{1638T}	Same as above, however, insertion of Neomycin gene inserted in SAME transcriptional orientation as *Apc* –resulting in stable expression of 182kD protein	0	*Apc* $^{1638T/1638T}$ are tumour free	*Smits et al.* 1999
Apc ^{1322T}	Truncating mutation at codon 1322	~ 190	Majority of adenomas in first and second segment of intestine, More polyps, and larger polyps than *Apc* $^{Min/+}$	*Pollard et al.* 2009
Apc $^{\blacktriangle 14}$	Frameshift mutation at codon 580	~ 65	Increase in colonic polyps, ACF and rectal prolapse. Tubular adenomas and invasive carcinomas in animals > 12 months. More severe than *Apc* $^{Min/+}$	*Colnot et al.* 2004
Apc $^{580/D}$	Frameshift mutation at codon 580 following adenoviral derived cre exposure	~ 6	Adenomas predominantly near anus	*Shiabta et al.* 1997
Apc $^{580s/+}$	Frameshift mutation at codon 580 following AhCre mediated recombination	~ 100	Adenomas predominantly in small intestine	*Sansom et al.* 2005

Table 1.8: Summary of Apc genetically engineered mice (adapted from McCart et al. 2008)

In all of these models, loss of the *wild type* allele of Apc is necessary to drive tumourigenesis. In the cases of the $Apc^{Min/+}$, Apc $^{\blacktriangle 716}$ and Apc $^{1638N/+}$, all three sets of genetically engineered mice develop histologically similar tumours, but a difference in tumour burden is observed between them, despite all being on the C57BL/6J background (Taketo, 2006). The Apc $^{1638N/+}$ mutant was believed to best recapitulate the FAP phenotype in terms of tumour progression. This was because this mutation resulted in a decrease in polyp burden (compared to $Apc^{min/+}$ and Apc $^{\blacktriangle 716}$ mice) within these mice, which allowed them to live longer and subsequently develop more advanced tumours (Fodde et al., 1994).

Although these genetically engineered mice have provided a great insight into the development of colorectal cancer *in vivo,* it must be noted however that important discrepancies exist between these mouse models and actual human intestinal neoplasia. One of the most obvious differences is tumour location. In all Apc mutational mouse models tumour development predominantly occurs in the small intestine, in contrast to human tumour development that arises predominantly in the colon. Another key discrepancy is that the Apc mouse models lack the key progressive features that are observed in human colorectal cancers, as tumours arising from these models are not invasive and most do not develop to adenocarcinomas. This can be due to environmental differences such as diet and genetics, which are controlled in a laboratory setting. Moreover, mouse models do not harbour the additional mutations in Smad4, KRAS or p53 that are observed in human tumour progression. This can also be explained by the

fact that they may reach a lethal tumour burden too quickly and need to be sacrificed before tumours can progress. (McCart et al., 2008).

In addition, these models have not allowed us to elucidate the key pathways driving the earliest stages of intestinal transformation. Also, they have not been able to allow us to decipher the major transforming properties of an Apc mutation in intestinal tumourigenesis. For example, why Apc mutations are almost exclusively within gastro-intestinal cancers.

Therefore in order to understand how Apc acts as a 'gatekeeper' against colorectal cancer, it was important to address the role Apc plays in normal colon cells and the immediate consequences of Apc loss *in vivo*. Complete genetic inactivation of *Apc* in the mouse leads to embryonic lethality at day E6.5 (Moser et al., 1995). To overcome this problem, the bacterial Cre-lox system has been used to allow conditional deletion of genes in specific tissues of the mouse. The Cre-Lox system uses the Cre Recombinase gene (causes recombination), which encodes a site specific bacteriophage recombinase called Cre. This allows the Cre protein to recombine DNA that is flanked by specific 34 base pair sites, called LoxP sites. Thus allowing genetic inactivation of your gene of interest by Cre-mediated excision. Intestinal inducible Cre can be driven using the Cyp1A1 (cytochrome p450 subfamily A1) promoter to deliver inducible Cre expression in the intestine (Ireland et al., 2004). The Cyp1A1 promoter is usually transcriptionally silent but is upregulated upon exposure to lipophilic xenobiotics, such as β-napthoflavone. These in turn bind to a cytoplasmic **A**ryl **H**ydrocarbon receptor (hence term **AH**Cre), allowing it to translocate to the nucleus where it complexes with other

factors to create a high affinity DNA binding protein. This transcriptional complex then binds to specific DNA recognition sequences present in the Cyp1A1 promoter and initiates transcription. Using the Cre-Lox based approach, *Sansom et al*. went on to cross *AhCre*$^{+}$ mice to mice carrying a *LoxP*-flanked *Apc* (*Apc* ^{580s}) and the Rosa 26 lacZ reporter allele (Shibata et al., 1997, Ireland et al., 2004, Sansom et al., 2004). The Rosa 26 lacZ reporter allele is used to determine the efficiency of Cre activity, and as a result, transgenic animals conditionally express lacZ from the constitutively active *ROSA26* locus after Cre-mediated excision of a PGKneo cassette. This is quite an attractive system for *in vivo* studies as the Rosa 26 gene product is not required for cell viability, with knockout mice expressing no phenotype. Most importantly, the Rosa 26 gene is ubiquitously expressed in embryogenesis and very widely expressed in adult tissue(Soriano, 1999)

From these crosses, *AhCre*$^{+}$*Apc*$^{+/+}$ as well as *AhCre*$^{+}$*Apc* $^{fl/fl}$ mice were generated and injected with four daily injections of β-napthoflavone (in order to induce recombination) at 8-10 weeks of age. This resulted in approximately 100% recombination in the intestine as scored through the Rosa26R allele. Induction of the transgene also results in recombination within the liver, and 'leaky' Cre expression, where no exposure to the inducer β-napthoflavone is needed, is observed within the renal epithelium (discussed in Chapter 4) (Ireland et al., 2004). By the 5th day after induction, *AhCre*$^{+}$*Apc* $^{fl/fl}$ mice were showing signs of illness and were killed. In contrast, *AhCre*$^{+}$*Apc*$^{+/+}$ showed no signs of illness (Sansom et al., 2004).

Histological examination revealed that *AhCre*$^{+}$*Apc* $^{fl/fl}$ mice displayed altered crypt-villus architecture, as a single crypt villus axis was no longer identifiable, with morphologically atypical 'crypt-like' cells now occupying the majority of the crypt villus axis (Sansom et al., 2004). Wholemount preparations of the intestine from *AhCre*$^{+}$*Apc* $^{fl/fl}$ mice stained for lacZ activity to report Cre mediated recombination at the Rosa26R locus, confirmed a direct overlay between the pattern of Apc loss and the pattern of histological change. Results went on to show that loss of Apc alters the normal pattern of differentiation, as Apc deficient cells no longer expressed villus cell markers such as alkaline phosphatase and Villin. Similarly goblet cells were lost in the absence of Apc and a re-distribution of paneth cells throughout the crypt-like area was observed. Moreover, inactivation of Apc was shown to completely abrogate migration along the crypt villus axis, and *AhCre*$^{+}$*Apc* $^{fl/fl}$ crypts displayed a 45% increase in the proliferation index, with proliferation now occurring independently of position (Sansom et al., 2004).

Following these studies that showed that Apc deletion *in vivo* leads to a hyper proliferative, 'crypt progenitor cell-like phenotype', it was only natural that studies then went on to examine one of the best pro-proliferative Wnt target genes, c-Myc.

Studies from our lab then went on to show that conditional deletion of both Apc and c-Myc within the intestinal epithelium *in vivo* rescued the phenotype of perturbed proliferation, migration, differentiation and apoptosis observed following loss of Apc (Sansom et al., 2004). Surprisingly however, despite the return of morphologically 'wild type' looking crypts in the double mutant mice, high levels of nuclear β-catenin were expressed throughout the crypt villus axis. Tissue expression array analysis from these

mice revealed that the majority of Wnt target genes were c-Myc dependent, arguing that the most important consequence of Apc loss and Wnt signalling was c-Myc activation (Sansom et al., 2007).

1.4 The role of c-Myc in colorectal cancer

1.4.1 An overview of Myc

The proto-oncogene c-Myc encodes a transcription factor c-Myc, which is the cellular homologue to the avian myelocytomatosis retrovirus (Vennstrom et al., 1982). The c-Myc gene was originally discovered in Burkitts lymphoma, and mapped to chromosome 8q, due to the observation of many chromosomal translocations at this site. The c-Myc protein belongs to the Myc group of transcription factors also including N-Myc and L-Myc genes.

Deregulation of Myc is observed in a variety of human tumours, which is due to the fact that Myc is required for a number of key events. These include ability to drive unrestricted cellular proliferation (Eilers et al., 1991), inhibit differentiation (Freytag and Geddes, 1992) and drive apoptosis (Askew et al., 1991, Evan et al., 1992). Myc is also known to play a role in ribosome biosynthesis, protein synthesis, mitochondrial function, metabolism and angiogenesis (Dang et al., 2006). Studies have also suggested a key role for Myc in DNA replication (Dominguez-Sola et al., 2007). Because of this, Myc is known to activate and repress a vast number of genes. Although a long list of genes activated by c-Myc exist, the majority include those involved in cellular growth, protein synthesis and mitochondrial function. Conversely the majority of genes inactivated by c-

Myc include those that would normally negatively regulate cell growth, metastasis and those that promote communication with cells external environment (O'Connell et al., 2003).

c-Myc is a basic helix–loop–helix zipper (bHLHZ) protein that forms a heterodimer with another bHLHZ, called Max. Once this heterodimer is formed, it is now able to recognize the hexameric DNA sequence CACGTG, known as E-boxes, and activate transcription of target genes. (Blackwood and Eisenman, 1991). Furthermore, studies have shown that c-Myc needs to form a heterodimer with Max in order to activate genes that contain Ebox binding sites (Amati et al., 1992). Studies have also shown that Myc recruits a histone acetyltransferase (HAT) in order to mediate its transactivation function (McMahon et al., 2000).

However Myc has also been shown to transcriptionally repress a number of target genes through the interaction with Miz-1. On its own, Miz-1 activates transcription on core promoters through the recruitment of the p300 histone acetyltransferase. However, the Myc-Max heterodimer is able to block this transcriptional activation by Miz-1, by disrupting its interaction with the p300 histone acetyltransferase, and through the recruitment of the DNA methyltransferase DNMT3a (Peukert et al., 1997).

Of great interest to this study is how c-Myc is able to induce cell growth and proliferation, particularly following Wnt signalling activation. c-Myc is known to induce G1- S phase progression through gene activation and repression. Of particular interest are the activation of CKD4/Cyclin D2 complexes and the repression of p21. In order to

mediate G1 to S phase progression, studies have shown that Myc/Max heterodimers induce Cyclin E/ CDK2 activity (Steiner et al., 1995). Similarly, studies have also shown that Myc is able to activate CDK4 and cyclin D2, which in turn results in sequestration of CDK inhibitor p27 (Bouchard et al., 1999). Further studies have gone on to show that Myc's role in cell cycle progression involves the transcriptional repression of various growth arrest genes, such as p15 and p21 (Steiner et al., 1995, Seoane et al., 2002). Studies have shown that when Myc levels are high, Myc is recruited to the p21 promoter by Miz-1 and inhibits p21 activation (Seoane et al., 2002). Micro-array analysis has confirmed this, implicating p21 as one of the major targets of c-Myc repression (Gartel and Radhakrishnan, 2005).

Given that c-Myc has been shown to be required for cellular and molecular changes that occur following Apc loss in the murine small intestine (Sansom et al., 2007), this thesis aims to investigate how c-Myc induces and controls proliferation following Apc loss in the murine small intestine *in vivo*. To do this the following mechanisms will be examined:

- The importance of the transcriptional repression of p21 by c-Myc through interaction with miz-1.
- The importance of the upregulation of the CDK4/cyclin D2 complexes by c-Myc

1.4.2 The role of Myc in apoptosis

Studies have now established a strong role for Myc in cell growth and proliferation, however it is also widely accepted that the Myc oncogene is also a potent inducer of programmed cell death (Askew et al., 1991, Evan et al., 1992). Moreover, the apoptotic function of Myc continued to require binding of its partner Max (Amati et al., 1992). Evan and colleagues then went on to suggest that Myc's opposing roles in proliferation and apoptosis, could be explained as a built- in 'safety mechanism', and that sensitization to apoptosis was a normal function of Myc (Evan et al., 1992). Moreover, it has been shown that the potent proliferative role of Myc is not unleashed unless apoptotic mechanisms are disabled. However if these mechanisms remain intact, Myc is able to increase sensitivity to DNA damage induced apoptosis (Arango et al., 2003). Studies have shown that Myc can potentiate apoptosis through both p53 dependent and independent mechanism (Sakamuro et al., 1995). The most extensively studied pathway for p53 dependent Myc induced apoptosis is the ARF-MDM2- p53 pathway. In this pathway, Myc activation leads to upregulation of the ARF tumour suppressor (a transcript from the alternative reading frame of the INK4a locus), which in turn inhibits Mdm2 function. Mdm2 is the most well known regulator of p53, as it binds and ubiquitinates p53, ultimately targeting it for proteosome degradation (Brooks and Gu, 2003). When Mdm2 is inhibited, p53 is now free and able to transcribe a number of pro-apoptotic genes such as *Bax*, *Bim*, *Noxa*, *Puma* and *Fas* (Benchimol, 2001). Similarly, Myc is postulated to effect the outcome of p53 activation following DNA damage, through its transcriptional repression of p21. Thus when Myc levels are low, activation of p53 leads to the induction of p21 and cell cycle arrest. In contrast, if Myc levels are high,

Myc is able to inhibit p21 through its interaction with Miz-1, thereby favoring the p53 induced apoptotic pathway (Seoane et al., 2002). Myc's induction of apoptosis in a p53 independent fashion, appears to be regulated through Myc's ability to regulate the balance between pro and anti-apoptotic BCL2 family members. Studies have shown that Myc is able to suppress anti-apoptotic BCL2 family members, such as BCL2 and BCL-xL, but is able to upregulate proapoptotic proteins such as Bax and Bim (Meyer and Penn, 2008). Moreover, studies have shown that cytochrome C is a direct transcriptional target of Myc, resulting in the cleavage of downstream caspases (Juin et al., 1999).

Recent studies in *Drosophila* have shown that cells which have high levels of Myc can outgrow and induce apoptosis in neighbouring cells (de la Cova et al., 2004). This may play a key role in the clonal expansion of neoplastic cells. Such a form of cell-cell competition may serve to explain the phenomenon known as field 'cancerisation', whereby a clone of neoplastic cells out grow and replace normal cells. Such dysplastic fields are often observed in colorectal neoplasia. Given that Myc is overexpressed in 70% of colorectal cancers, studies have suggested that the inhibition of apoptosis in wild type cells may prove effective in limited apoptotic transformation induced by Myc, and therefore result in a decrease in tumour growth (Secombe et al., 2004, Donaldson and Duronio, 2004).

1.4.3 Deleting c-Myc from the intestinal epithelium *in vivo*

Deletion of c-Myc by gene targeting in mice causes mid-gestation lethality . To overcome this problem, the bacterial Cre-lox system has been used to allow conditional deletion of genes in specific tissues of the mouse . Conditional deletion of c-Myc was achieved by crossing mice which express the *Ah*Cre-recombinase, to mice with a *loxP*-flanked c-Myc allele. Cre^{+} $Myc^{fl/fl}$ mice were generated, and conditional deletion of the c-Myc allele was obtained by inducing Cre-recombination with intraperitoneal injections of β-naphthoflavone. To generate 100% recombination, mice were given three injections in a single day, which induces Cre activity within the crypts of the small intestine, including the stem cells (Muncan et al., 2006b). The effect of *in vivo* deletion of c-Myc from the intestinal epithelium has been of much debate. Studies performed by *Muncan et al.* showed that deletion of c-Myc from the intestinal epithelium, resulted in a reduction in size and in proliferation of c-Myc deficient enterocytes compared to wildtype. It was suggested the absence of c-Myc resulted in reduced levels of its target gene nucleophosim; a key regulator of cell growth and ribosome biosynthesis. Moreover deletion of c-Myc within these mice resulted in the repopulation of c-Myc deficient enterocytes with wild type ones over the longer term, resembling the phenomenon of 'cell-cell' competition, observed in *Drosophila* (Muncan et al., 2006b). However other studies performed by *Bettess et al.* showed c-Myc to be essential for intestinal crypt formation but not for intestinal homeostasis (Bettess et al., 2005). The suggested difference in outcomes between these two models is thought to be the method of transgene recombination used and genetic background. For example, *Mucan et al.* performed studies using the *AhCre* transgene as described above, whereas *Bettess et al.*

used the *VillinCreER* transgene to delete c-Myc. Regardless of different outcomes, these studies illustrated the key point that deletion of c-Myc from the intestinal epithelium results in mice that are viable, and allows for short term retention of c-Myc deficient enterocytes.

As a result, one of the key aims of this study is to use $AhCre^{+}$ $Myc^{fl/fl}$ mice in order to examine the interaction between c-Myc and p21.

1.4.4 The role of c-Myc as a critical mediator of the 'crypt progenitor' phenotype

Studies from this lab have showed that c-Myc is required for all the immediate phenotypic changes that are observed following loss of Apc *in vivo*. Crossing $AhCre^{+}$ $Apc^{fl/fl}$ mice to $AhCre^{+}$ $Myc^{fl/fl}$ mice, to generate double mutant $AhCre^{+}$ $Apc^{fl/fl}$ $Myc^{fl/fl}$ mice, we have shown that double mutant intestinal enterocytes are able to proliferate, migrate and differentiate like wild type cells (Sansom et al., 2007). These studies confirmed complete gene loss of both Apc and c-Myc, illustrating these effects were not due to repopulation of wild type cells. Surprisingly however, high levels are nuclear β-catenin were continued to be observed in double mutant cells, demonstrating that in the absence of c-Myc, nuclear β-catenin is insufficient to drive the Apc 'crypt progenitor' phenotype (Sansom et al., 2007).

Following these key findings, various studies have been aimed at identifying the effects of Apc loss and the requirement of c-Myc, with many studies showing that effects are cell context specific. For example, studies have shown that overexpression of c-Myc in pancreatic islets leads to a large induction of proliferation and apoptosis (Pelengaris et al., 2002). Therefore one would predict that targeted Apc gene deletion, and hence c-Myc

activation, using a pancreatic specific Cre would produce similar effects. However this was not the case. When Apc was deleted within the islets of the pancreas through the use of a pancreatic specific Cre (Pax6-Cre), no phenotype was observed, despite efficient gene deletion (Strom et al., 2007). This lack of phenotype was due to a complete failure of upregulation of Wnt/TCF target genes, such as c-Myc. However, using the pancreatic and duodenal homeobox to drive recombination throughout the entire pancreas, Apc gene deletion lead to pancreatomegaly due to the hyperproliferation of acinar cells but did not lead to tumourigenesis (Strom et al., 2007). Moreover, similar to the intestine, inactivation of c-Myc and Apc completely rescued this phenotype. Taken together these results highlight the importance of cellular context.

However in not all cell contexts is Wnt-mediated hyperplasia c-Myc dependent. This can be observed in the liver where loss of Apc leads to hepatomegally, however the loss of c-Myc is unable to rescue this phenotype (Sansom et al., 2007). Therefore, studies have gone on to examine whether the simple overexpression of c-Myc within the intestinal epithelium is sufficient to recapitulate those observed following activation of the Wnt/β-catenin pathway. Studies from *Finch et al.* have used mice which have a transgenically targeted expression of the reversibly switchable form of Myc, termed $MycER^{TAM}$, to determine if acute activation of Myc is able to mimic the 'crypt progenitor' phenotype observed following Apc loss (Finch et al., 2009). Results from these studies showed that direct activation of c-Myc within the intestinal epithelium did result in phenotypic changes that overlapped those observed following acute loss of Apc, but were not identical. As observed following loss of Apc, c-Myc activation induces an increase in proliferation and apoptosis as well as a loss of the goblet cell lineage (Finch et al., 2009).

Following Apc loss, paneth cells are redistributed, however c-Myc activation results in the complete ablation of paneth cell, presumably through *in situ* dedifferentiation. However the most important distinction observed was the robust activation of the ARF/p53 pathway within $MycER^{TAM}$ mice, which is not normally observed following indirect c-Myc activation via deregulated Wnt signalling (Sansom et al., 2004). The authors of this study suggest that it is the levels of c-Myc expression that account for these discrepancies. Results showed that $MycER^{TAM}$ mRNA was expressed 40-fold higher than endogenous c-Myc mRNA and 5 fold higher than those levels observed following loss of Apc. It is postulated that these levels are high enough in $MycER^{TAM}$ mice to allow activation of the ARF/p53 pathway, resulting in high levels of apoptosis and tumour suppression. However in the case of direct c-Myc activation following aberrant Wnt activation, c-Myc levels are not high enough to activate the ARF/p53 pathway, but instead are able to repress p21. The ability of c-Myc to repress p21 in this context, may serve to explain why p53 activation is unable to significantly affect the hyperproliferation that is observed following loss of Apc (Reed et al., 2008). This notion was furthered investigated by studies performed by *Murphy et al.* which used the Rosa26 promoter to drive low-level deregulated expression of the switchable form of Myc, Myc^{ERT2}, in target tissues (Murphy et al., 2008b). Results showed that acute activation of Myc lead to an induction of ectopic proliferation in many tissues. However this low level of Myc induction was not sufficient to activate the ARF/p53 pathway. These results show that although c-Myc is required, c-Myc independent Wnt/β-catenin functions exist that may be critical for intestinal tumourigenesis (Finch et al., 2009). Taken together these results show that following degregulated Wnt signalling, c-Myc is specifically

upregulated only to levels which are high enough to lock crypt stem and progenitor cells in a proliferative and undifferentiated state, but are crucially not high enough to induce the ARF/p53 pathway, which would result in tumour suppression.

1.5 The cell cycle

Deregulation of the cell cycle in human cancer has been well documented in a vast array of studies over the past two decades. Some of the key hallmarks of cancer are uncontrolled proliferation, due to constitutive mitogenic signalling and defective anti-mitogenic signalling, genomic and chromosomal instability; all of which can be attributed to the misregulation of **C**yclin **D**ependent **K**inases (**CDK**s)

CDK's are regulated by a group of regulatory subunits known as cyclins, and therefore require binding of these cyclins to be active. In order to regulate the cell cycle, cyclins are synthesized and destroyed at the correct times in order to activate their appropriate kinase [reviewed in (Malumbres and Barbacid, 2009)]

Initial studies investigating the control of the cell cycle were performed in yeasts which found that regulation of the cell cycle was through a single CDK, known as Cdc28 in *Saccharomyces cerevisiae* and Cdc2 in *Saccharomyces Pombe* (Russell and Nurse, 1986), and was shown to bind to a number of different cyclins throughout the cell cycle. In human cells however, the number of CDKs and cyclins are significantly more (Malumbres and Barbacid, 2005). However despite this increase, there is only a subset of CDKs and cyclins that complex together to drive the cell cycle. These include : CDK2, CDK4, CDK6, CDK1 and ten cyclins belonging to four different classes; A, B, D, E.

The cell cycle is comprised of two main stages; interphase and M(mitosis) phase. Interphase encompasses G1, S and G2 phases. In G1 (Gap 1) cells increase in size and produce a significant amount of RNA and protein. In S (synthesis) phase, DNA replication occurs. In G2 (Gap 2) phase the cell continues to grow and produces more proteins and the production of microtubules is now observed. In M phase, cell growth and

protein production stop, and the division into two daughter cells is now observed. In human cells, interphase lasts anywhere between 12-24 hours, whilst mitosis is much shorter, which a duration of only 1-2 hours. Cells that exit mitosis either go on to proliferate and enter the cell cycle again in G1, or enter what is know as a resting or quiescent state, known as G0. Cells that are non-proliferative may stay in this state for long periods of time, such is observed in terminally differentiated cells; example neuronal cells.

As mentioned above, there are key CDK/Cyclin complexes are responsible for certain stages of progression throughout the cell cycle (Figure 1.9)

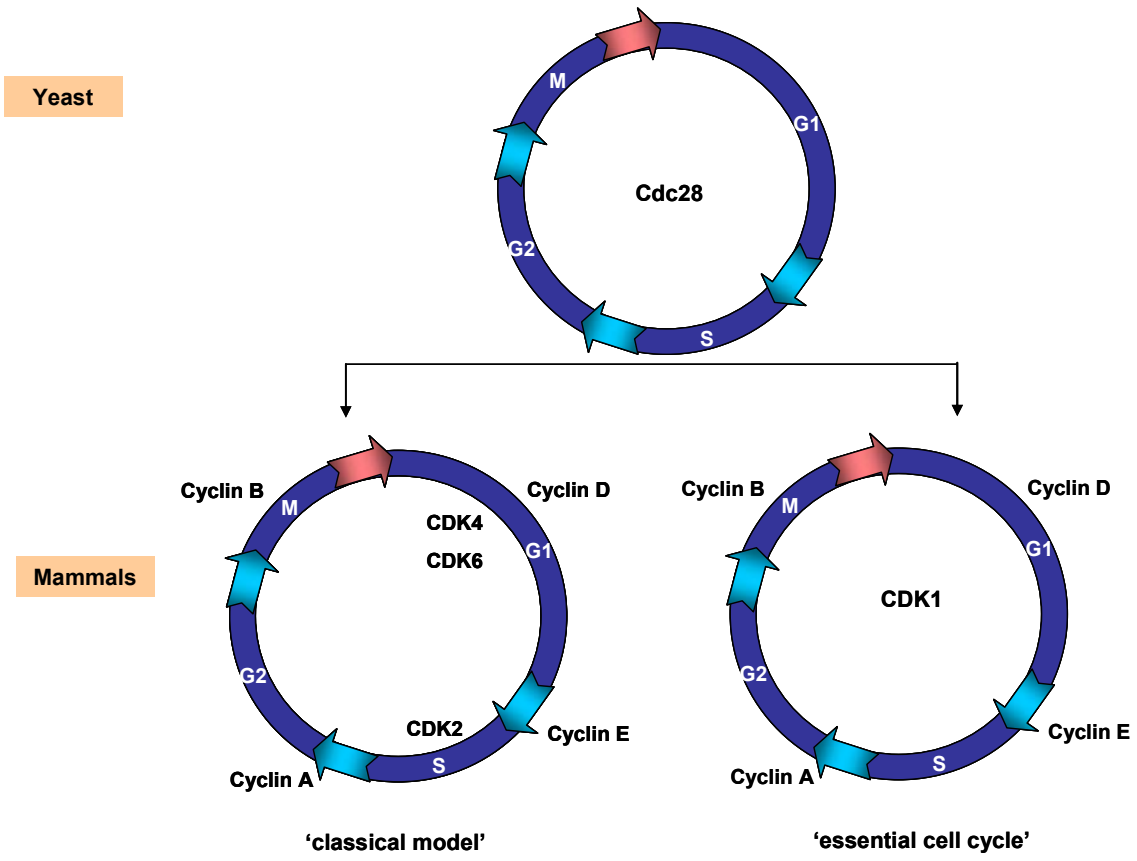

Figure 1.9: The cell cycle (adapted from Mumbres et al. 2009)

Cartoon showing the *S.cerevisiae* cell cycle and two models of the mammalian cell cycle, 'the classical' and 'essential' cell cycle. The yeast cell cycle is simply governed by Cdc28. The 'classical' mammalian cell cycle depicts each main event of interphase (G1, S, G2) is driven by unique cyclin-dependent kinases(CDKs) bound to specific cyclins. The 'essential' cell cycle is based on genetic evidence indicating that CDK1 is sufficient to drive proliferation of all cell types up to mid gestation.

During G1, D-type cyclins (cyclin D1, D2 and D3) sense initial mitogenic signals and bind preferentially to CDK4 and CDK6. The binding of these cyclins results in the activation of CDK4 and CDK6, which in turn leads to the partial inactivation of RB proteins; RB1 (known as p107) and RB2 (known as p130). Under normal conditions, RB serves as a transcriptional repressor through its interaction with various transcription factors such as E2F, histone deacetylases and chromatin remodelling complexes (Cobrinik, 2005). Importantly, one of the main repressional targets of the Rb/E2F complex are the E cyclins, which bind to and activate CDK2. Active CDK2/Cyclin E complexes are now able to further phosphorylate Rb proteins, leading to their complete inactivation. (Harbour et al., 1999, Lundberg and Weinberg, 1998). This inactivation is key to G1 progression, as active CDK2/Cyclin E complexes are believed to be required to drive effective G1-S transition (Vandenheuvel and Harlow, 1993). CDK2 is then activated by cyclin A2 in order to drive the progression through G2. At the end of G2, CDK1 is believed to be activated by cyclin A, and this complex now initiates mitosis. A-type Cyclins are then degraded and subsequently B-type Cyclins are able to bind to CDK1. An active CDK1/cyclin B complex is believed to be necessary to drive cells through mitosis [reviewed in (Malumbres and Barbacid, 2009)].

1.5.1 Evaluation of the CDK/Cyclin D complexes in vivo

The 'classical' model of the mammalian cell cycle dictates that specific CDKs are responsible for certain stages of progression of the cell cycle. However recent animal models with germline deletions of various CDKs have shown this not to be the case [reviewed in (Santamaria and Ortega, 2006)]. Germline deletions of interphase CDK2, CDK4 and CDK6 have shown redundancy for cell cycle progression in the majority of murine cells, as mice with germline deletions for any one of these CDKs remain viable. However, deletions of these CDKs result in developmental defects of specific cell types, which in turn can result in a reduced life span. (Table 1.10)

Targeted gene	Functional effect	Life span	Major phenotype	Reference
CDK4	knockout	Viable. Reduced life span due to diabetes	Pancreatic beta cell proliferation impaired: diabetes. Anterior pituitary cell proliferation impaired, particularly lactotrophs. Leydig cell numbers reduced. Defective spermatogenesis.	*Rane et al.* 1999
CDK6	knockout	Viable. Normal life span	Hypoplastic thymus and spleen. Reduced erythrocyte and megakaryocyte numbers. Reduced body size only in females.	*Malumbres et al.* 2004
CDK2	knockout	Viable. Normal life span	Spermatocytes die in pachytene. Oocytes die in diplotene. 100% esterility in males and females. Strain dependent reduced body size.	*Ortega et al.* 2003; *Berthet et al.* 2003
CDK4 & CDK6	Double knockout	Embryonic lethality at E14.5-E18.5	Defective fetal hematopoiesis. Severe anemia.	*Malumbres et al.* 2004
CDK2 & CDK6	Double knockout	Viable. Normal life span.	Addition of individual knock-out phenotypes.	*Malumbres et al.* 2004

Table 1.10: Major phenotypes of gene targeted or spontaneous mutant mice lacking one or more CDK (adapted from *Santamaria et al.* 2009)

For example, mice with targeted gene deletion of CDK4 are viable but have a reduced life span due to an impairment in pancreatic beta cell proliferation, which results in the onset of diabetes (Rane et al., 1999). Similarly, knockouts of CDK6 and CDK2 result in mice that are viable, however these mice have a normal life span due to only minor defects observed in the erythroid lineage in CDK6 mice (Malumbres et al., 2004), whilst deletion of CDK2 results in defective meiotic division of male and female germline cells (Ortega et al., 2003, Berthet et al., 2003).

Further studies have gone on to show that the lack of any major cell cycle defects cannot just simply be attributed to the compensation of the CDKs. For example the combined knockout of CDK4 and CDK6 results in embryonic lethality at day E14.5-18.5 which is attributed to defective fetal hematopoiesis, resulting in severe anaemia (Malumbres et al., 2004). Surprisingly however, no major defects in entry to or cell cycle progression are observed in any other cell lineage other than the haematopoietic lineage. Moreover, mice with combined knockouts of CDK2 and CDK6 were completely viable and reached adulthood. The only defects observed were those seen in the single knockout strains (Malumbres et al., 2004).

More recent studies have shown that *in vivo* of deletion of CDK2, CDK4 and CDK6 results in mice that are able to undergo organogenesis but die by mid gestation due to hematopoietic defects (Santamaria et al., 2007). Results from the same lab also went on to show that CDK1 is sufficient to drive the mammalian cell cycle, as within these triple knockout mice, CDK1 was sufficient to bind to all cyclins, resulting in the phosphorylation of Rb, and subsequent transcription of E2F target genes. Moreover, mice that were homozygous for a CDK1 mutant allele resulted in embryonic lethality due

to impaired cell division in very early development, illustrating the essential role of CDK1, and the inability of all other CDKs to compensate for its loss (Santamaria et al., 2007). Therefore these findings suggest that although CDK1 may be the only CDK that is completely required to drive the cell cycle, the presence of additional CDKs may have evolved in mammalian cells as a higher regulatory mechanism for a variety of specific cell types.

In vivo studies have also examined the effect of the D-type cyclins (D1, D2, D3) on cell cycle progression through gene targeting (Table 1.11).Similarly to CDK2/4/6 deletion, deletion of individual D-type cyclins results in the cell type specific developmental defects. Although the expression of D-type cyclins throughout embryonic development is tissue specific, it is widely accepted that there is co-expression of the three cyclins in a number of tissues, and therefore redundancy may explain the failure to observe embryonic development (Santamaria and Ortega, 2006). Knockouts of cyclin D1, D2 or D3 all result in viable mice, with cell type specific defects. Deletion of cyclin D1 results in defects in eye and mammary gland development (Fantl et al., 1995), whilst deletion of cyclin D2 results in defective pancreatic beta cell proliferation (Sicinski et al., 1996b), similar to that observed in CDK4 knockout mice. Deletion of cyclin D3 results in a hypoplastic thymus (Sicinska et al., 2003). Consistent with the hypothesis of genetic redundancy of cyclin D proteins, *in vivo* deletion of a single D-type cyclin often results in the upregulation of the other two D-type cyclins. The exact mechanism for this compensation appears to be embryonic tissue specific. For example in some cases altered translation is observed with an increase in mRNA of the given cyclin, by various post-

translational mechanisms (Ciemerych et al., 2002). The exact mechanism of compensation is unknown, however studies have shown that during embryogenesis, cellular proliferation is reliant on the 'net' cyclin D activity, with all D-type cyclins being interchangable for proliferation in most cell types. Tissue specific defects occur in double knockout mice, when specific transcription factors are unable to upregulate the remaining cyclin, when normally expression of that cyclin in that particular tissue is either absent, or expressed at much lower levels. For example combined cyclin D1 and cyclin D2 knockout mice are viable but die within the first three weeks of birth due to reduced body size and hypoplastic cerebellum. Combined knockouts of all three D-type cyclins have resulted in embryonic lethality at day E16.5 due to megaloblastic anemia and defective fetal hematopoiesis (Kozar et al., 2004), similar to those defects observed in CDK4/CDK6 knockout mice (Malumbres et al., 2004)

Studies examining the 'overlap' between D-type cyclins have found that in the case of cyclin D1 deletion, knock in replacement with cyclin D2 is insufficient to rescue all the phenotypes observed in cyclin D1 null mice (Carthon et al., 2005). Taken together these results illustrates that although there is co-expression and redundancy of D-type cyclins during embryonic development, small functional differences and expression patterns between D-type cyclins exist which may serve to provide additional regulation of proliferation of specific cell types.

Targeted gene	Functional effect	Life span	Major phenotype	Reference
Cyclin D1	knockout	Viable	Neurological abnormalities. Impaired mammary epithelial proliferation during pregnancy. Retinal hypoplasia. Reduced body size.	*Fantl et al.* 1995; *Sicinski et al.* 1995 *Antanasoski et al.* 2001 *Ciemerych et al.* 2005
Cyclin D2	knockout	Viable	Impaired pancreatic beta cell proliferation. Impaired granulosa cell proliferation in response to FSH. Female sterility. Hypoplastic testes, decreased sperm counts. Impaired proliferation of B-lymphocytes.	*Sicinski et al.* 1996; *Georgia et al.* 2004 *Huard et al.* 1999 *Solvason et al.* 2000 *Kowalczyk et al.* 2004
Cyclin D3	knockout	Viable. Normal life span	Impaired cerebellar cell development. Hypoplastic thymus.	*Sicinska et al.* 2003;
Cyclin D1 & D2	Double knockout	Viable. Die in the first three weeks	Hypoplastic cerebellum. Reduced body size	*Ciemerych et al.* 2002
Cyclin D1, D2, D3	Triple knockout	Embryonic lethality at E16.5	Megaloblastic anemia. Defective fetal hematopoiesis.	*Kozar et al.* 2004

Table 1.11 : Major phenotypes of gene targeted or spontaneous mutant mice lacking one or more D-type Cyclin (adapted from *Santamaria et al.* 2006)

1.5.2 The role of Cyclin D1 and Cyclin D2 following activation of Wnt signalling

Previous studies have shown that CyclinD/CDK4/6 complexes may act as key modulators of c-Myc dependent proliferation, due to the fact that CDK4, Cyclin D1 and Cyclin D2 have been proposed as direct c-Myc target genes (Haas et al., 1997). Although Cyclin D1 has been proposed as a canonical Wnt target gene, studies from our lab have confirmed that Cyclin D1 is not immediately upregulated in the murine small intestine following loss of Apc (Sansom et al., 2005b). Furthermore, genetic deletion of Cyclin D1 did not impact any of the immediate phenotypes observed following Apc loss, and most importantly did not effect proliferation. However, Cyclin D1 was observed to be upregulated at later stages following Apc loss and was shown to be required for efficient adenoma formation (Sansom et al., 2005b). Studies from our lab have gone on to show that Cyclin D2 is immediately upregulated following Apc loss (Sansom et al., 2004). This may be explained by the fact that Cyclin D2 levels are most highly expressed in the base of the crypt, coinciding with high areas of Wnt signalling, arguing that Cyclin D2 expression is driven by Wnt signalling (Yang et al., 2006). Given that the induction of proliferation following Apc loss is c-Myc dependent, this thesis aims to investigate the importance of the upregulation of cyclin D2 following Apc loss, as well as the dependence of Apc deficient cells on CyclinD/CDK4/6 complexes.

1.5.3 CDK's and CDK inhibitors in cancer

Given that one of the key hallmarks of cancer is uncontrolled cellular proliferation it is not suprising that studies have found pRb, the major regulator of entry into the cell cycle, to be one of the most frequent targets of genetic alterations in tumours. Given that CDK2, CDK4 and CDK6 control the G0 to S transition, they have been the main focus of CDK drug targeted inhibition (Malumbres and Barbacid, 2001). Therefore due to the important regulatory role that CDKs play in controlling the cell cycle, CDKs are often upregulated in cancer, resulting in the unscheduled cellular division of either stem or possibly progenitor cells (Malumbres and Barbacid, 2009). Both CDK4 and CDK6 are overexpressed in wide range of tumours, including sarcoma, glioma, breast tumours, lymphoma and melanoma. Studies have shown that D-type cyclins are frequently deregulated in tumours, resulting in hyperactivation of CDK4 and CDK6. CDK6 has been shown be preferentially hyperactivated in mesenchymal tumours such as leukaemia and sarcomas. Whereas CDK4 tends to be upregulated in epithelial tumours. [reviewed in (Malumbres and Barbacid, 2009)]. Targeting CDKs for cancer therapy relies on the notion that cancer cells, due to their highly proliferative nature are more dependent on CDK/Cyclin complexes than most normal cells that are in a quiescent, non proliferative state (Santamaria and Ortega, 2006). However this principle has not held up in clinical trials of initial CDK inhibitors such as flavopiridol and UCN-01, which target CDK 1,2,4 and 6 (Shapiro, 2006). The most plausible reason for their failure is the fact that these inhibitors were not targeted to tumours which were dependent on CDKS. Various *in vivo*

studies have shown that the tumour dependence on a certain CDK, is reliant not only on the oncogenic mutation that drives tumour development but also on the tissue specificity of the tumour. This can be observed in mouse tumour models that have shown that deletion of cyclin D1 (and therefore CDK4/CDK6 activity) prevents breast cancer when driven by Erbb2 and Hras oncogenes, but not in the case of those that are driven by Wnt1 or Myc driven pathways (Yu et al., 2001). However in a model of Myc induced skin tumourigenesis, inhibition of CDK4 proved to be effective (Miliani de Marval et al., 2004). Therefore these results suggest that each tumour type dependent on its mutational and tissue origins may require inhibition of a specfic CDK to effectively inhibit cell growth.

Although mouse models with germline or conditional deletion of various CDK's exist, these models do not accurately mirror pharmacological inhibition. This is due to the fact that pharmacological inhibition results invariably in incomplete inhibition of the designated target and usually is accompanied by off target effects. It has therefore been suggested that knock in mutations that express dead CDKs or non-activating cyclins be used in order to better predict the consequences of *in vivo* targeting as well as off target effects (Malumbres and Barbacid, 2009).

1.6 Regulators of the cell cycle

In order to regulate the cell cycle, two families of CDK inhibitors exist. These include the INK4 proteins, namely INK4a, INK4b, INK4c and INK4d, and the Cip and Kip family which are composed of $p21^{Waf/CIP1}$, p27 and p57 (Sherr and Roberts, 1999).

Ink4a proteins inhibit CDK4 and CDK6 complexes by competing with D-type cyclins for binding. In contrast, Cip and Kip family members bind to active CDK/cyclin complexes, resulting in inactive trimeric complexes. Besides binding to CDK4 and CDK6, Cip/Kip inhibitors are also known to inhibit complexes between CDK2/cyclin A, CDK2/cyclin E, CDK1/cyclin A and CDK1/cyclin E [reviewed in (Malumbres and Barbacid, 2005)]. In order to investigate the effects of these CDK inhibitors *in vivo*, mouse models harbouring germline mutations for these inhibitors have been made. Mice which harbour knockout mutations in $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$ are viable, however mice with deletions in $p15^{INK4b}$, $p16^{INK4a}$ and $p18^{INK4c}$ have an increased risk of tumourigenesis (Krimpenfort et al., 2001, Sharpless et al., 2001, Franklin et al., 1998, Zindy et al., 2001, Bai et al., 2003). However $p19^{INK4d}$ mice display no susceptibility to tumourigenesis, they exhibit only minor defects such as testicular atrophy and progressive hearing loss (Zindy et al., 2000, Chen et al., 2003).

Mutations in INK4 genes, including $p15^{INK4b}$, $p16^{INK4a}$ or $p18^{INK4c}$ have been found in a wide variety of human cancers, including melanoma and pancreatic cancers. These mutations include either gene deletion, promoter methylation or point mutations (Ruas and Peters, 1998).

Unlike INK4a genes, mutations in the Cip/Kip family of CDK inhibitors are much more rare. $p21^{Waf1/Cip1}$ and $p57^{Kip2}$ are rarely found altered in human cancer, however reduced

levels of $p27^{kip1}$ are associated with poor prognosis in a wide range of cancers, including those of breast, prostate and gastric carcinomas (Lloyd et al., 1999). Similarly, p21 levels are downregulated in human colon tumours, and is linked to decreased survival rates. p21 deficiency also decreases the number of mature goblet cells, which is significant as the loss of this lineage and mucin secretion is characteristic of early, preneoplastic aberrant crypt foci in patients who are at risk for developing colon cancer (Zirbes et al., 2000).

1.6.1 The role of p21 in the cell cycle

$p21^{waf/cip}$ was originally discovered as a key target of the p53 tumour suppressor gene following DNA damage. Induction of p21 by p53 induced cell cycle arrest by a block in G1 (Deng et al., 1995). Studies have also shown that p21 can be induced in a p53 independent fashion, such as through oxidative stress, cytokines, tumour viruses and anticancer agents [reviewed(Abbas and Dutta, 2009)]. In order to inhibit cell cycle progression, p21 binds to CDK2/cyclin E complexes in G1, which inhibits the phosphorylation of Rb by CDK2, further inhibiting the transcription of E2F target genes, and ultimately inhibiting the progression from G1 to S phase. p21 also inhibits the progression through S phase by binding to and inhibiting CDK2/Cyclin A and CDK1/cyclin A complexes. Moreover, p21 inhibits progession through G2 phase and into M phase by inhibiting CDK1/Cyclin B complexes.

P21 also directly inhibits DNA synthesis by binding directly to PCNA (**P**roliferative **C**ell **N**uclear **A**ntigen), which inhibits binding of DNA polymerase δ as well as other proteins involved in DNA synthesis (Abbas and Dutta, 2009).

p21 has also been shown to suppress cell cycle progression through mechanisms that are CDK and PCNA independent. p21 has been shown to bind and directly inhibit the transcription activity of various transcription factors such as E2F-1 (Devgan et al., 2005), Stat3 (Coqueret and Gascan, 2000) and Myc; which can in turn disrupt the binding of p21 to PCNA and alleviate p21's inhibition of DNA synthesis (Kitaura et al., 2000).

1.6.2 The role of p21 in apoptosis

Studies have shown that p21 is also implicated in protection from p53-dependent and independent apoptosis (Weiss, 2003, Gartel and Radhakrishnan, 2005).

In response to DNA damage, the cell undergoes repair or dies by apoptosis. If the cell is to be repaired, p53 induces p21 and it is postulated that p21 is the crucial survival factor. Therefore the increase in p21 that is seen in some cancers may give these cells a survival advantage, by placing cells on a repair pathway instead of an apoptotic one. This anti-apoptotic effect of p21 makes it an attractive target for cancer therapy as attenuation of p21 in malignant cells may subvert the normal repair process induced by DNA damaging agents (chemotherapeutic drugs) and thus make such drugs more effective (Weiss, 2003). Therefore it appears that p21 has two roles; 1) in regulating cell cycle transit as a universal inhibitor of cyclin dependent kinases and 2) in preventing apoptosis of DNA damaged cells via a p53 induced repair pathway.

It is thought that this switch between cell survival and cell cycle inhibition might occur through the localization of p21.When p21 is cytoplasmically located, it is able to bind and inhibit a number of key pro-apoptotic genes such as procaspase 8, caspase 3 and caspase

10. Moreover p21 can directly bind to and inhibit Myc and E2F-1 which inhibits the transcriptional activation of various pro-apoptotic genes (Dotto, 2000).

1.6.3 The role of p21 in senescence

Cellular senescence is defined as the terminal growth arrest of cells, which is accompanied by changes in cellular adhesion, morphology and gene expression (Campisi, 1997). Two forms of senescence are believed to exist; replicative and accelerated senescence. Replicative senescence is referred to as the 'classical' form of senescence, where senescence is induce by the shortening of telomeres (DePinho, 2000), whereas accelerated senescence occurs in response to DNA damage (Dileonardo et al., 1994) or the activation of the Ras oncogene (Serrano et al., 1997). A role for p21 in permanent growth arrest and replicative senescence was first identified in studies in human fibroblasts which showed a knockout for p21 in these cells dramatically increased their lifespan (Brown et al., 1997). Studies have now shown a link between p21 and senesence in tumour cells, as overexpression of p21 was shown to be sufficient to drive tumour cells into senescence (Fang et al., 1999). Moreover, studies have shown that p21 is key in the induction of senescence following treatment with anti-cancer agents (Chang et al., 1999). It has now become clear that in order to induce a state of permenant growth arrest and senescence, p53, p21 and p16 need to be activated. In the response to telomere shortening, or DNA damage, p53 becomes activated and subsequently induces p21. However studies have observed that the expression of p21 in senescence is transient, and suggests that p21 is only expressed in the early stages of cell cycle arrest, but is not

involved in the long term maintenance of this state. However studies have shown that following the upregulation of p21, p16 also becomes upregulated, and it is the upregulation of p16 that maintains the state of permanent growth arrest (Alcorta et al., 1996, Roninson, 2002).

1.6.4 The effect of p21 deletion in vivo and it's role in cancer

In order to investigate the role of p21 in tumour formation *in vivo*, mouse models which harbour knockout mutations for p21 have been studied. Despite the numerous studies which have shown key roles for p21 in cell cycle regulation, apoptosis, differentiation and senescence, p21 knockout mice are viable and furthermore were tumour free up to seven months of age (Deng et al., 1995). However studies showed that p21 knockout mice spontaneously developed tumours by 16 months of age, with the majority of tumours consisting of haematopoietic and endothelial origin, with a small percentage consisting of epithelial origins (Martin-Caballero et al., 2001). In accordance with these findings, previous studies have also shown that deletion of p21 results in an increase in tumourigenesis following irradiation (Jackson et al., 2003). Deletion of p21 has been shown to increase tumourigenesis in Apc $^{1638\ N/+}$ mice (Yang et al., 2001a) , and has also been shown to be essential for the mitotic arrest and inhibition of Apc-initiated tumour formation by sulindac in Apc $^{1638\ N/+}$ mice (Yang et al., 2001b).

In order to further understand the role of p21 in p53 dependent cell cycle control *in vivo*, *Barboza et al.* utilized a mouse model harboring the point mutation $p53^{R172P}$ (referred to as $Trp53^{515c/515c}$), which is unable to induce apoptosis but retains it's ability to regulate

the cell cycle, through the induction of p21 (Barboza et al., 2006). *Trp53*$^{515c/515c}$ mice were then crossed to *p21*$^{-/-}$, to determine the role of p21 in tumour formation. Results from these studies showed that lymphomas and sarcomas that arouse in *Trp53*$^{515c/515c}$ *p21*$^{-/-}$ mice displayed aneuploidy and chromosomal aberrations that were absent from *Trp53*$^{515c/515c}$ mice (Barboza et al., 2006). Taken together, these studies show that p21 is key for delaying tumour onset by preservation of chromosomal instability.

In contrast to these results, some studies have suggested an oncogenic role for p21. For example, p21 deficiency reduced tumour formation in radiation induced ATM deficient and wild type mice (Wang et al., 1997, Martin-Caballero et al., 2001)

A small set of p21 mutations have been observed in human cancers such as Burkitt's lymphoma (Bhatia et al., 1995), prostate (Gao et al., 1995), melanoma (Vidal et al., 1995) and breast cancers (Balbin et al., 1996). However, in comparisons to p53 and p16 mutations which are frequently mutated in a wide range of cancer, the mutation rate for p21 in human cancer is very rare (Shiohara et al., 1997).

Many large studies have been conducted, examining the expression pattern of p21 in human cancers. Although one would predict that loss of p21 would result in greater tumourgenicity and reduced survival, this was not always the case. In small-cell lung, colorectal, cervical and head and neck cancers, a decrease in p21 expression did in fact correlate with tumour progression and poor prognosis. In the majority of these cases, this correlation was strongest when p53 expression was also lost. However, studies also showed that in some cases of prostate, ovarian, cervical, breast, esophageal squamous cell carcinomas as well as in brain tumours, increased expression of p21 correlated with

tumour progression and poor prognosis. Moreover, some studies found p21 to have no prognostic value [reviewed in (Roninson, 2002)]. Taken together these studies suggest that the role of p21 in tumourigenesis may be highly tissue specific, and it may therefore act as a tumour suppressor or promoter.

1.7 Thesis aims

Previous studies from this lab have shown that c-Myc is essential for the proliferation that occurs following Apc loss. In this thesis I will address how important Myc's repression of p21 is for the phenotypes following Apc loss. I will also examine the importance of the CDK4/Cyclin D2 complexes for c-Myc dependent proliferation. Moreover, these studies led me to address how important p21 is in initiating senescence of Apc deficient cells *in vivo*. Therefore the aims for this thesis are:

- To investigate the role of c-Myc in inducing apoptosis within the intestinal crypt, and whether this is p21 dependent?

- To investigate the role of p21 in causing senescence of Apc deficient cells, and whether this is c-Myc dependent?

- To determine the functional importance of repression of p21 by c-Myc in Apc deficient cells.

- To determine the significance of Cyclin D2 upregulation within Apc deficient cells.

Chapter 2: Material and Methods

2.0 Material and Methods

2.1 Generation of Mice colonies

All Experiments were performed under the UK Home Office guidelines. All mice were maintained under non-barrier conditions and given a standard diet (Harlan) and water *ad libitum*.

The alleles used for this thesis were as follows: *c-Myc*^{fl} (Baena et al., 2005), *AhCre*, A*pc*^{580S} (Ireland et al., 2004),(Sansom et al., 2004), p21$^{-/-}$ (Brugarolas et al., 1995), *Lgr5-EGFP-IRES-creER*^{T2} (Barker et al., 2007), *Apc* $^{Min/+}$ (Moser et al., 1990), *p16*$^{-/-}$ (Serrano, 1997), *Cyclin D2*$^{-/-}$ (Sicinski et al., 1996a)

2.1.1 Mouse experiments for Chapter 3

For Cre induction, *AhCre*$^{+}$ *c-Myc*$^{++}$ and *AhCre*$^{+}$ *c-Myc*$^{fl/fl}$ were given 3 injections intraperitoneally (IP) of 80mg/kg β-napthoflavone (Sigma, #N3633) in a single day, which yields nearly 100% constitutive recombination in the murine small intestine (Muncan et al., 2006b).

Mice were then exposed to DNA damaging agents (gamma irradiation or cisplatin treatment) 4 days after gene loss. Previous experiments have shown that using this protocol, no significant induction of apoptosis is seen in induced (*AhCre*$^{+}$ *c-Myc*$^{++}$) versus uninduced *AhCre*$^{+}$ *c-Myc*$^{++}$ or induced wild type (mice not carrying the AhCre transgene) at day 4 after induction (Muncan et al., 2006b).

2.1.2 Mouse experiments for Chapter 4

To address the effect of *p21* and *Apc* deletion within the renal epithelium *AhCre*$^{+}$, A*pc*$^{fl/fl}$ mice were bred to *p21*$^{-/-}$ mice to generate *AhCre*$^{+}$ A*pc*$^{fl/fl}$ *p21*$^{-/-}$ mice. As previously described, in the absence of the inducer β-napthoflavone, sporadic Cre mediated recombination occurs in the renal epithelium in the S and Comma shaped bodies (Sansom et al., 2005a). Cohorts containing *AhCre*$^{+}$ A*pc*$^{fl/fl}$, *AhCre*$^{+}$ A*pc*$^{fl/fl}$ *p21*$^{+/-}$ and *AhCre*$^{+}$ A*pc*$^{fl/fl}$ *p21*$^{-/-}$ mice (n>15 for each genotype) were aged , and examined three times a week for signs of renal disease or failure. These included blood in the urine, hunching, and swollen kidneys; which was investigated through scruffing the mouse and gently feeling for an enlarged kidney, which was indicative of a tumour.

In order to address the effect of p21 and Apc deletion within the intestinal epithelium, *AhCre*$^{+}$ A*pc*$^{fl/fl}$ *p21*$^{-/-}$ mice were given 3 injections (IP) of 80mg/kg β-napthoflavone in a single day, which yields nearly constitutive recombination in the murine small intestine. Analysis of all intestinal phenotypes were examined at day 4 post induction.

For tumourigenic studies, cohorts containing *AhCre*$^{+}$ A*pc*$^{fl/+}$, *AhCre*$^{+}$ A*pc*$^{fl+}$ *p21*$^{+/-}$ and *AhCre*$^{+}$A*pc*$^{fl/+}$ *p21*$^{-/-}$ (n> 15 for each genotype) mice were induced at 6 weeks of age with 3 injections (IP) of 80mg/kg β-napthoflavone and left until developing signs of intestinal illness; rapid weight loss, anaemia, hunching and blood in faeces.

In order to address the effect of *INK4A* deletion on an intestinal tumourigenic model, *Apc* $^{Min/+}$ mice were crossed to *INK4A* $^{-/-}$ mice. Cohorts containing *Apc* $^{Min/+}$ *INK4A* $^{+/+}$, *Apc*

Min/+ INK4A +/-, *Apc Min/+ INK4A -/-* mice (n>15 for each genotype) were aged until developing signs of intestinal illness (as described above).

Lgr5-EGFP-IRES-creER^{T2} Apc$^{fl/fl}$ mice were generated by breeding *AhCre^{+} Apc fl/fl* and the *Lgr5-EGFP-IRES-creER^{T2}* mice as previously described (Barker et al., 2009). To induce recombination of Apc within the LGR5 stem cell, mice 6 weeks of age were induced with a single (IP) injection of tamoxifen (Sigma # T5648-1G) 10mgml^{-1} in sunflower oil and left until showing signs of intestinal illness.

To generate non-stem cell intestinal adenomas, *AhCre^{+} Apc fl/fl* mice were orally gavaged with 1mgkg^{-1} β-napthoflavone in corn oil and left until showing signs of intestinal illness (Barker et al., 2009)

2.1.3 Mouse experiments for Chapter 5

In order to address the effect of combined Apc, c-Myc and p21 loss within the intestinal epithelium, *AhCre^{+} Apc$^{fl/fl}$ Myc fl/fl* mice were crossed to *p21$^{-/-}$* mice. *AhCre^{+} Apc$^{fl/fl}$ Myc fl/fl p21$^{-/-}$* mice were given 3 injections (IP) of 80mg/kg β-napthoflavone in a single day, which yields nearly constitutive recombination in the murine small intestine. Analyses of all intestinal phenotypes were examined at day four post induction.

For tumourigenic studies, cohorts containing *AhCre^{+}*A*pc$^{fl/+}$, AhCre^{+} Apc^{fl+} Myc fl/fl p21$^{-/-}$* (n>15 for each genotype) mice were induced at 6 weeks of age with 3 injections of 80mg/kg β-napthoflavone (IP) and left until developing signs of intestinal illness; rapid weight loss, anaemia, hunching and blood in faeces.

2.1.4 Mouse experiments for Chapter 6

In order to address the importance of Cyclin D2 upregulation following loss of Apc within the intestinal epithelium, *AhCre*$^{+}$ *Apc*$^{fl/fl}$ mice were crossed to *Cyclin D2* $^{-/-}$ mice. *AhCre*$^{+}$ *Apc*$^{fl/fl}$ *Cyclin D2* $^{-/-}$ mice were given 3 injections (IP) of 80mg/kg β-napthoflavone in a single day, which yields nearly constitutive recombination in the murine small intestine. Analyses of all intestinal phenotypes were examined at day four post induction.

In order to address the effect of Cyclin D2 deletion on an intestinal tumourigenic model, *Apc* $^{Min/+}$ mice were crossed to *Cyclin D2* $^{-/-}$. Cohorts containing *Apc* $^{Min/+}$ *Cyclin D2*$^{+/+}$ *Apc* $^{Min/+}$ *Cyclin D2* $^{+/-}$, *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ (n>15 for each genotype) were aged until developing signs of intestinal illness; rapid weight loss, anaemia, hunching and blood in faeces. A second cohort containing the same set of mice were culled at a timepoint of 110 days in order to address the onset of tumourigenesis.

2.2 Tissue isolation.

For the analysis of kidney tumourigenic cohorts, at the appropriate time, mice were culled and both kidneys (including cystic tumours) were removed and fixed in 4% formalin, overnight at 4^{o}C for no more than 24 hours before processing and were then paraffin embedded. For Senescence associated β-galactosidase staining, kidney or intestinal tissues were placed on a cork disk and covered in OCT before being submerged into liquid nitrogen. For the analysis of intestinal phenotypes at four days post induction;

the small intestine was removed and flushed with water. Intestines were dissected as follows: The proximal 7cm was mounted 'en face' and fixed overnight in methacarn (methanol, chloroform and acetic acid; 4:2:1) and paraffin embedded. The following 5cm was divided into 1cm lengths, bundled using surgical tape and then fixed in 4% formaldehyde at 4^{o}C for no more than 24 hours before processing. The remainder was fixed in methacarn and then paraffin embedded.

For tumourigenic studies, the entire intestine and colon was removed and flushed with water. Both intestine and colon were mounted 'en face' and fixed overnight in methacarn. Lesions were then scored macroscopically. Intestine were then wound into a "swiss" roll which were subsequently embedded in paraffin, sectioned at 10μM and stained with haematoxylin and eosin prior to microscopic analysis.

2.3 Genotyping of mice

2.3.1 DNA Extraction from tails

DNA was extracted from tails using the PUREGENE DNA EXTRACTION kit. Tails were lysed overnight in 500μl of cell lysis solution (Puregene) and 10μl of proteinase K (20mg/ml, Sigma), shaken at 37ºC. Tails were left to cool at room temperature, 200μl of protein preciptation solution (Puregene) was added to each tube. These were vortexed and centrifuged at top speed for 5 minutes in a microfuge.

The supernatant was removed into a clean tube containing 500μl of isopropanol, vortexed and centrifuged at top speed for 5 minutes. The supernatant was poured and off and the

DNA pellet was left to dry overnight. DNA was resuspended in 500µl DNA hydration solution (Puregene).

2.3.2. Genotyping of Mice via PCR

All genotyping of mice was done by genomic PCR (Polymerase Chain Reaction) from DNA extracted from tails (2.3.1). All PCR reactions were done in 50µl volumes using 2.5µl of the tail DNA preparation.

Apc flox PCR Protocol

PCR mix

	µl
5x Colorless GoTaq Flexi Buffer*	10
$MgCl_2$ (25mM)	5
dNTPs (10mM)	0.4
Primer (100µM)	0.2 (of each)
Go Taq*	0.2
H_2O to final volume of 47.5 µl	

Add 2.5µl gDNA.
*GoTaq Flexi DNA Polymerase from Promega.

Primers
APC P3 = GTT CTG TAT CAT GGA AAG ATA GGT GGT C
APC P4 = CAC TCA AAA CGC TTT TGA GGG TTG

PCR Program: 95°C, 3min (95°C, 30s; 60°C, 30s; 72°C 1min) $_{30}$ 72°C, 5min. 4°C, hold.

Run PCR products on a 2% gel.
Bands FLOX = 314bp
WT = 226bp

c-Myc flox PCR Protocol

PCR mix

	µl
5x Colorless GoTaq Flexi Buffer*	10
$MgCl_2$ (25mM)	5
dNTPs (10mM)	0.4
Primer (100µM)	0.2 (of each)
Go Taq*	0.2
H_2O to final volume of 47.5 µl	

Add 2.5µl gDNA.
*GoTaq Flexi DNA Polymerase from Promega.

Primers (5' to 3')
MYC FL A = CCG ACC GGG TCC GAG TCC CTA TT
MYC FL S = GCC CCT GAA TTG CTA GGA AGA CTG

PCR Program: 94°C, 3min (94°C, 1min; 60°C, 1min; 72°C, 1min) $_{30\ cycles}$
72°C, 10min. 15°C, hold.

Run PCR products on a 2% gel.

Bands
FLOX = ~500bp
WT = ~400bp
HET = 500p and 400bp (+ sometimes a non-specific band c600bp)

Cre LacZ PCR Protocol (Go Taq)

PCR mix

	µl
5x Colorless GoTaq Flexi Buffer*	10
$MgCl_2$ (25mM)	5
dNTPs (10mM)	0.4
Primer (100µM)	0.2 (of each)
Go Taq*	0.2
H_2O to final volume of 47.5 µl	

Add 2.5µl gDNA.
*GoTaq Flexi DNA Polymerase from Promega.

Primers
CRE A = TGA CCG TAC ACC AAA ATT TG

CRE B = ATT GCC CCT GTT TCA CTA TC
LACZ A = CTG GCG TTA CCC AAC TTA AT
LACZ B = ATA ACT GCC GTC ACT CCA AC

PCR Program: 95°C, 3min (95°C, 30s; 55°C, 30s; 72°C 1min) $_{30}$
72°C, 5min. 15°C, hold.

Bands CRE = ~1000bp
LacZ = ~500bp

Cyclin D2 PCR Protocol (Split PCR)

PCR Mix

		µl
Buffer		5
$MgCl_2$	(50mM)	2.5
dNTPs	(10mM)	0.4
Primer	(100µM)	0.1 (of each)
Platinum Taq		0.2
H_2O to final volume of		*47.5*

Add 2.5µl gDNA.
Note: Is a split PCR
WT Reaction use D2-D & N2
Null Reaction use D2-D & D2-G

Primers
D2-D = GCTGGCCTCCAATTCTAATC
N2 = CTAGTGAGACGTGCTACTTC
D2-D = GCTGGCCTCCAATTCTAATC
D2-G = CCAGATTTCAGCTGCTTCTG

PCR Program: 94°C, 3min (94°C, 1min; 60°C, 1min; 72°C 1min) $_{36}$
72°C, 7min. 15°C, hold.

Run products on a 2% gel
Bands WT = 400bp
NULL = 250 bp

MIN PCR Protocol

PCR MIX

		µl
Buffer		5
$MgCl_2$	(50mM)	2.5
dNTPs	(10mM)	0.4
Primer	(100µM)	0.1 (of each)
Pic Taq		0.4
H_2O to final volume of 47.5		

Add 2.5µl gDNA.
Primers
MIN1 = TCT CGT TCT GAG AAA GAC AGA AGC T
MIN2 = TGA TAC TTC TTC CAA AGC TTT GGC TAT

PCR Program: 94°C, 2min (94°C, 1min; 60°C, 1min; 72°C, 1min) $_{30\ cycles}$
72°C, 10min.

Digest 20µl of PCR product. Add 1.5µl Hind III, 2.5µl buffer.
Incubate for 12h 37°C. 15°C on hold.
Run digest products on a **4%** gel
Bands WT = 111bp
MIN = 123bp

P16 PCR Protocol

PCR mix

	µl
5x Colorless GoTaq Flexi Buffer*	10
$MgCl_2$ (25mM)	5
dNTPs (10mM)	0.4
P16 WT F (100µM)	0.2
P16 WT R (100µM)	0.2
P16 Null neo (100µM)	0.2
P16 Null R1 (100µM)	0.2
Go Taq*	0.2
H_2O	31.1

Add 2.5µl gDNA.
*GoTaq Flexi DNA Polymerase from Promega.

Primers (5' to 3')
P16 WT F = ATG ATG ATG GGC AAC GTT C
P16 WT R = CAA ATA TCG CAC GAT GTC
P16 Null neo = CTA TCA GGA CAT AGC GTT GG
P16 Null R1 = AGT GAG AGT TTG GGG ACA GAG

PCR Program: : 94°C, 3min (94°C, 1min; 60°C, 1min; 72°C 1min) $_{30}$; 72°C, 10min. 15°C, hold.

Run PCR products on a 2% gel.

Bands
WT = 236bp
NULL = 723bp

LacZ PCR Protocol

PCR MIX

		µl
Buffer		5
$MgCl_2$	(50mM)	2.5
dNTPs	(10mM)	0.4
Primer	(100µM)	0.1 (of each)
Platinum Taq		0.2
H_2O to final volume of		*47.5*

Add 2.5µl gDNA.
Primers
LACZ A = CTG GCG TTA CCC AAC TTA AT
LACZ B = ATA ACT GCC GTC ACT CCA AC

PCR Program: 95°C, 3min (95°C, 30s; 55°C, 30s; 72°C 1min) $_{30}$
72°C, 5min. 15°C, hold.

Bands LacZ = ~500bp

2.3.3 Summary of PCR reactions

PCR	Primer Sequences (5' to 3')	PCR Program	Expected Products
APC Flox	APC P3 = GTT CTG TAT CAT GGA AAG ATA GGT GGT C APC P4 = CAC TCA AAA CGC TTT TGA GGG TTG ATT C	95°C, 3min (95°C, 30s; 60°C, 30s; 72°C 1min) $_{30}$ 72°C, 5min. 4°C, hold.	FLOX = 314bp WT = 226bp
cMYC Flox	MYC FL A = CCG ACC GGG TCC GAG TCC CTA TT MYC FL S = GCC CCT GAA TTG CTA GGA AGA CTG	94°C, 3min (94°C, 1min; 60°C, 1min; 72°C, 1min) $_{30}$ 72°C, 10min. 15°C, hold	FLOX = ~500bp WT= ~400bp
Cyclin D2 (Split PCR)	D2-D = GCTGGCCTCCAATTCTAATC N2 = CTAGTGAGACGTGCTACTTC	94°C, 3min (94°C, 1min; 60°C, 1min; 72°C 1min) $_{36}$ 72°C, 7min. 15°C, hold.	NULL = 400bp
	D2-D = GCTGGCCTCCAATTCTAATC D2-G = CCAGATTTCAGCTGCTTCTG	94°C, 3min (94°C, 1min; 60°C, 1min; 72°C 1min) $_{36}$ 72°C, 7min. 15°C, hold.	WT = 250bp

Cre PCR	CRE A = TGA CCG TAC ACC AAA ATT TG CRE B = ATT GCC CCT GTT TCA CTA TC	95°C, 3min (95°C, 30s; 55°C, 30s; 72°C 1min) $_{30}$ 72°C, 5min. 15°C, hold.	CRE = ~1000bp
Lacz PCR	LACZ A = CTG GCG TTA CCC AAC TTA AT LACZ B = ATA ACT GCC GTC ACT CCA AC	95°C, 3min (95°C, 30s; 55°C, 30s; 72°C 1min) $_{30}$. 72°C, 5min. 15°C, hold.	LacZ = ~500bp
P16 PCR	P16 WT F = ATG ATG ATG GGC AAC GTT C P16 WT R = CAA ATA TCG CAC GAT GTC P16 Null neo = CTA TCA GGA CAT AGC GTT GG P16 Null R1 = AGT GAG AGT TTG GGG ACA GAG	94°C, 3min (94°C, 1min; 60°C, 1min; 72°C 1min) $_{30}$; 72°C, 10min. 15°C, hold.	WT = 236bp NULL = 723bp
P21 Null (Split PCR)	P21 F = TCT TGT GTT TCA GCC ACA GGC P21 R = TGT CAG GCT GGT CTG CCT CC (WT PCR)	95ºC, 2min (95ºC, 30s; 59ºC, 30s; 72ºC, 1min) $_{35}$ 72ºC, 3min. 4ºC hold.	WT = 430bp
	P21-5. = ATT TTC CAG GGA TCT GAC TC 3' R1N-1A = CCA GAC TGC CTT GGG AAA AGC 3' (P21 interrupted PCR)	95ºC, 2min (95ºC, 30s; 59ºC, 30s; 72ºC, 1min) $_{35}$ 72ºC, 3min. 4ºC hold.	NULL = 150bp
Min PCR	MIN1 = TCT CGT TCT GAG AAA GAC AGA AGC T MIN2 = TGA TAC TTC TTC CAA AGC TTT GGC TAT	94°C, 2min (94°C, 1min; 60°C, 1min;	WT = 111bp

72°C, 1min) $_{30\ cycles}$

72°C, 10min. MIN = 123bp

2.3.4 Controls for PCR experiments

For all PCR reactions, a negative control was used. This included a sample which contained PCR mix but with water in the place of mouse tail DNA. This was used to ensure that there was no genomic contamination. Secondly, in some cases a positive control was used, where the DNA from a mouse known to carry the mutation/deletion in question was used along side the tested samples.

2.4 Wholemount Method for assaying recombination through LacZ expression

For assaying LacZ expression, the whole gut was removed and flushed with ice-cold PBS. Following which, the gut was flushed with ice-cold X-gal fixative (2% formaldehyde, 0.1% gluteraldehyde in PBS). The whole gut was then cut into 4 sections and pinned out 'en face' onto a wax plate. Once gut sections were pinned out they were fixed with X-gal fixative for 1 hour. They were then washed once with PBS and incubated in DTT demulcifying solution for 30 minutes (50ml/plate: 5 ml glycerol, 5ml 0.1 M Tris pH 8.2, 10mls 100% ethanol, 30 ml saline [0.9% NaCl], 170mg DTT. Plate was then washed 2x with PBS and incubated overnight with gentle agitation in X-gal stain: 200µl solution A (5% X-gal in DMF) in 50ml solution B (0.1g $MgCl_2$, 0.48g K-ferricyanide, 0.64 K-ferrocyanide in 500ml PBS). The following day examined presence or absence of blue/green colour. Wash plate with PBS and fix in 10% formalin.

2.5 DNA Damage inducing agents

For assessing whether c-Myc deficiency affects the DNA damage response following gamma irradiation 'wild type' *AhCre*$^{+}$ *c-Myc*$^{++}$ and c-Myc deficient *AhCre*$^{+}$ *c-Myc*$^{fl/fl}$ mice were irradiated with 14 Gy irradiation using a Cs^{137} source delivered at a dose rate of 0.423 Gy/min. Mice were then harvested at 30 mins, 1, 2, 3, 6, 12, 24 and 48 hour timepoints following the irradiation. At least 3 mice were used for each timepoint. For cisplatin treatment, mice were given a single IP injection of 10mg/kg Cisplatin and harvested 6 hours later (Purchased from David Bull Laboratories and distributed by Faulding Pharmaceuticals).

2.5.1 Nutlin Treatment

For assessing whether MDM2 upregulation abrogated apoptosis following c-Myc deletion, 'wild type' *AhCre*$^{+}$ *c-Myc*$^{++}$ and c-Myc deficient *AhCre*$^{+}$ *C-Myc*$^{fl/fl}$ mice were gavaged with either 200µl of vehicle or 200 mg/kg of Nutlin-3a (synthesized at the Roche Research Center, Nutley, NJ) twice a day as previously described (Tovar et al., 2006). On day 4 post Cre induction, mice were given a single application of Nutlin and irradiated with 14Gy and harvested 6 hours following the irradiation.

2.5.2 Assaying apoptosis, mitosis and crypt size in vivo.

At each indicated time point following either induction of β-napthoflavone and or following DNA damage, a minimum of three animals were killed and the small intestine was removed, flushed with water and fixed overnight in methacarn. Samples were wound into 'swiss rolls' and stored in 70% ethanol prior to staining.

Haematoxylin and Eosin (H&E) stained sections were made and apoptosis was scored through the use of the Highly Optimised Microscopic Environment (HOME) microscope (Clarke *et al.,* 1994).

Apoptosis was identified through its morphological appearance. Apoptosis is recognisable in the intestine through the appearance of smooth membrane bound apoptotic bodies. Cells shrink to produce a halo around the apoptotic bodies with clear chromatin condensation within the nuclei. The nuclei also stains a much redder colour (see Kerr *et al.,* 1972, Wyllie *et al.,* 1980). Apoptosis was independently confirmed by immunohistochemical staining with an antibody against active caspase 3.

Crypt size was scored from H&E stained sections by counting the total number of cells contained within one crypt. For each analysis, 25 full crypts were scored from at least 3 mice of each genotype. Similarly, mitosis was scored from H&E stained sections by counting the total number of mitotic cells per crypt. Mitotic cells were identified morphologically to be undergoing division. Once again, for each analysis, 25 full crypts were scored from at least 3 mice of each genotype.

2.5.3 Assaying proliferation and migration in vivo

In order to examine levels of proliferation, mice were injected with 250µl of bromodeoxyuridine (BrdU) (Amersham) two hours prior to being sacrificed. Similarly, in order to score migration, mice were injected with BrdU 24 hours prior to being sacrificed. Immunohistochemical staining for BrdU was then performed using an anti-BrdU antibody. At least 3 mice were used for each genotype and timepoint. To examine levels of proliferation, following a two hour BrdU pulse chase, position and total number of BrdU positive cells per crypt were counted. To examine migration levels, following a 24 hour BrdU pulse chase, the position of total BrdU positive cells were scored and compared to those at 2 hours by plotting their cumulative frequency.

To quantify levels of proliferation between adenomas from *Apc* $^{Min/+}$ and *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice, 3 mice of each genotype were injected with BrdU two hours prior to be sacrificed. BrdU immunohistochemistry was then performed on paraffin embedded intestinal sections of these mice. For each mouse, 3 different adenomas were identified, and for each adenoma, the total number of BrdU positive cells per 500 tumour cells was counted and scored as a percentage. The average between these 3 adenomas was taken as the final percentage of BrdU positive cells per 500 tumour cells for that mouse.

2.6 Immunohistochemistry

2.6.1 Immunohistochemistry on frozen sections:

Immunohistochemistry for Senescence associated β-galactosidase:

Frozen sections of either kidney or intestinal gut parcels were cut into 3μM sections on Poly-L-lysine slides (Sigma). Sections were then thawed at room temperature for 30 minutes. The senescence β-galactosidase staining kit from Cell Signalling (# 9860) was used for this IHC. Slides were then fixed with 1x fixative solution from the kit for 10 minutes at room temperature. Slides were then washed 2x in dH20. A 1x staining solution from the kit was then prepared and the pH was adjusted to 5.5. Slides were incubated with the staining solution overnight at 37^{o}C. A small piece of parafilm was applied to each slide to ensure that the staining solution did not evaporate. The following day, excess staining solution was removed from the slides and the slides were counter stained with nuclear fast red (Sigma # N8002) for 5 minutes. Slides were then washed in dH20 for 5 minutes. Slides were mounted using DPX (TCS biosciences # HC8610) mounting medium.

2.6.2 Immunohistochemistry on paraffin sections:

For all immunohistochemistry on paraffin sections, except for β-catenin, antigen retrieval was performed with citrate buffer either in the microwave or in the water bath (detailed protocols below)

Microwave antigen retrieval:

Make up the following solutions:

Solution A:

10.5g of citric acid

500 mls dH20

Solution B:

29.4g SodiumCitrate

1 litre dH20

To make up a 1.5L solution: Mix : 27mls of solution A + 123mls of solution B and complete to 1.5L with dH20 and adjust to pH 6.0. Add 1.5 L solution to pressure cooker and microwave for 20 minutes or until solution is boiling. Add slides and microwave until pressure is optimized. Continue to boil for another 3-4 minutes.

Remove pressure cooker and place into a sink filled with cold water. Remove lid and allow slides to cool in solution for 20 minutes.

Water Bath antigen retrieval:

Dilute Citrate Buffer (Labvision) 1/10 in distilled water and place 50ml of diluted citrate buffer solution into a glass coplin jar. Immerse coplin jar into a water bath and preheat to 99.9^{o}C . Immerse slides into preheated solution and boil for 20 minutes. Remove coplin jar from water bath and allow to cool for 30 minutes at room temperature in the solution.

2.6.3 Immunohistochemistry for p21:

The staining was done on paraffin embedded, 'quick fixed' (for less than 24hrs) formalin fixed sections. 3μM sections of tissue were cut onto Poly-L-lysine slides (Sigma). Sectioned were dewaxed by placing into xylene for 20 minutes. They were rehydrated through graded ethanol solutions (absolute alcohol, 70 % ethanol) and then into water. Antigen retrieval was performed as per the microwave method. Slides were washed 2-3x 5 minutes in PBS. Blocked slides for 30 minutes with 5% goat serum in PBS. Wash 2-3x 5 minutes with PBS. Incubate slides with Santa Cruz anti-p21 (M-19) (rabbit polyclonal) diluted 1/500 in 5% goat serum /PBS for 1 hour at room temperature. Wash 2-3x 5 minutes with PBS. Block again for 10 minutes with 5% goat serum in PBS Incubate slides 30 min with biotinylated secondary antibody from rabbit ABC kit (Vector Laboratories). Washed slides 2-3x 5 minutes with PBS. Incubated slides with ABC solution for 30 minutes; 2 drops of solution A + 2 drops of solution B in 5 ml 5% goat serum/ PBS. (Leave for 30 minutes to warm up). Mix DAB reagents in the ratio 1ml substrate buffer to 1 drops chromogen. Apply to slides and incubated for 5-10 minutes. Washed slides 2-3x 5 minutes with PBS. Transferred slides to dH2O. Counterstain slides in Haematoxylin for approximately 60 seconds. Wash in running tap water for 5 minutes. Dehydrate slides by washing in increasing concentrations of alcohols (1x 5minute wash in 70% alcohol, 2x 5minute washes in 100% alcohol). Slides were then placed in xylene for 2x 10 minute washes. Slides were then mounted using DPX mounting medium.

2.6.4 Immunohistochemistry for p16:

Immunohistochemistry was performed as in 2.6.3. Exceptions: Slides were incubated with primary antibody to p16 (Santa Cruz M-156) 1/25 in 5% goat serum PBS for 1 hour at room temperature. Slides were then incubated for 30 minutes with biotinylated secondary antibody from rabbit ABC kit (Vector Laboratories).

2.6.5 Immunohistochemistry for p19:

Immunohistochemistry was performed as in 2.6.3. Exceptions: Antigen retrieval was performed as per the water bath method. Slides were incubated with primary antibody p19/ARF 1/300 (Upstate) in 5% goat serum PBS overnight at 4^0C. Slides were then incubated for 30 minutes with biotinylated secondary antibody from rabbit ABC kit (Vector Laboratories).

2.6.6 Immunohistochemistry for Caspase 3:

Immunohistochemistry was performed as in 2.6.5. Exceptions: Antigen retrieval was performed as per water bath method. Slides were incubated with primary antibody anti-active caspase 3 (rabbit polyclonal; AF835, R&D systems) 1/750 in 5% goat serum PBS overnight at 4^0C. Slides were then incubated for 30 minutes with biotinylated secondary antibody from mouse ABC kit (Vector Laboratories).

2.6.7 Immunohistochemistry for y-H2ax:

Immunohistochemistry was performed as in 2.6.3. Exceptions: Slides were incubated with primary antibody γ-H2ax 1/300 (Upstate) in 5% goat serum PBS for one hour at room temperature. Slides were then incubated for 30 minutes with biotinylated secondary antibody from mouse ABC kit (Vector Laboratories).

2.6.8 Immunohistochemistry for p-ATM:

Immunohistochemistry was performed as in 2.6.3. Exceptions: Slides were incubated with primary antibody P-ATM (1/500 pS1981 ROCKLAND 200-301-500) in 5% goat serum PBS for one hour at room temperature. Slides were then incubated for 30 minutes with biotinylated secondary antibody from mouse ABC kit (Vector Laboratories).

2.6.9: Immunohistochemistry for P-Chk-1:

Immunohistochemistry was performed as in 2.6.3. Exceptions: Antigen retrieval was performed as per the water bath method. Slides were incubated with primary antibody P-Chk1 (1:100 Phospho Chk-1 ser 345 Cell Signalling) in 5% goat serum PBS for one hour at room temperature. Slides were then incubated for 30 minutes with biotinylated secondary antibody from rabbit ABC kit (Vector Laboratories).

2.6.10: β-Catenin immunohistochemistry:

Slides were cut and rehydrated as in 2.6.3. Slides were blocked for 30-45 minutes in the following block; 1 litre stock = 4.16g citric acid, 10.76g DiSodium Hydrogen Phosphate

2 hydrate, 1g NaAz; add fresh H_2O_2 to 1.5%. For antigen retrieval, slides were boiled in a water bath for 50 minutes in Tris EDTA; 1 litre stock = 242g Tris, 18.6g EDTA. For working solution, dilute 30ml stock in 1500ml DDW, adjust pH to 8.0 with HCl (3-4 pasteur pipettes). Slides were allowed to cool for 1 hour. Slides were then blocked for 30 minutes in 1% BSA in PBS. Slides were then incubated with β-catenin antibody (mouse monoclonal; C19220, Transduction Laboratories) 1/50 in 1%BSA/PBS for 2 hours at room temperature. Washed slides 3x in PBS. Incubate slides with HRP-labelled polymer from Mouse Envision+ system (Dako systems) for 1 hour at room temperature. Washed slides 3x in PBS. Slides were then developed with DAB, rehydrated and mounted as 2.6.3.

2.6.11 c-Myc Immunohistochemistry:

Slides were cut and rehydrated as in 2.6.3. Antigen retrieval was performed as per the water bath method for 50 minutes. Slides were allow to cool for 30 minutes at room temperature in solution. Slides were then rinsed in dH20. For prevention of endogenous staining, slides were blocked for 15 minutes in 1.5% H_2O_2 solution in PBS. Slides were then rinsed in water and then in PBS. Slides were blocked for 30 minutes in 5% goat serum in TBST. Slides were then rinsed 3x in TBST. Slides were incubated with primary antibody rabbit anti-c-Myc (Santa Cruz N-262. Lot # C1309) 1:200 in Tris Buffered Saline+ 1% Tween (TBST)/5% goat serum for 48 hours at 4^oC. Slides were then washed 3x 10 minutes in TBST, and incubated with secondary PowerVision poly HRP-anti-rabbit IgG for 2 hour at room temperature.

Washed slides 3x 10 minutes TBST. Slides were then developed with DAB, rehydrated and mounted as in 2.6.3.

2.6.12 BRDU Immunohistochemisty:

Slides were cut and rehydrated as in 2.6.3. Antigen retrieval was performed as per the water bath method for 20 minutes. Slides were allowed to cool for 30 minutes at room temperature in solution. Slides were then rinsed in dH_2O. For prevention of endogenous staining, slides were blocked for 15 minutes in 1.5% H_2O_2 solution in dH_2O. Slides were then blocked for 30 minutes in 1%BSA in PBS. Slides were then incubated with a 1/500 dilution of mouse anti-BRDU [Becton, Dickinson and Company (BD) :cat N 347 580] diluted in PBS/1%BSA overnight at 4^0C. Slides were then washed 3x in PBS and incubated with secondary polymer HRP-conjugated Envision+ (Mouse Envision+ system Dako Systems) for 1 hour at room temperature. Slides were then washed 3x 5 minutes in PBS. Slides were then developed with DAB, rehydrated and mounted as in 2.6.3.

2.6.13 P53 Immunohistochemistry:

Slides were cut and rehydrated as in 2.6.3. For epitope retrieval slides were steamed for 40-45 minutes in 10 mM sodium citrate (pH 6.0). Cooled slides slowly at room temperature for 20-30 minutes. Slides were then incubated in methanol/ H_2O_2 (180 mL methanol /20 mL 30% H_2O_2) for 20 min at room temperature. Rinse profusely with dH_2O. Slides were rinsed in PBS three times. Slides were then blocked for 30 minutes at room temperature in normal serum from VECTASTAIN kit (4 drops serum/10 mL 1X

PBS-0.1% Tween-20). Slides were then incubated with primary antibody diluted in blocking solution and incubated for 1-2 hours at room temperature (anti-p53 (CM5 from Vector laboratories) at 1:200 dilution). Slides were then washed 3x in PBS, and incubated with secondary antibody from Vectastain Universal kit (1 drop/10 mL blocking solution) at room temperature for 30 minutes.

Slides were washed 3x in PBS. Incubated slides with ABC solution for 30 minutes; 2 drops of solution A + 2 drops of solution B in 5 ml 5% goat serum/ PBS. Washed slides 3x in PBS. Slides were then developed with DAB, rehydrated and mounted as in 2.6.3.

2.6.14 MCM2 Immunohistochemistry:

Slides were cut and rehydrated as in 2.6.3. Antigen retrieval was performed as per the microwave method. Slides were then blocked in 10% H202 made with dH20 for 10 minutes at room temperature. Slides were then washed with PBS 3X 5 minutes. Slides were then blocked in 5% goat serum PBS for 30 minutes. Incubated slides with MCM2 (Cell Signalling # 4007) rabbit polyclonal antibody at 1/200 with 5% goat serum PBS. Incubated overnight at 4^{o}C. Washed slides with PBS 3x 5 minutes. Slides were then incubated with secondary polymer HRP-conjugated Envision+ (Rabbit Envision+ system Dako Systems) for 1 hour at room temperature. Slides were then washed 3x 5 minutes in PBS. Slides were then developed with DAB, rehydrated and mounted as in 2.6.3.

2.6.15 MDM2 Immunohistochemistry:

IHC was performed as in MCM2 staining. Exceptions: Antigen retrieval was as per the water bath method. Incubate with primary antibody: (Lab Vision MDM2 smp14 ms- 291-p1) at 1/200 with 20% rabbit serum in PBS for 1hr at room temperature. Wash slides with PBS 3x 5 minutes. Slides were then incubated with secondary polymer HRP-conjugated Envision+ (Mouse Envision+ system Dako Systems) for 1 hour at room temperature. Slides were then washed 3x 5 minutes in PBS. Slides were then developed with DAB, rehydrated and mounted as in 2.6.3.

2.6.16 Ki-67 Immunohistochemistry:

IHC was performed as per MCM2 IHC. Incubate with primary antibody: LabVision (now Thermo; RM-9106) 1/250 in 5% goat serum PBS for 1 hour at room temperature. Wash slides with PBS 3x 5 minutes. Slides were then incubated with secondary polymer HRP-conjugated Envision+ (Rabbit Envision+ system Dako Systems) for 1 hour at room temperature. Slides were then washed 3x 5 minutes in PBS. Slides were then developed with DAB, rehydrated and mounted as in 2.6.3.

2.6.17 Control for Immunhistochemistry

For all immunohistochemical stains performed both positive and negative controls were used. Positive controls included samples that were known to have high expression of that particular protein. For example, when performing immunohistochemistry for β-catenin, a

section of mouse intestine that was homozygous for *Apc*, which displays high levels of nuclear β-catenin throughout the crypt was used. Negative controls included samples of tissue from mice that were known (through genotyping) to have a complete genetic deletion for that particular gene. For example when staining for p16, c-Myc and p21, control samples from genetically knockout (p16, p21) and c-Myc (floxed) mice were used along side tested samples.

2.7 Epithelial extractions

In order to obtain a population of cells enriched for epithelial cells, an epithelial extraction protocol based on that of Bjerknes & Cheng (Bjerknes and Cheng, 1981) was performed on freshly harvested intestine. Briefly, a 10cm section of small intestine was flushed well with water before being tied off at one end and everted over a 4mm glass rod. Vibration was then applied to the glass rod, and the intestine placed in 10mM EDTA in Hanks' Balanced Salt Solution (HBSS; Gibco) at 37°C for 15min. The intestine was then moved into a clean tube of 10mM EDTA/HBSS and incubated in the same fashion for a further 15min. Centrifugation (2700 x *g*, 4°C, for 15 minutes) yielded a pellet containing predominantly epithelial cells.

2.8 Protein extraction from epithelial extracts

Protein was extracted from intestinal epithelial extracted samples by standard methods using lysis buffer (20mM Tris-Hcl pH8.0, 2mM EDTA [pH8.0], 0.5% [v/v] NP-40) containing protease inhibitors (Complete Mini Protease inhibitor tablets, Roche) and

phosphatase inhibitors (25mM sodium β-glycerophosphate, 100mM sodium fluoride, 20nM Calyculin A, 10mM sodium pyrophosphate).

2.8.1 Determination of protein concentration

For estimation of protein concentration a microscale variant of Bradford's dye of binding method was used (Bradford 1976). 5-10µl of protein sample was added to 1ml of Bradford reagent (BioRad), mixed and allowed to stand for 10 minutes before measurement of A595. In each case absorbance measurements were performed against blanks containing an equal volume of the Bradford reagent.

These absorbance measurements were then correlated with freshly generated calibration curve (0-25µg of bovine serum albumin in 1ml of bovine serum albumin in 1ml Bradford Reagent) to estimate the protein concentration of the unknown sample.

2.8.2 Western Analysis

For Western analysis, proteins were run on a 10% denaturing polyacrylamide gel.

Protein samples were equalised with RIPA buffer so that all samples were 20µg and of equal volume (20µl). They were then boiled for 5 minutes in 4 x Loading Buffer containing β-mercaptoethanol, quenched on ice, centrifuged and loaded onto the gel. Gels were run for 2 hours at 125V in running buffer or until the protein markers (Gibco) had separated.

Gels were then blotted on PDVF membrane (Millipore) in transfer buffer overnight at 15 mA. Prior to transfer PDVF membrane was soaked in methanol (Fisher) for 30 minutes. After transfer, blots were blocked in TBS/0.1% Tween/10% Marvel (TTM) for one hour. Primary antibodies and conditions used to probe blots were rabbit anti-MDM2 (1:1000; R&D systems AF1244) and mouse anti-β-actin (1:5000; Sigma). Appropriate HRP-conjugated secondary anti-rabbit or anti-mouse antibodies were used (Amersham Biosciences).

Blots were then washed in TBS and visualised using ECL plus (Amersham) on ECL film (Amersham). To confirm equal loading after blotting, blots were then stained with Ponso Red (Sigma).

Reagents

15% Loading Gel	10% loading Gel	10% Stacking Gel
3.33ml DDW 11.69ml 30% acrylamide (1:29) 9.37ml 1M Tris HCL pH 8.8 250µl of 10% SDS (Fisher) 72µl of 25% APS (Fisher) 13.2µl of Temed (Sigma)	6.65ml DDW 8.35ml 30% acrylamide (1:29) 9.37ml 1M Tris HCL pH 8.8 250µl of 10% SDS 72µl of 25% APS 13.2µl of Temed	3.57ml DDW 1.70ml 30% acrylamide (1:29) 0.62ml 1M Tris HCL pH6.8 50µl of 10% SDS 33µl of 25% APS 3.6µl of Temed

4x Loading Buffer

200mM Tris HCl pH 6.8

400mm Dithiothreitol (DTT)

8% SDS

0.4% Bromophenol blue

40% Glycerol

10x Running Buffer: For 1 L

30.2g Tris

188g Glycine (Fisher)

Transfer Buffer For 1L:

800ml DDW

200ml methanol

2.9g Tris

14.5g Glycine

2.9 Quantitative PCR

Reverse transcription was performed using the SuperscriptII reverse transcriptase kit (Invitrogen) and Random hexamers (Invitrogen) as per the manufacturer's instructions. qRT-PCR was performed in order to assess the expression of p19ARF from c-Myc deficient intestinal enterocytes, as well as to assess the expression of Cyclin D2 and

CDK4 from both *AhCre*$^{+}$*Apc*$^{fl/fl}$ and *AhCre*$^{+}$*Apc*$^{fl/fl}$ *Myc* $^{fl/fl}$ from intestinal enterocytes. DyNAmo HS (hot-start) SYBR green supermix (Finnzymes, GRI) was added to appropriate cDNA samples and primers. Samples were loaded onto a white one-piece thin-wall 96-well PCR plate (BIOplastics) and the PTC-200 Peltier thermal cycler and Chromo4 continuous fluorescence detector (both MJ Research) were used in conjunction with Opticon Monitor analysis software (Version 2.03, MJ Research) to calibrate and run the reaction.

Primers used for ARF were:

F – CTGGACCAGGTGATGATGA

R – ACCAGCGTGTCCAGGA

Primers used for Cyclin D2:

F- CTACCGACTTCAAGTTTGCC

R- GCTTTGAGACAATCCACATCAG

Primers used for CDK4 :

F- AATGTTGTACGGCTGATGGA

R- AGAAACTGACGCATTAGATCCT

Chapter 3: C-Myc is essential for p53 induced apoptosis in response to DNA damage *in vivo*

3.0 Introduction

One of the best known functions of the c-Myc protein is its ability to drive apoptosis in numerous cellular contexts (Askew et al., 1991, Evan et al., 1992, Hoffman and Liebermann, 2008). Of the studies performed, most have concentrated on the ability of c-Myc overexpression to drive apoptosis, unless accompanied by other mutations such as p53 loss (Hermeking and Eick, 1994). The suppression of apoptosis is thought to be a key factor in driving tumourigenesis *in vivo*, for example overexpression of c-Myc in the pancreatic islets alone does not induce tumourigenesis unless apoptosis is blocked e.g. by p53 loss, BCL-XL overexpression or ARF knockout (Pelengaris et al., 2002, Finch et al., 2009). The studies examining combined c-Myc overexpression and p53 loss have implicated p53 is directly downstream of c-Myc, but whether this is through direct transcriptional control or indirect (e.g. through c-Myc induction of the DNA damage response) is still controversial (Hermeking and Eick, 1994). The most cited model linking c-Myc overexpression to the p53 pathway is via transcription induction of ARF by c-Myc, which in turn inhibits MDM2 (a key negative regulator of p53) (Eischen et al., 1999, Braig et al., 2005). Indeed recent studies overexpressing c-Myc from the Rosa 26 locus showed that c-Myc induced apoptosis only in the colon. This was due to the high expression of the Rosa26 locus and hence overexpression of c-Myc within the colon compared to other tissues led to the induction of ARF and apoptosis. Genetic deletion of ARF rescued this c-Myc induced apoptosis (Murphy et al., 2008b).

Much less well studied is the importance of c-Myc in signalling apoptosis following DNA damage. Thus far no study has examined this *in vivo,* though *in vitro* studies have

suggested it may be of vital importance. There are 3 lines of evidence for this; first (and most importantly) Seoane *et al* (Seoane et al., 2002) have shown that in colorectal cancer cell lines depletion of c-Myc reduces apoptosis as a consequence of altering the balance of downstream effectors of p53 signalling. Thus, in the absence of c-Myc, there are increased levels of the anti-apoptotic cell cycle arrest protein p21 (a target of p53 which is also transcriptional repressed by c-Myc in a complex with Miz), and reduced levels of pro-apoptotic genes such as *bax,* resulting in cell arrest rather than apoptosis. Secondly, numerous transcriptional c-Myc targets (either activated or repressed by C-Myc) such as *bax, gadd45a* and *onzin* have been shown to be crucial for DNA damage signalling *in vitro* (Mitchell et al., 2000, Barsyte-Lovejoy et al., 2004, Rogulski et al., 2005). Thirdly, c-Myc has been shown to augment apoptosis in fibroblasts following gamma irradiation (Maclean et al., 2003).

One of the most tractable systems for studying the DNA damage response *in vivo* is the intestinal crypt. Previously, numerous cytotoxic agents such as cisplatin, ionizing radiation and NMNU have been shown to induce apoptosis with a peak induction normally 6-12 hours following DNA damage (Sansom et al., 2003, Sansom and Clarke, 2000). This early wave of apoptosis is completely dependent on the nuclear accumulation of p53. The tractability of this system in conjunction with the our previous data showing that c-Myc deletion is not immediately deleterious to intestinal enterocytes makes this an ideal system to determine whether c-Myc is important for signalling apoptosis in normal cells following DNA damage (Muncan et al., 2006a). Importantly neither of the two studies which conditionally deleted c-Myc from the normal intestine saw any changes in

the physiological levels of apoptosis, which could have possibly confounded any analysis (Muncan et al., 2006a, Bettess et al., 2005). Both studies showed that c-Myc deficient enterocytes could proliferate, however our study showed that both the level of proliferation and cell size was reduced compared to wild type intestinal enterocytes.

In this study we show that c-Myc is essential for the induction of apoptosis within the intestinal crypt due to the inability of c-Myc deficient cells to upregulate p53. Mechanistically this was associated with high levels of MDM2 in Myc deficient cells and treatment with the MDM2 inhibitor nutlin restored the upregulation of p53 and induced apoptosis.

3.1 Results

3.1.1: c-Myc deficient crypts do not undergo apoptosis following treatment with DNA damaging agents

We first examined whether c-Myc deletion could alter the DNA damage response to ionizing radiation. To induce Cre mediated gene deletion, *AhCre*$^{+}$ *c-Myc*$^{fl/fl}$ mice and control *AhCre*$^{+}$ *c-Myc*$^{+/+}$ mice were given 3 injections ip of 80mg/kg β-napthoflavone within a single day. This protocol leads to near constitutive levels of c-Myc deletion from the intestinal epithelium 4 days following Cre induction (Muncan et al., 2006a) (Figure 3.1 b). At this stage, no Cre recombinase expression can be detected (gene loss remains as the deletion event occurs within the stem cell population) (Ireland et al., 2004). *AhCre*$^{+}$ *c-Myc*$^{fl/fl}$ mice and control *AhCre*$^{+}$ *c-Myc*$^{+/+}$ mice were then exposed to 14Gy of

gamma irradiation and the induction of apoptosis was scored 6 hours following the irradiation.

Analysis of H&E stained sections of the small intestine of wild type mice clearly showed a significant induction of apoptosis following 14Gy gamma irradiation (Figure 3.2a-b), compared to their un-irradiated littermates as has been previously reported. However, the number of apoptotic figures was significantly reduced in c-Myc deficient mice given the same dose of irradiation (Figure 3.2 b, Mann-Whitney U test, $p= 0.001$, $n=6$).

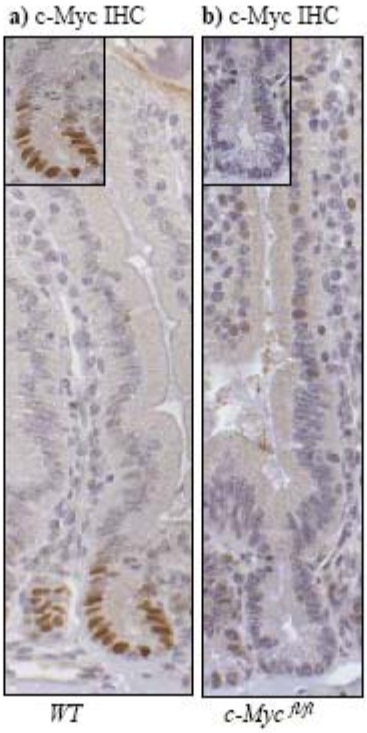

Figure 3.1: Efficient c-Myc deletion 4 days following Cre induction.

c-Myc immunohistochemistry performed on intestinal sections from wild type (*AhCre*$^{+}$ *Myc*$^{+/+}$) **(a)** and c-Myc deficient (*AhCre*$^{+}$ *c-Myc*$^{fl/fl}$) **(b)** mice. Note complete loss of c-Myc protein from the intestinal crypt **(b).**

To confirm the scoring of apoptosis on H&E sections, immunohistochemistry was performed against cleaved ('active') Caspase 3 (Marshman et al., 2001) and once again the number of caspase 3 positive cells was significantly lower in irradiated c-Myc deficient mice when compared to wild type mice given the same dose of gamma irradiation (14Gy) (Figure 3.2 c-d).

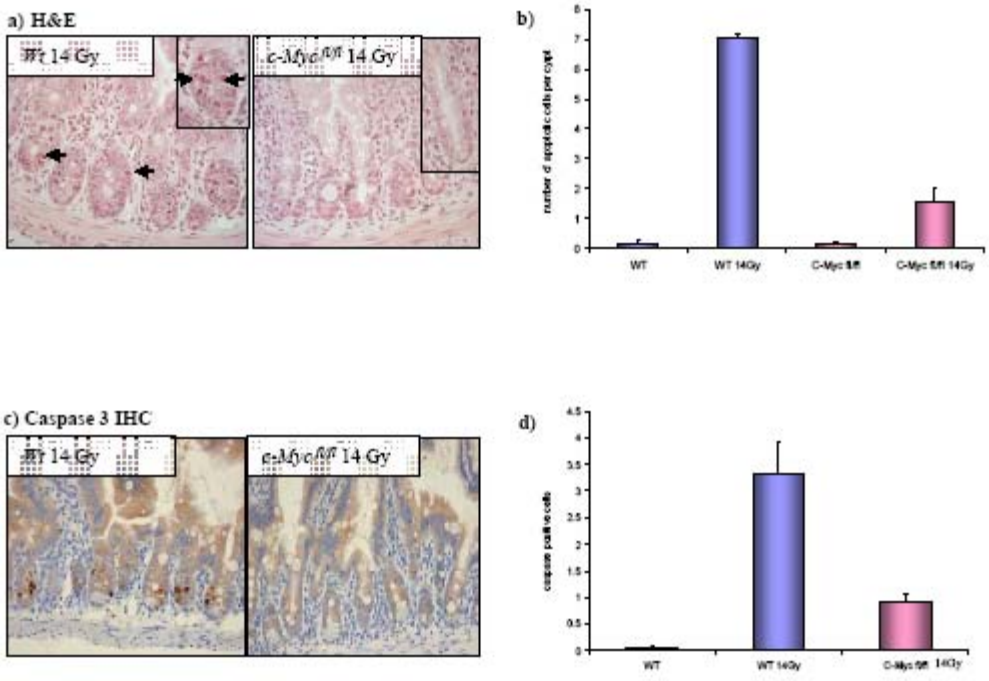

Figure 3.2: c-Myc deficient crypts do not undergo apoptosis following treatment with 14Gy irradiation. **(a)** H&E staining of wild type (*AhCre*$^{+}$ *Myc*$^{+/+}$) and c-Myc deficient (*AhCre*$^{+}$ *c-Myc*$^{fl/fl}$) intestines six hours following 14Gy irradiation, arrows shows apoptotic figures in wild type mice. **(b)** Scoring of apoptotic figures from H&E sections shows a significant decrease in apoptosis in c-Myc deficient mice following 14Gy irradiation compared to wild type (p=0.001, Mann-Whitney U test,n=6). **c)** Immunohistochemical staining for cleaved ('active') Caspase 3 was performed on intestinal sections of wild type and c-Myc deficient mice. **(d)** Quantification of these sections revealed a significant decrease in the number of Caspase-3 positive cells in c-Myc deficient mice following 14Gy irradiation compared to wild type (p=0.0001, Mann-Whitney U test,n=3).

To extend this analysis beyond a single timepoint, we next scored apoptosis at a series of different times following gamma irradiation and found significantly lower levels of apoptosis at all timepoints subsequent to 2 hours in c-Myc deficient intestinal crypts (Figure 3.3 a).

The resistance of apoptosis of c-Myc deficient enterocytes was not restricted to high doses of γ irradiation, as apoptosis following either a lower dose of irradiation (5Gy) or cisplatin treatment was also found to be c-Myc dependent (Figure 3.3 b-c). This failure to undergo apoptosis was not simply because c-Myc deficient cells were not cycling, as we and others have previously shown that c-Myc deficient intestinal enterocytes can undergo proliferation (Muncan et al., 2006a).

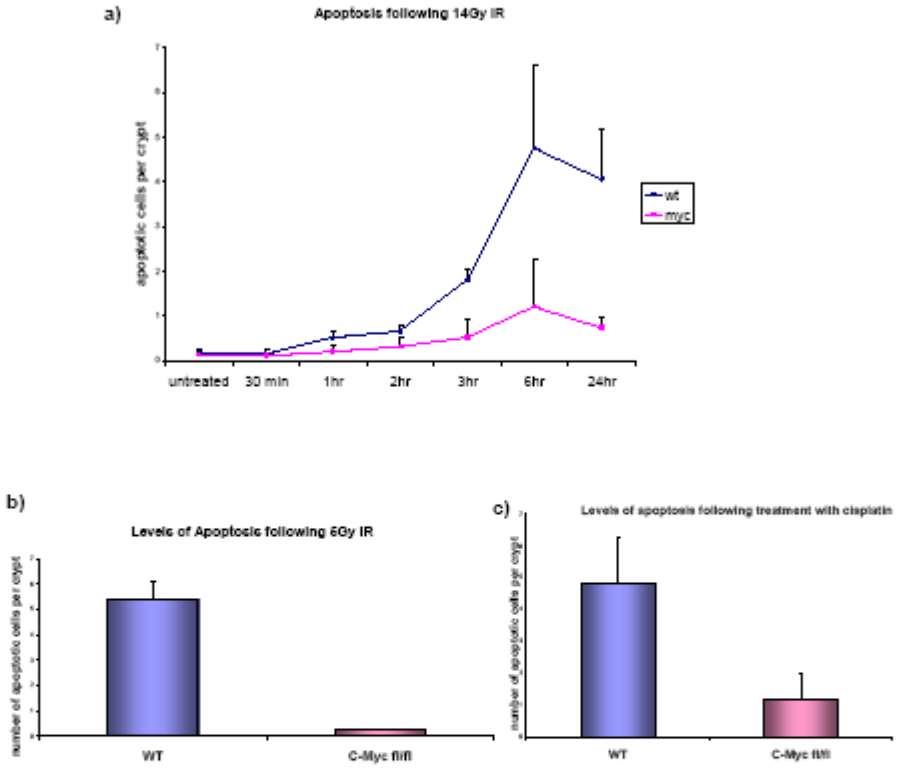

Figure 3.3: c-Myc deficient crypts do not undergo apoptosis following treatment with DNA damaging agents.

(a) Graph showing that c-Myc is essential for the induction of apoptosis following 14Gy irradiation. Each timepoint represents at least 3 mice, illustrating significantly lower levels of apoptosis in c-Myc deficient mice at all timepoints after 2 hours. **(b-c)** Graphs showing c-Myc is essential for apoptosis 6 hours following 10 mg/kg of cisplatin (Mann-Whitney U test, $p < 0.01$, n=3). treatment or lower levels of irradiation (5Gy) (Mann-Whitney U test, $p < 0.05$, n=3).

3.1.2 c-Myc deficient enterocytes do not upregulate p53

The phenotype of c-Myc deficiency paralleled the well established phenotype of p53 deficiency in the intestine, namely a strong suppression of the immediate wave of apoptosis (Sansom and Clarke, 2000, Sansom and Clarke, 2002, Clarke et al., 1994) . Taking this information with previous data suggesting direct links between c-Myc and p53, we investigated the kinetics of p53 induction in the murine small intestine (Hoffman and Liebermann, 2008). Figure 3.4a-c shows that p53 levels rise sharply in intestinal crypts following gamma irradiation, but in c-Myc deficient mice this upregulation does not occur. Therefore this provided a ready mechanism for the abrogated apoptosis.

An alternative mechanism for failed apoptosis is that deletion of c-Myc causes a derepression of p21 which could also block apoptosis in this system (Peukert et al., 1997, Seoane et al., 2002). We and others have previously shown that c-Myc deficiency alone is not sufficient to trigger p21 upregulation in the intestine, however following combined Apc and c-Myc deletion a clear induction of p21 was observed (Muncan et al., 2006a, Sansom et al., 2007, Wilkins and Sansom, 2008). Following irradiation (despite the failed p53 activation) p21 was still upregulated in c-Myc deficient intestinal enterocytes (Figure 3.4d-f). To determine whether the lack of transcriptional repression of p21 by c-Myc was sufficient to block apoptosis we intercrossed p21 knockout mice to mice carrying the *Ah Cre* transgene and loxp flanked *C-Myc* alleles to generate $AhCre^{+}$ $c\text{-}Myc^{fl/fl}$ $p21^{-/-}$ mice. Cre was induced in these mice as described above, and these mice were irradiated four days after Cre induction and apoptosis was scored 6 hours following 14Gy irradiation. As expected, p21 was no longer upregulated in the intestinal crypts of these mice following

irradiation and importantly they displayed the same lack of apoptosis in response to irradiation as the single c-Myc deficient intestinal crypts (Figure 3.4g). Therefore this demonstrates that the induction of p21 was not responsible for the block of apoptosis and that the failed p53 upregulation was the most likely cause of our phenotype.

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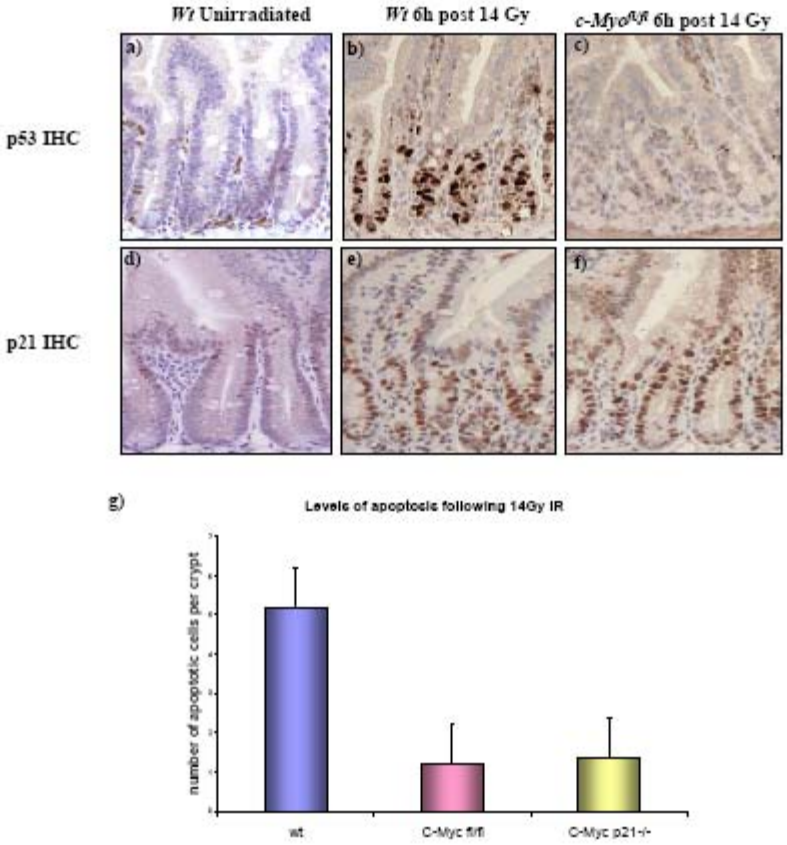


Figure 3.4: c-Myc deletion prevents p53 accumulation after DNA damage. p53 IHC showing p53 expression in intestinal crypts in unirradiated **(a)** and after 6 hours following 14Gy irradiation **(b)** in wild type *(AhCre^{+} c-Myc$^{+/+}$)* mice. Note the large induction of nuclear p53 following irradiation in these mice **(b)**. **(c)** p53 IHC showing lack of p53 expression in intestinal crypts of c-Myc deficient *(AhCre^{+} c-Myc$^{fl/fl}$)* mice 6 hours post 14Gy irradiation. **d-e)** p21 IHC showing p21 expression in intestinal crypts in unirradiated **(d)** and after 6 hours following 14Gy irradiation **(e)** in wild type mice. **(f)** p21 IHC showing p21 expression in intestinal crypts of c-Myc deficient mice 6 hours post 14Gy irradiation. Note the large induction of nuclear p21 following irradiation in a p53 independent manner. **(g)** Graph showing *AhCre^{+} Myc$^{fl/fl}$ p21$^{-/-}$* mice display the same lack of apoptotic response to 14Gy irradiation as the c-Myc deficient mice, illustrating that the induction of p21 in these mice is not responsible for the failure to upregulate p53 and induce apoptosis.

3.1.3 c-Myc deficient enterocytes sense the DNA Damage Stimuli

From the literature there are numerous potential mechanisms which could explain the failure to see increased levels of p53 protein in c-Myc deficient enterocytes. These include: failure to detect DNA damage, reduced p53 protein stability, reduced transcription or translation of p53 mRNA (Hoffman and Liebermann, 2008). Therefore we decided to test a number of these potential mechanisms.

First, we investigated if DNA damage recognition was functioning in the c-Myc deficient intestinal enterocytes, and examined whether there was efficient activation of the DNA damage sensing proteins γH2AX and ATM. This is particularly important in terms of ATM as it has previously been suggested that ATM is required for c-Myc to activate p53 (Pusapati et al., 2006). γH2AX becomes phosphorylated by ATM at the sites of double strand breaks in DNA, and is essential for their recognition and repair (Paull et al., 2000). We performed immunohistochemistry using an antibody which specifically recognises the activated, phosphorylated form of γH2AX to determine if this mechanism of DNA damage response was still intact in c-Myc deficient mice. In wild type mice, the level of P-γH2AX is dramatically increased 30 minutes after irradiation and this level decreases 6 hours later as DNA damage is repaired. This activation of γH2AX following either γ-irradiation or cisplatin was also observed in c-Myc deficient intestinal enterocytes (Figure 3.5 a-h).

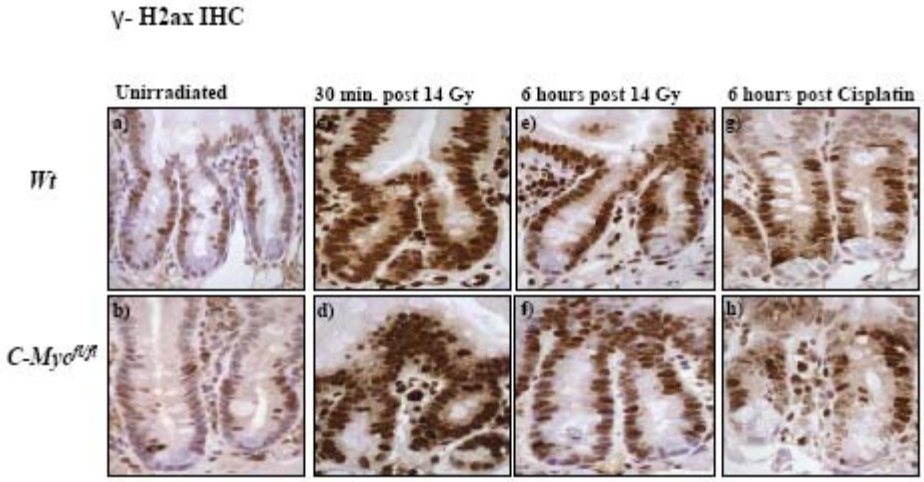
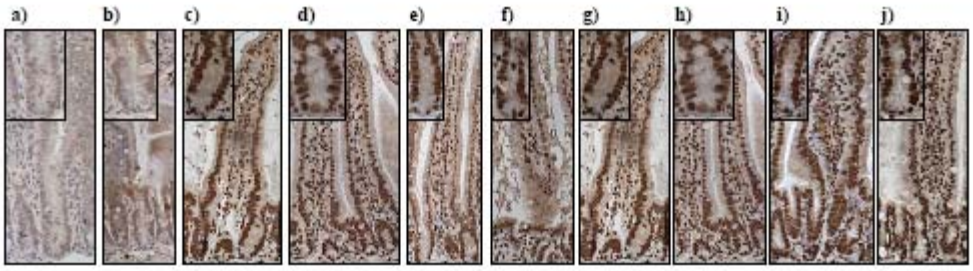


Figure 3.5: c-Myc deficient enterocytes are able to sense DNA damage

a-b) Immunohistochemisty for γ-H2ax showing low levels in the intestinal crypts of unirradiated wild type and c-Myc deficient mice. **c-h)** Immunohistochemisty for γ-H2ax showing a large upregulation in both wild type and c-Myc deficient mice 30 minutes after 14 Gy irradiation, illustrating that c-Myc deficient enterocytes are able to sense DNA damage stimuli.

We also used an antibody specific to the activated, phosphorylated form of ATM. ATM is a regulator of cellular response to DNA damage, and is autophosphorylated and associates with other proteins such as p53, MDM2 and Chk2 to arrest cell cycle at G1. In common with the γH2AX results, p-ATM and Chk1 are still activated in response to DNA damage in wild type and c-Myc deficient mice (Figure 3.6a-t). Taken together these results demonstrate that the DNA damage response is still intact in c-Myc deficient enterocytes, and suggests that the mechanism behind the failure to induce apoptosis in response to DNA damage is through the control of p53 levels (either at the RNA or protein level).

P-ATM IHC



CHK-1 IHC

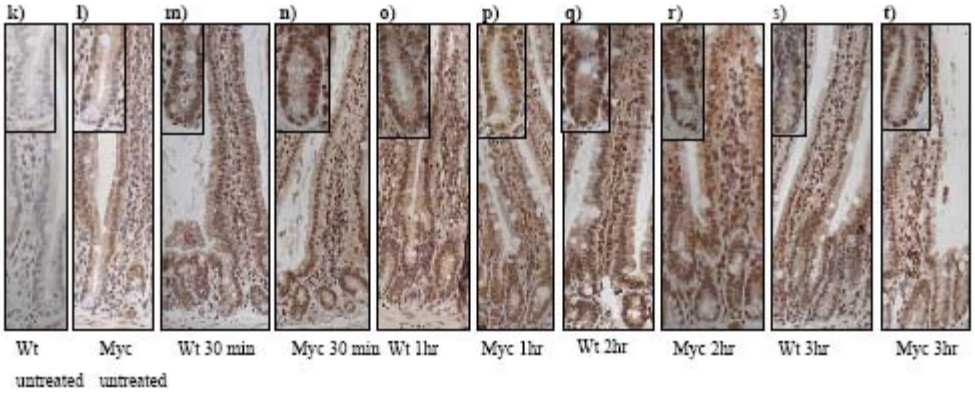


Figure 3.6: p-ATM and Chk-1 is still activated in response to DNA damage in wild type and c-Myc deficient mice. a-b) IHC for p-ATM showing low levels of expression in the intestinal crypts of wild type *(AhCre^{+} c-Myc$^{+/+}$)*and c-Myc deficient *(AhCre^{+} C-Myc$^{fl/fl}$)* mice. **c-j)** IHC for p-ATM showing equal levels of upregulation in wild type and c-Myc deficient mice, 1- 3 hours following 14Gy irradiation. **k-l)** IHC for Chk-1 showing low levels of expression in the intestinal crypts of wild type and c-Myc deficient mice. **m-t)** IHC for Chk-1 showing equal levels of upregulation in wild type and c-Myc deficient mice, 1- 3 hours following 14Gy irradiation.

3.1.4 MDM2 upregulation and P19ARF and nucleolin downregulation in c-Myc deficient enterocytes.

Given the number of studies in the literature that link Myc to control of p53 stability, we next investigated the ARF-MDM2-P53 pathway (Eischen et al., 1999, Braig et al., 2005). One of the key regulators of p53 protein stability is the MDM2 E3 ubiquitin ligase. Loss of MDM2 *in vivo* leads to embryonic death due to the activation of high levels of p53 which can be rescued by co-deletion of p53 (Montes de Oca Luna et al., 1995, Jones et al., 1995). First we examined the levels of MDM2 following c-Myc deletion by immunoblotting and immunohistochemistry and found a marked upregulation of MDM2 in c-Myc deficient cells (Figure 3.7 a-e).

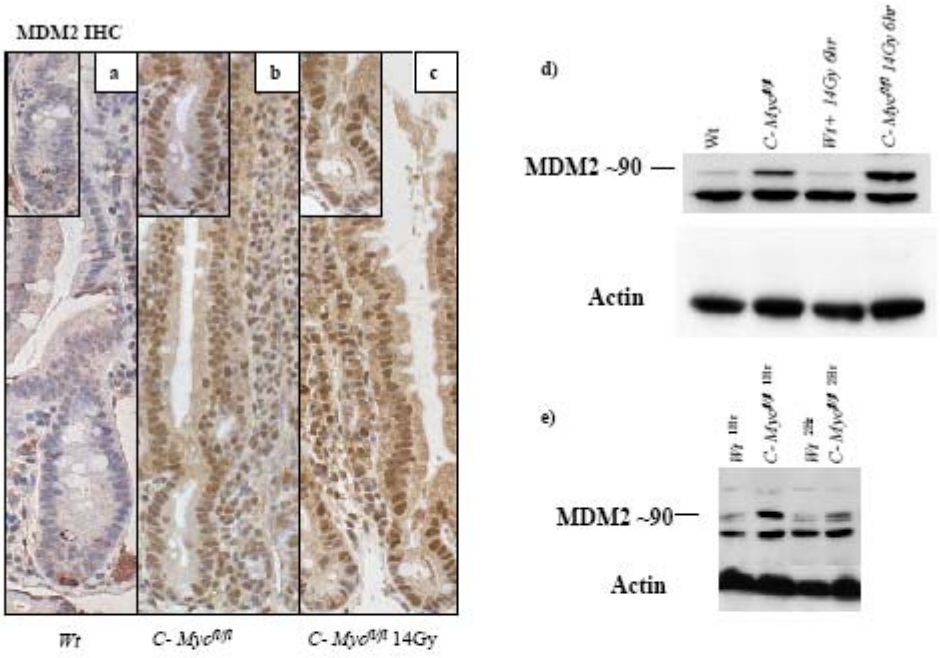


Figure 3.7: c-Myc deletion causes an accumulation of MDM2. **(a)** MDM2 IHC showing no expression in intestinal crypts of wild type *(AhCre^{+} c-Myc$^{+/+}$)* mice. **b-c)** MDM2 IHC showing a large increase of MDM2 expression in c-Myc deficient (*AhCre^{+}c-Myc$^{fl/fl}$*) mice in both unirradiated and 14Gy irradiated mice.**(d)** Immunoblotting for MDM2 shows a marked increase in MDM2 protein levels in both unirradiated and 14Gy irradiated mice c-Myc deficient intestines. **(e)** Western blot for MDM2 shows an increase of MDM2 protein levels at both 1 and 2 hours following 14Gy irradiation in c-Myc deficient mice.

To test if this was functionally important for blocking apoptosis in the c-Myc deficient mice we employed the MDM2 antagonist, Nutlin-3a. Nutlin is a selective small-molecule inhibitor of the p53-MDM2 interaction that releases p53 from MDM2 control, leading to accumulation of the tumour suppressor protein and activation of the p53 pathway (Tovar et al., 2006, Vassilev, 2007). Treatment of cancer cells with wild type p53 induces cell arrest and apoptosis *in vitro* and suppressed the growth of human tumour xenografts in nude mice (Vassilev et al., 2004, Tovar et al., 2006).

c-Myc deficient mice were treated with Nutlin twice daily on days 1-3 post Cre induction and a final time 3 hours prior to 14Gy irradiation on day 4. This methodology was employed as it has been shown previously that Nutlin can knockdown MDM2 for approximately 12 hours *in vivo* and a number of doses are required for full functional inhibition (Tovar et al., 2006). Apoptosis was scored in wild type and c-Myc deficient mice 6 hours following irradiation and a restoration of the apoptotic response was observed in c-Myc deficient mice treated with Nutlin though not vehicle (Figure 3.8a). Most importantly, this restoration of the apoptotic response correlated with the induction of p53 in the c-Myc deficient mice treated with Nutlin (Figure 3.8d). Consistent with previous reports, Nutlin treatment had no obvious impact on proliferation or apoptosis of wild type intestinal enterocytes (either alone or treated with 14Gy) (Vassilev et al., 2004). Unirradiated c-Myc deficient intestines showed increased levels of p21 and small but significant decrease in proliferation though no changes in apoptosis. Consistent with other reports, no gross changes in p53 levels could be observed which could reflect either transient low level p53 activation or that p53 levels were below our threshold of detection.

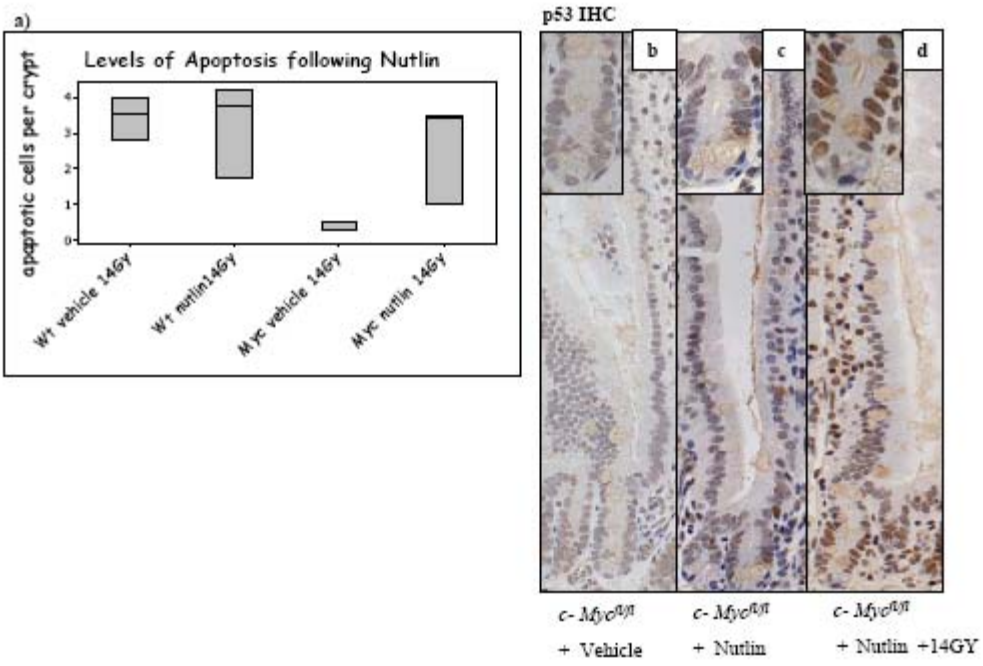


Figure 3.8:Treatment of c-Myc deficient mice with Nutlin restores apoptosis and p53 response

(a) c-Myc deficient mice treated with Nutlin exhibited a full restoration of the response (p= 0.04, Mann-Whitney U test, n=3), which was not observed in c-Myc deficient mice treated with vehicle. Note this restoration of apoptosis correlates with an induction of p53 in c-Myc deficient mice treated with Nutlin **(d)**. **b-d)** Immunohistochemistry for p53 shows that p53 now accumulates in Nutlin treated c-Myc deficient intestines following gamma irradiation (d) but not vehicle treated (b) and non-irradiated c-Myc deficient intestines **(c)**.

A number of potential pathways affecting MDM2 stability could be affected by c-Myc deletion. Recent studies have implicated the c-Myc transcriptional target gene nucleolin in regulation of p53 stability in two opposing manners. Saxena *et al* (2006) (Saxena et al., 2006) showed that nucleolin inhibits MDM2 by multiple mechanisms including direct binding and reduction of protein levels, and thus is a positive regulator of p53 stability. Contrastingly, Takagi et al (2005) (Takagi et al., 2005) showed that nucleolin can bind to the 5' UTR of p53 causing degradation of the p53 message. To determine if nucleolin was a c-Myc target in the intestine we performed immunhistochemistry, which clearly shows that nucleolin levels were much lower in intestines of c-Myc deficient mice (Figure 3.9a-d). Thus this could be responsible for the altered levels of MDM2 seen in c-Myc deficient intestines. Moreover, we have also previously shown that nucleophosmin levels are also downregulated following c-Myc deletion (Muncan et al., 2006a) and nucleophosmin has also been shown to regulate p53 levels (both positively and negatively) (Colombo et al., 2002).

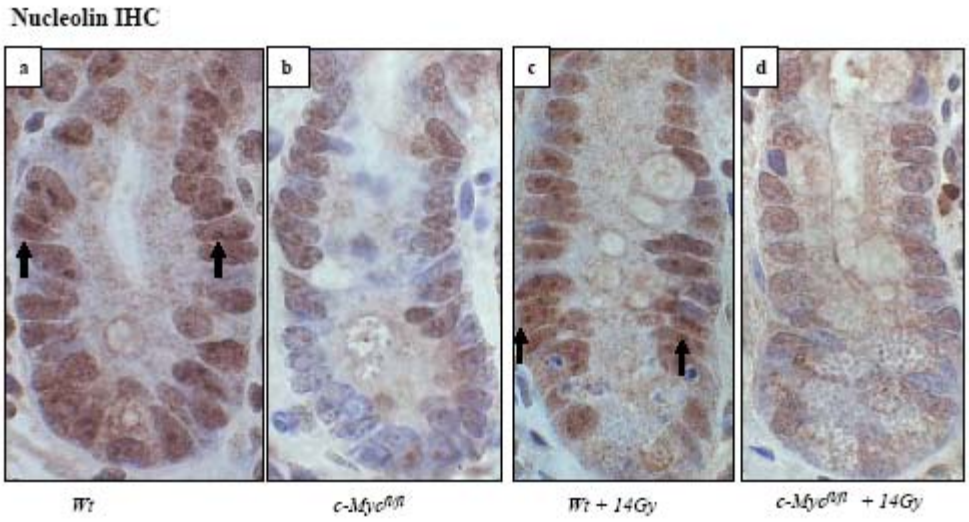


Figure 3.9 : c-Myc deficient enterocytes are unable to upregulate nucleolin

(a-d) Nucleolin IHC showing lack of the c-Myc target gene nucleolin in c-Myc deficient mice compared to wild type, in both unirradiated as well as 14Gy irradiated mice.

Additionally, given the recent studies linking Myc to ARF expression in the intestinal epithelium (Murphy et al., 2008b) we also investigated whether levels of ARF were changed following Myc deletion in the intestine. Immunohistochemistry for p19ARF showed a reduction in p19ARF levels following 14Gy irradiation in c-Myc deficient mice (Figure 3.10a-b). Similarly, qRT-PCR showed that following c-Myc deletion there was a clear downregulation of ARF in response to irradiation (Figure 3.10c). Therefore this data also suggest that the failure to upregulate ARF in Myc deficient intestinal enterocytes following gamma irradiation could also be contributing to the failure of the intestinal enterocytes to undergo apoptosis.

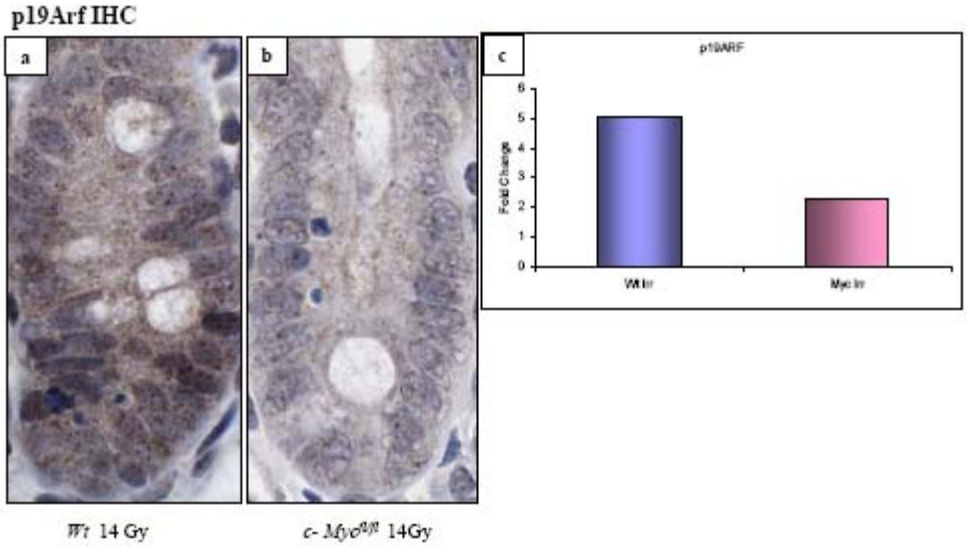


Figure 3.10 : Following 14Gy irradiation, c-Myc deficient enterocytes are unable to upregulate p19ARF

(a) p19ARF IHC showing reduced expression in the intestinal crypts of c-Myc deficient *(AhCre^{+} c-Myc$^{fl/fl}$)* mice. **(c)** Similarly, qRT PCR shows significant reduction in p19Arf levels in c-Myc deficient mice 6 hours following 14Gy irradiation.

3.2 Discussion:

In this study we have examined the role of c-Myc induced apoptosis following DNA damage *in vivo.* To do this we utilized Cre Loxp technology to conditionally delete c-Myc from the murine intestinal epithelium (Muncan et al., 2006b). Following exposure to DNA damage, *c-Myc*$^{fl/fl}$ mice displayed a dramatic decrease in apoptotic levels, which was due to an inability to upregulate p53. In this study we confirm that following DNA damage, *c-Myc*$^{fl/fl}$ mice are unable to undergo apoptosis due to a strong upregulation of MDM2, as well as an inability to regulate ARF, resulting in the ablation of p53 levels. This was mechanistically confirmed by treatment with the MDM2 antagonist Nutlin3a, which restored apoptosis and p53 levels following DNA damage in *c-Myc*$^{fl/fl}$ mice.

It is tempting to speculate that the levels of c-Myc allow cells to be permissive for apoptosis *in vivo*. For example, differentiated villus enterocytes which do not express c-Myc do not induce p53 or undergo DNA damage induced apoptosis following gamma irradiation (despite showing the activation of the γH2AX). Likewise epithelial tissues that do not undergo DNA damage induced apoptosis such as the liver or the pancreas also fail to upregulate p53 and have very low levels of c-Myc. *In vivo* studies have shown that it is the threshold of Myc expression that determines its biological output. For example, studies by *Murphy et al.* have used the Rosa26 promoter to drive low-level deregulated expression of the switchable form of Myc, Myc^{ERT2}, in target tissues. Results showed that acute activation of Myc lead to an induction of ectopic proliferation in many tissues, including endocrine and exocrine pancreas, liver, kidney epithelium, lung, skin, and lymphoid organs (Murphy et al., 2008a). However, within the colon, higher levels of Myc expression resulted in activation of the ARF/p53 pathways, and induced apoptosis. This

phenotype was rescued by ARF deletion. *In vivo* studies from Finch et al. used the switchable form of Myc, MycER^{tam} under control of the villin promoter (a regulatory element active in all epithelial lineages) to drive high levels of deregulated Myc expression within the intestinal epithelium. In contrast to the previous study, induction of Myc within these mice lead to acute activation of the ARF/p53 pathway and a significant increase in apoptosis. Taken together, these two studies show that an important threshold of Myc expression exists whereby low levels of Myc induce proliferation, whilst higher levels of Myc expression need to be obtained in order to induce an apoptotic program, such as activation of the ARF/p53 pathway.

In summary we have shown for the first time in an *in vivo* setting that endogenous c-Myc is absolutely required for induction of p53 dependent apoptosis through an MDM2 dependent pathway.

Chapter 4: p21 loss blocks senescence following Apc loss and provokes tumourigenesis in the renal but not the intestinal epithelium

4.0 Introduction

The Apc tumour suppressor is mutated in approximately 80% of colorectal cancers (CRC) where it is thought to be the key initiating event (Kinzler and Vogelstein, 1996). The major tumour suppressor function of Apc gene is thought to be as a negative regulator of Wnt Signalling.

Outwith colorectal cancer, mutations in the Apc gene are much more rare, however Wnt pathway activation is observed in cancers such asHepatocellular Carcinoma (HCC) through β-catenin activating mutations or loss of negative regulators such as Axin or Axin2 (Satoh et al., 2000, Giles et al., 2003). Similarly activating or stabilizing mutations in β-catenin have been observed in several cancers including melanoma, ovarian carcinomas, childhood hepatoblastomas and medulloblastomas, desmoid tumours as well as non-ductal solid pancreatic tumours (Giles et al., 2003). However in these cancers activating mutations of the Wnt pathways are not thought to be the intitating event. Recently a role for Wnt signalling in renal carcinoma has been proposed as the key renal tumour suppressor protein (Von Hippel-Lindau) VHL, acts through JADE, an E3 ubiquitin ligase to target β-catenin for degradation (Zhou et al., 2005). Therefore, a mutation in VHL results in the stabilization and activation of the oncogenic β-catenin pathway (Behrens, 2008). This link between these 2 pathways helps to explain why FAP (Familial Adenomatous Polyposis) patients, with a germline mutation of Apc rarely present with renal cell carcinoma, but mice with a renal specific deletion of Apc develop cancer similar to those with VHL mutations (Sansom et al., 2005a). Moreover the

promoter of the Apc gene is hypermethylated in up to 30% of renal carcinomas (Dulaimi et al., 2004), suggesting Apc loss/reduction may play an important role in the progression of renal carcinoma. Proof of principle experiments in the mouse using tissue specific deletion of Apc have suggested that Apc loss can predispose to renal carcinoma but Apc gene loss alone is a very poor initiating event (Sansom et al., 2005a). Indeed using the *Ah Cre* transgene which yields sporadic constitutive Cre expression within all cell lineages of the kidney, only 1/3 of mice develop renal carcinoma, despite showing the presence of small premalignant lesions from 2 months of age (Sansom et al., 2005a). In contrast deletion of Apc within the intestinal epithelium rapidly leads to a marked 'crypt-progenitor cell-like' phenotype (Sansom et al., 2004) and moreover, deletion of Apc within the $LGR5^{+}$ stem cell zones leads to adenoma formation in as little as 2 weeks (Barker et al., 2009).

Over the past few years there has been a great interest in the role of senescence as a *bona fide* tumour suppression mechanism *in vivo*. Of particular note is oncogene induced senescence, where activation of oncogene leads to senescence often in association with a DNA damage response and the expression of β-galactosidase, hence Senescence Associated β-galactosidase (SAβgal) (Collado et al., 2005) (Collado and Serrano, 2006). The evidence to support this has come from both humans and mice (Collado et al., 2005, Chen et al., 2005). Staining of pre-malignant lesions such as benign nevi, adenomas of the lung and PANINs have shown the expression of SAβgal and cell cycle arrest proteins such as p16 and p21, which are key components of senescence *in vitro*. Conditional activation of oncogenes such as BRAF or KRAS in mice has led to the formation of

premalignant lesions which have been associated with markers of senescence that either fail to progress or rarely progress (Dhomen et al., 2009), (Dankort et al., 2007). However controversy remains in the literature over whether these premalignant lesion really exhibit a permenant irreversible growth arrest and indeed other studies have shown proliferation in equivalent premalignant lesions of the lung (Tan et al., 2001). This raises the question of whether these lesions are truely senescent or more comparable to a reversible growth arrest.

There is very little evidence linking activated Wnt signalling to driving a senescence programme *in vivo*. This is despite the fact that Apc loss drives adenoma formation, and tumours from patients are often thought to progress over years rather than months. Most tissue culture and *in vivo* studies have instead shown that Wnt signalling is either required for or cooperates with other mutations to overcome senescence (Delmas et al., 2007). However in lymphoid cells β-catenin activation has been shown to drive senescence *ex vivo*, suggesting that the Wnt pathway has the capacity to cause senescence albeit not in a physiological setting (Xu et al., 2008).

In this study, we show that Apc loss drives a context dependent senescence response. Within the kidney, Apc loss triggers a p21 dependent senescence programme, abrogation of which rampantly drives renal carcinoma. Within the intestine, Apc loss drives a p21 independent proliferative response hence leading to rapid tumourigenesis.

4.1 Results

4.1.1 Apc loss leads to senescence in the renal epithelium

Our previous studies investigating Apc loss in the renal epithelium using the AHCRE to drive constitutive Cre expression within the kidney have suggested that Apc loss is a very poor tumour initiating event (Sansom et al., 2005a). To investigate the mechanism underpinning this, we investigated the long term fate of Apc deficient cells within the kidney . To do this we intercrossed AHCRE transgenic mice to mice carrying conditionally inactivatable 'floxed' Apc580s alleles (*AhCre* $Apc^{fl/fl}$) and the Z/EG GFP Cre reporter transgene. Unlike *AhCre* $Apc^{+/+}$ Z/EG^{+} (Novak et al., 2000) mice, which showed GFP expression throughout the kidney both on wholemount examination and through immunohistochemical (IHC) staining for GFP, *AhCre* $Apc^{fl/fl}$ Z/EG^{+} showed very low or no GFP expression via wholemount examination (Figure 4.1 a) and only a small number of cells that expressed GFP by IHC staining on sections (data not shown). This small number of GFP positive cells correlated to the small number of cells which showed nuclear β-catenin (as a marker of Apc loss) and the very low levels present of the Apc recombined allele in the kidneys of *AhCre* $Apc^{fl/fl}$ Z/EG^{+} mice (Figure 4.1b). This data suggested that most of the cells where Apc was being deleted were being lost from the kidney. Histological examination for apoptotic cells and IHC staining for cleaved caspase 3 failed to reveal any evidence for apoptosis, suggesting cells were not being cleared by apoptosis (Data not shown).

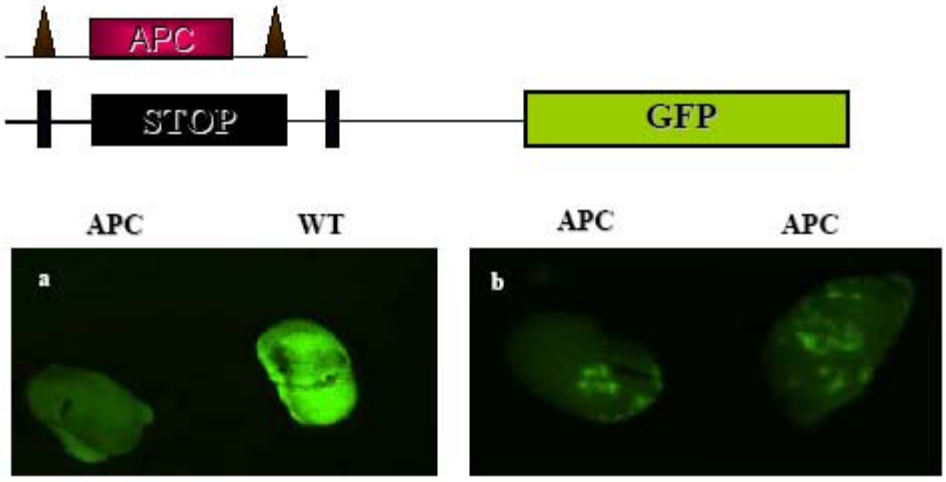


Figure 4.1: Apc deletion within the renal epithelium leads to rapid deletion of recombined cells.
a-b) cartoon showing GFP activation following deletion of the stop codon (and *Apc*) upon Cre mediated deletion. Note absence of green recombined cells in Apc (Cre^{+} $Apc^{fl/fl}$) **(b)** kidney compared to wild type ($WT/\ Cre^{+}\ Apc^{+/+}$) **(a)**, suggesting that Apc deficient cells are being deleted.

The cells that remained following Apc loss fell into two classifications either single cells that were scattered throughout the kidney and those that had formed small premalignant lesions. Given these lesions rarely progress to cancer we next sought to investigate if Apc loss was inducing senescence within these lesions. Currently a number of OIS (oncogene induced senescent) markers have been proposed (Collado and Serrano, 2006), though these are thought to have tissue specific expression so a definite set of markers that work robustly in all tissues are still lacking. Therefore we tested a number of these markers and found upregulation of p21, p16 and Saβgal in *AhCre Apc*$^{fl/fl}$ kidneys. p21 (Figure 4.2 d-f) and p16 (Figure 4.2 g-i) showed upregulation in occasional normal looking renal epithelium nuclei (but only ever in *AhCre Apc*$^{fl/fl}$, which had nuclear β-catenin in coincident section) (Figure 4.2 a-c) and in small premalignant lesions. Saβgal was also present in the pre-malignant renal lesions (Figure 4.2 j). Importantly the rare tumours that formed lost the expression of Saβgal (Figure 4.2 k) and p21/p16 (data not shown).

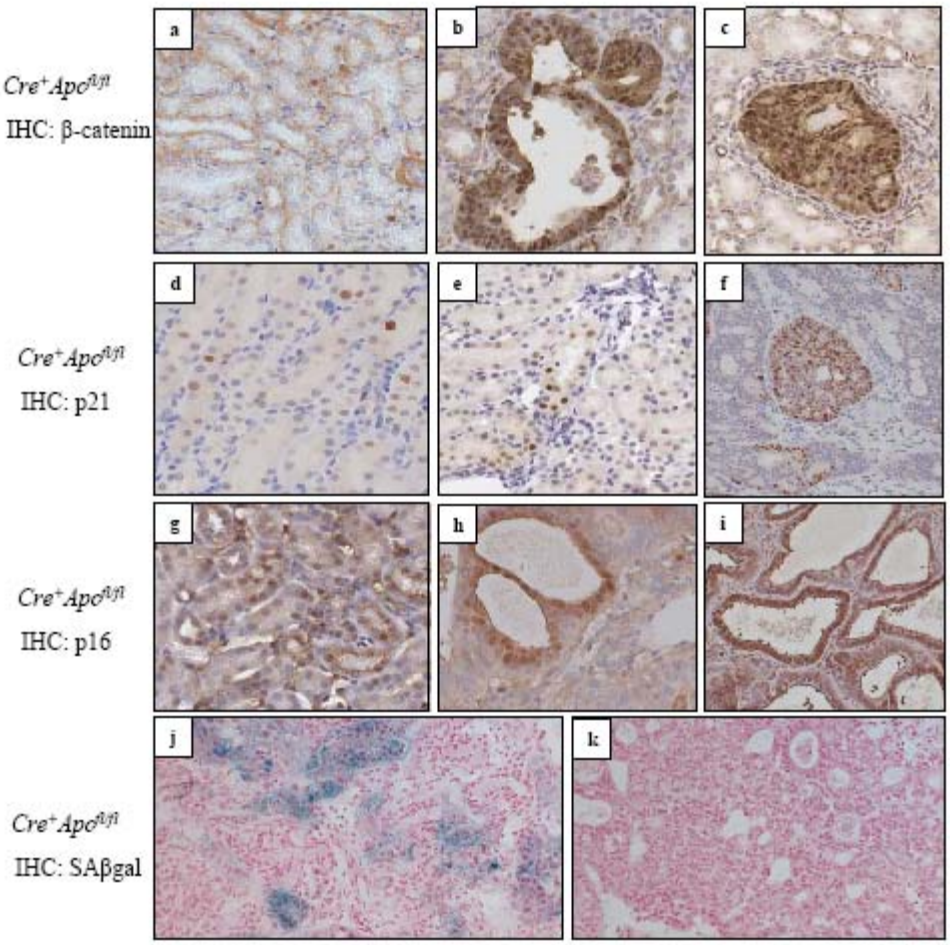


Figure 4.2: Deletion of Apc within the renal epithelium leads to an upregulation of senescent markers

a) β- catenin IHC showing only a small subset of Apc (*Cre*$^{+}$ *Apc* $^{fl/fl}$) deficient cells are retained. **b-c)** β- catenin IHC showing continued expression in small renal lesions. **d-f)** IHC showing that the same subset of cells expressing β-catenin (Apc deficient cells) express senescent markers p21 and p16 (**g-i**). **j-k)** SA-βgal IHC showing positivity in small Apc deficient lesions (**k**), whilst no SA-βgal positivity in large adenoma (**l**).

Given the lack of markers that are exclusive to senescence the most definitive read out should be a strong reduction/total ablation of proliferation within the premalignant lesions. To test this we performed IHC staining for 2 independent markers of proliferation, first the cell cycle antigen Ki-67, second the replication licensing factor MCM2. Staining of premalignant lesions show little if any expression of these proliferative markers, with staining being restricted to surrounding stromal or immune cells (Figure 4.3 c,f). Figure 4.4 shows back to back staining in a cluster of premalignant cells, showing a coinciding upregulation for β-catenin (Figure 4.4 a) and p21 (Figure 4.4 b), however a marked absence of proliferative markers Ki-67 (Figure 4.4 c) and MCM2 (Figure 4.4 d). Importantly, a strong increase in proliferation can now be seen in the few Apc deficient premalignant lesions that grow out to become larger renal carcinomas (Figure 4.4 e). Taken together this data strongly suggests that Wnt pathway activation is leading to senescence within the renal epithelium.

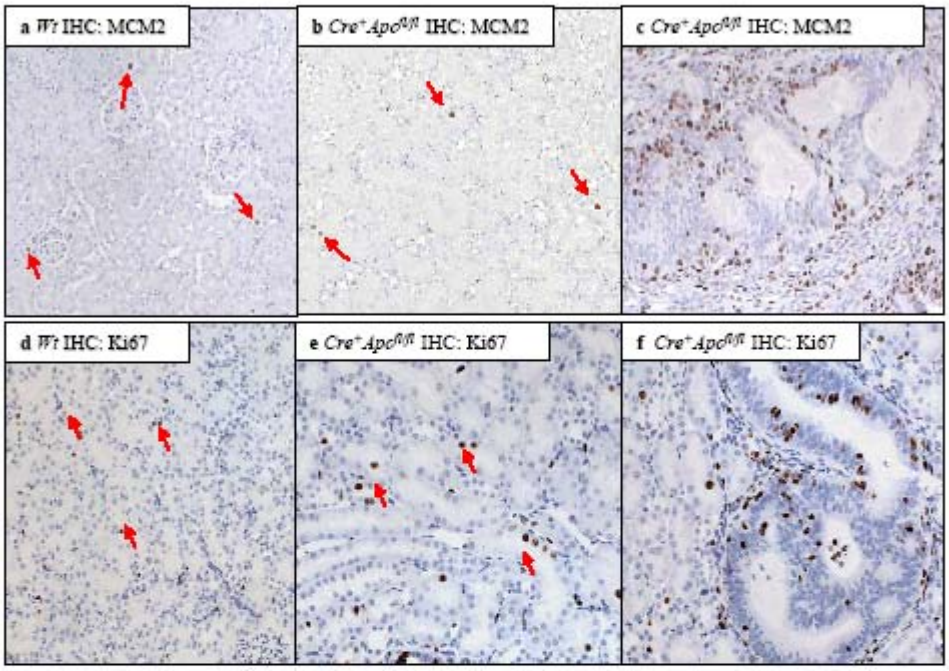


Figure 4.3: Apc deficient lesions are not proliferative.

a-b) MCM2 IHC showing only a small subset of cell expressing MCM2 in both wild type (*Wt*) **(a)** and Apc deficient (*Cre*$^{+}$ *Apc* $^{fl/fl}$) **(b)** mice. **c)** MCM2 IHC showing low levels of proliferation in small Apc deficient renal lesions. **d-f)** Ki-67 IHC showing only a small subset of cell expressing Ki-67 in both Wt **(d)** and Apc deficient **(e)** mice. **f)** Ki-67 showing low levels of proliferation in small Apc deficient renal lesions.

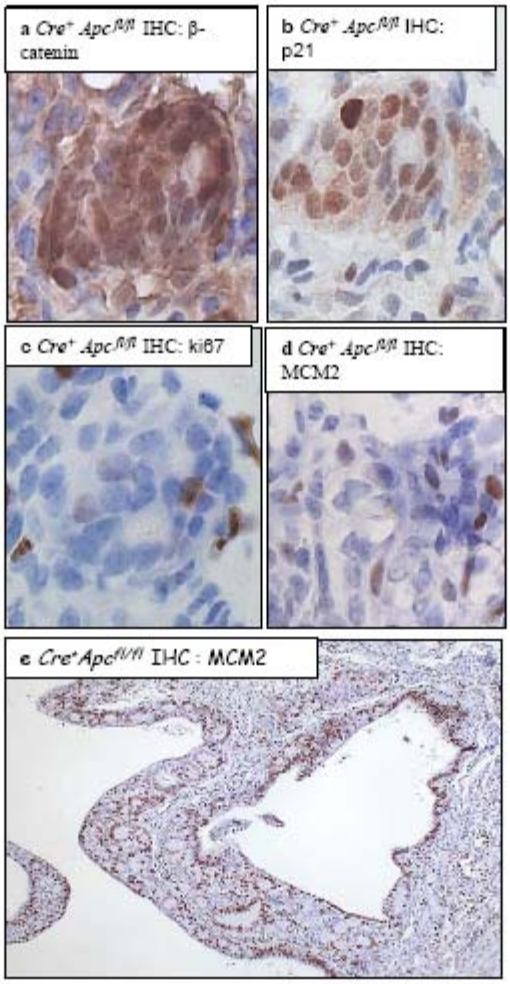


Figure 4.4 Renal carcinomas that grow out from Apc deficient cells become highly proliferative: a-b) IHC showing high levels of β-catenin **(a)** and p21**(b)** in small Apc (*Cre*$^{+}$ *Apc* $^{fl/fl}$) deficient renal lesions.**c-d)** However these lesions not proliferative as seen through a lack of expression of Ki67 **(c)** and MCM2 **(d)**. **(e)** IHC for MCM2 showing that renal carcinomas that grow out from Apc deficient mice are highly proliferative.

4.1.2 p21 loss robustly cooperates with Apc deletion to cause renal carcinoma

To test the functional significance of the upregulation of OIS markers, we intercrossed *AhCre Apc$^{fl/fl}$* mice to p21 knockout mice and aged until mice developed renal carcinoma. Previous studies looking at the effect of p21 deletion have shown that p21 knockout mice have a weak predisposition to tumourigenesis, with an average onset of tumour development of 16 months, with the majority of tumours consisting of sarcomas, lymphomas and tumours of the vascular and endothelial origins (Martin-Caballero et al., 2001). Similar studies have gone on to show that p21 null mice are more susceptible to irradiation induced tumourigenesis (Jackson et al., 2003), and that p21 deletion increases tumour formation in Apc 1638 $^{+/-}$ mice when placed on a western diet of high fat and phosphates and low calcium and vitamin D (Yang et al., 2001a). Previous studies have also shown that in an Apc 1638$^{+/-}$ model of intestinal tumourigenesis, p21 was essential for the mitotic arrest as well as the inhibition of Apc-initiated tumour formation by sulindac (Yang et al., 2001b). In all of the three studies mentioned above, loss of one copy of p21 was sufficient to accelerate tumourigenesis or inhibit sulindac efficacy (Jackson et al., 2003) (Yang et al., 2001a) (Yang et al., 2001b). However this may due to the fact that p21 knockout mice have not been intercrossed to models where p21 is upregulated in premalignant lesions. Remarkably within the renal epithelium the impact of p21 was marked. All *AhCre Apc$^{fl/fl}$ p21$^{-/-}$* mice rapidly developed signs of renal tumourigenesis (hunching, swelling, anaemia due to blood in the urine) and all had to be sacrificed by 100 days of age (median death 63 days Figure 4.5 a). Histological examination of these kidneys from these mice show the development of renal carcinoma in all mice (often multiple foci) (Figure 4.5 b). No control *AhCre Apc$^{fl/fl}$* mice showed

signs of renal tumourigenesis until 180 days, and indeed by 1 year of age only 8% (2 out of 24 mice) had developed renal tumours. Renal carcinomas from *AhCre Apc$^{fl/fl}$ p21$^{-/-}$* mice did not express Saβgal (Figure 4.5 c), p16 (Figure 4.5 d) or as expected p21 (Figure 4.5e), showing that p21 loss is sufficient to overcome the senescence block induced by Apc loss. Importantly, these carcinomas continued to express high levels of β-catenin (Figure 4.5f), showing the retention of Apc deficient cells. Immunohistochemistry for Ki-67 (Figure 4.5g) and MCM2 (Figure 4.5h) show strong upregulation of both of these proliferation markers, illustrating that deletion of p21 now renders these carcinomas highly proliferative. Given the rampant acceleration of tumourigenesis by p21 nullizygosity we also assessed whether haploinsufficiency for p21 could accelerate tumourigenesis. Remarkably haploinsufficiency for p21, resulted in a rapid acceleration of tumourigenesis in *AhCre Apc$^{fl/fl}$ p21$^{+/-}$* compared to *AhCre Apc$^{fl/fl}$ p21$^{+/+}$* (median age of death 109 days Figure 4.5a). To further confirm that escape from a senescene like program was occuring we also aged a small cohort of *AhCre Apc$^{fl/fl}$ INK4A$^{-/-}$* mice. Once again all of these developed renal carcinoma by 6 months of age (n=10)(data not shown). Thus this data provides definitive functional evidence that p21 acts as a key block to tumour progression in the kidney in the absence of Apc.

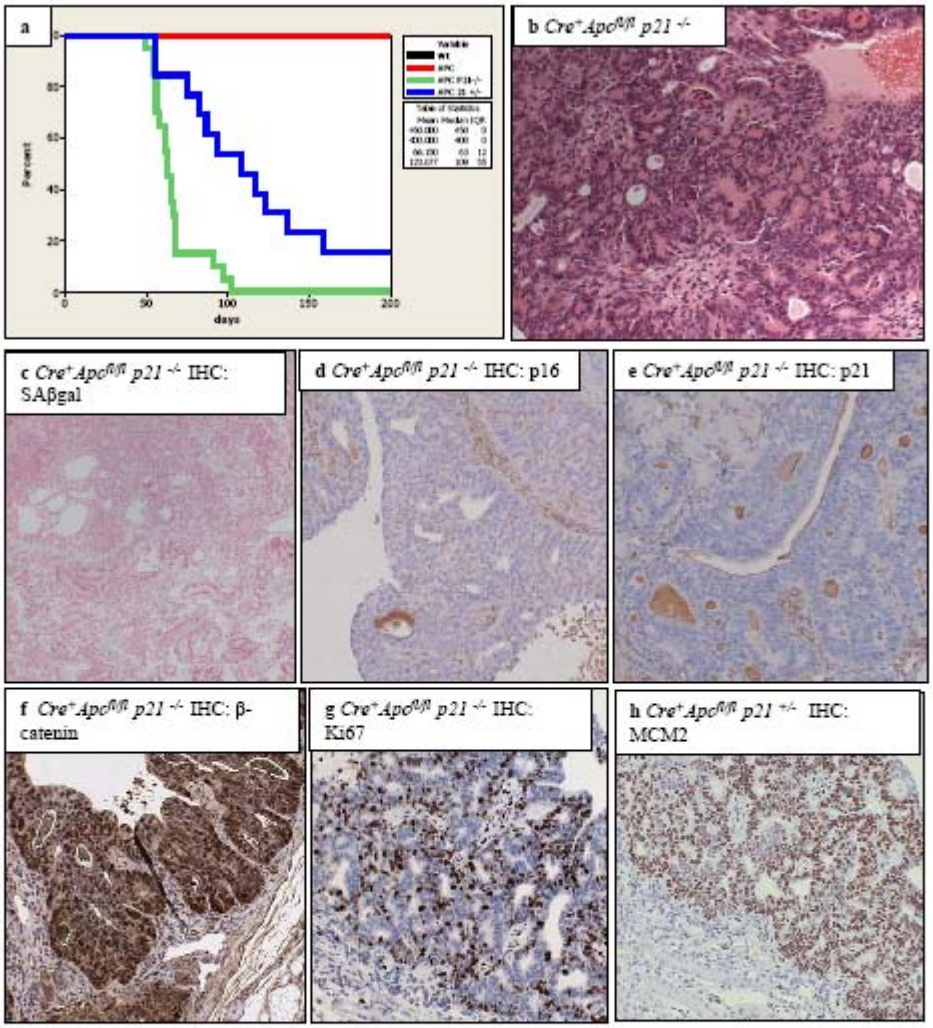


Figure 4.5: p21 loss following Apc deletion leads to rapid onset of renal tumourigenesis. Kaplan Meier survival graph showing a dramatic acceleration of renal tumourigenesis in Apc p21 deficient mice (*Cre*$^{+}$ *Apc*$^{fl/fl}$ *p21*$^{-/-}$, n= 31, median lifespan 63 days) compared with wt (n=20) or Apc deficient mice (*Cre*$^{+}$ *Apc*$^{fl/fl}$, n=23, Log rank p<0.001). Note a marked acceleration of tumourigenesis in *Cre*$^{+}$ *Apc*$^{fl/fl}$ *p21*$^{+/-}$ mice (median lifespan 117 days, n=17, Log rank p<0.001). **b)** H&E of a renal tumour in Apc p21 deficient mice. **c-e)** Staining shows abscence of senescent markers SA β-gal **(c)**, p16 **(d)** and p21 **(e)** in Apc p21 deficient renal tumours. **f)** β-catenin IHC showing continued Wnt signaling activation in Apc p21 deficient renal tumours. **g-h)** Ki67 IHC **(g)** and MCM2 IHC **(h)** showing a marked increase in proliferation in Apc p21 deficient tumours.

4.1.3 Apc loss does not lead to senescence within the intestine, even within slowly progressing lesions

These data suggest that the reason why Apc loss is not an initating tumour suppressor in the kidney is due to the induction of senescence. Implicit in this hypothesis is that Apc loss in the intestine should not induce senescence. We therefore investigated this by examining whether Apc loss within the intestinal epithelium provoke senescence. First, we conditionally deleted Apc within the intestinal epithelium by injecting *AhCre* $Apc^{fl/fl}$ mice with 3 injections of β-napthoflavone in a single day. This induces nearly 100 percent recombination within the small intestinal crypt. This regime produces a marked crypt-progenitor cell like phenotype within 4 days of Apc gene deletion(Sansom et al., 2004). Our previous studies have shown that a small subset of cells following Apc loss upregulated p21 at the leading edge of the phenotype (Figure 4.6b) (Sansom et al., 2007). To test whether this might be a senescent population of cells we stained for SAβ-gal and found no evidence of positivity (Figure 4.6c). Likewise no upregulation of p16 was observed (Figure 4.6f). Next we stained for the proliferation markers MCM2 and Ki-67 and found that every Apc deficient cell in the small intestine was MCM2 (Figure 4.6g) and Ki-67 (Figure 4.6i) positive, highlighting that there is no senescence following Apc loss in the intestine (albeit over the short term). This fits with our previous studies showing increased MCM2 staining and BrdU incorporation following Apc loss (Sansom et al., 2004).

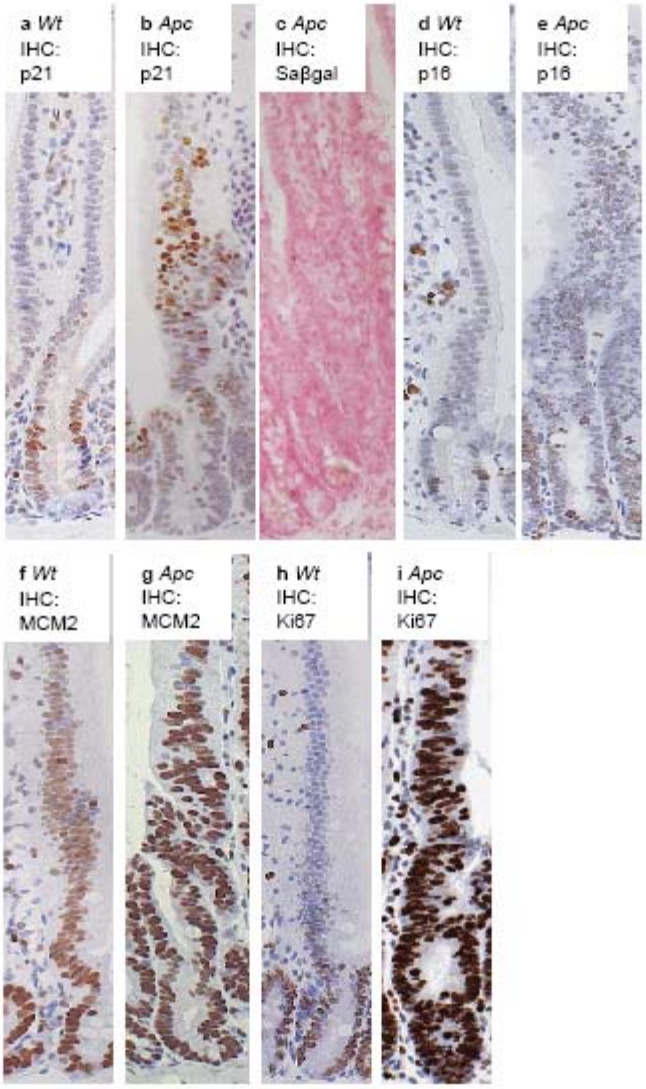


Figure 4.6: Deletion of Apc does not induce senescence within the intestinal epithelium
a-b) p21 IHC showing a small upregulation of p21 in wild type (*wt*) crypts at the crypt villus junction **(a),** as well as strong upregulation in a subset of cells in the villus following Apc loss (Cre^{+} $Apc^{fl/fl}$). **c)** Apc deficient crypts are not senescent as shown by lack of Saβgal staining. **d-e)** p16 IHC showing no major upregulation between wt **(d)** and Apc deficient crypts **(e)** **(f-g)** IHC showing an upregulation of MCM2 staining in the proliferative crypts of wt mice **(f)**, MCM2 is upregulated in all Apc deficient cells **(g)**.**(h-i)** IHC showing an upregulation of Ki-67 staining in the proliferative crypts of wt mice **(h)**MCM2 is upregulated in all Apc deficient cells **(i).**

To confirm that there was not a functional role that p21 was playing we induced *AhCre* $Apc^{fl/fl}$ $p21^{-/-}$ mice at 6 weeks of age with 3 injections of β-napthoflavone to yield near constitutive inducible intestinal recombination and investigated the phenotype of doubly mutant Apc p21 knockout intestines. Confirming the lack of any senescence or arrest at the early stages of Apc loss, Apc p21 double knockout intestines were indistinguishable to single Apc knockout intestines, in terms of proliferation, crypt size, apoptotic or mitotic levels (Figure 4.7 a-d).

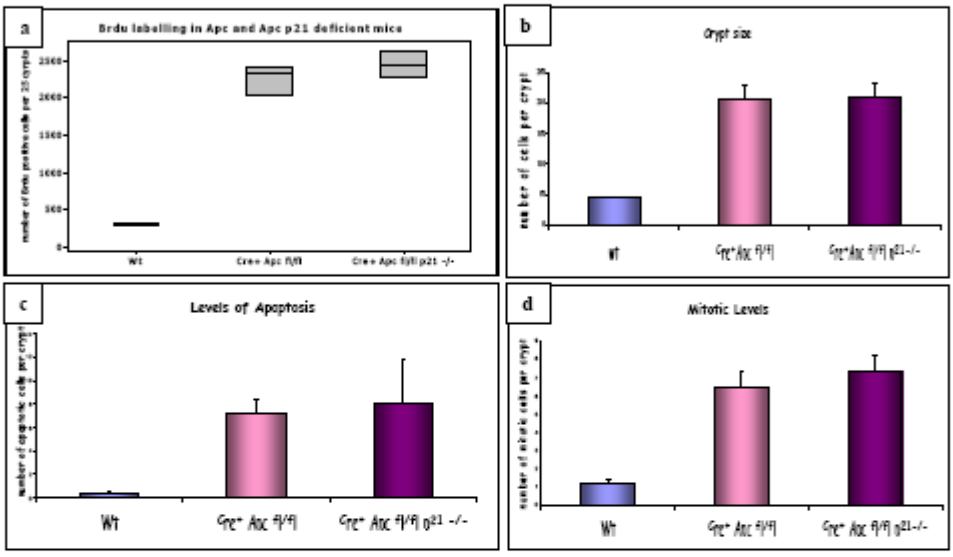


Figure 4.7: Deletion of p21 does not affect the Apc intestinal phenotype

a) p21 deletion following Apc loss does not affect the intestinal phenotype as illustrated by a lack of difference in proliferation , marked by BRDU labelling **(a,** Mann-Whitney U test, p= 0.3807). p21 deletion does not affect crypt size **(b)**, levels of apoptosis **(c)** or mitosis**(d)**

As the Apc mutation occurs in 80% of colorectal cancer as the initiating event and there has been some evidence to suggest benign adenomas may exhibit some features of senescence we next investigated whether adenomas that are formed following Apc loss show signs of senescence. To do this we examined oncogene induced senescence (OIS) in small lesions and adenomas generated from 3 different models of Apc loss , first the $Apc^{Min/+}$ mouse, second adenomas formed through stem cell deletion of Apc and thirdly single crypt lesions from the non stem cell deletion of Apc. Our previous studies have shown that when Apc is deleted outwith the stem cell zone this leads to protracted tumourigenesis and although adenomas can form, most lesions remain small lesions and do not progress (over a 200-300 day period)(Barker et al., 2009). In all three scenarios we failed to see upregulation of p16, Saβgal, though p21 was expressed within the adenomas of the $Apc^{Min/+}$ mouse (Figure 4.8a-d). To examine the proliferative capacity of the lesions and the adenomas we stained for Ki-67 and MCM2 (Figure 4.8g-h, k-l). Once again both lesions and adenomas exhibited high levels of staining showing that Apc loss within the intestinal epithelium does not provoke senescence within the intestinal epithelium. Therefore even in our protracted models we again fail to see senescence in the premalignant lesions. The major difference we see between the small lesions and adenomas is levels of apoptosis, within the small lesions both proliferation and apoptosis is high whilst in adenomas levels of apoptosis are low; consistent with the notion that apoptotic escape is important for colorectal cancer progression.

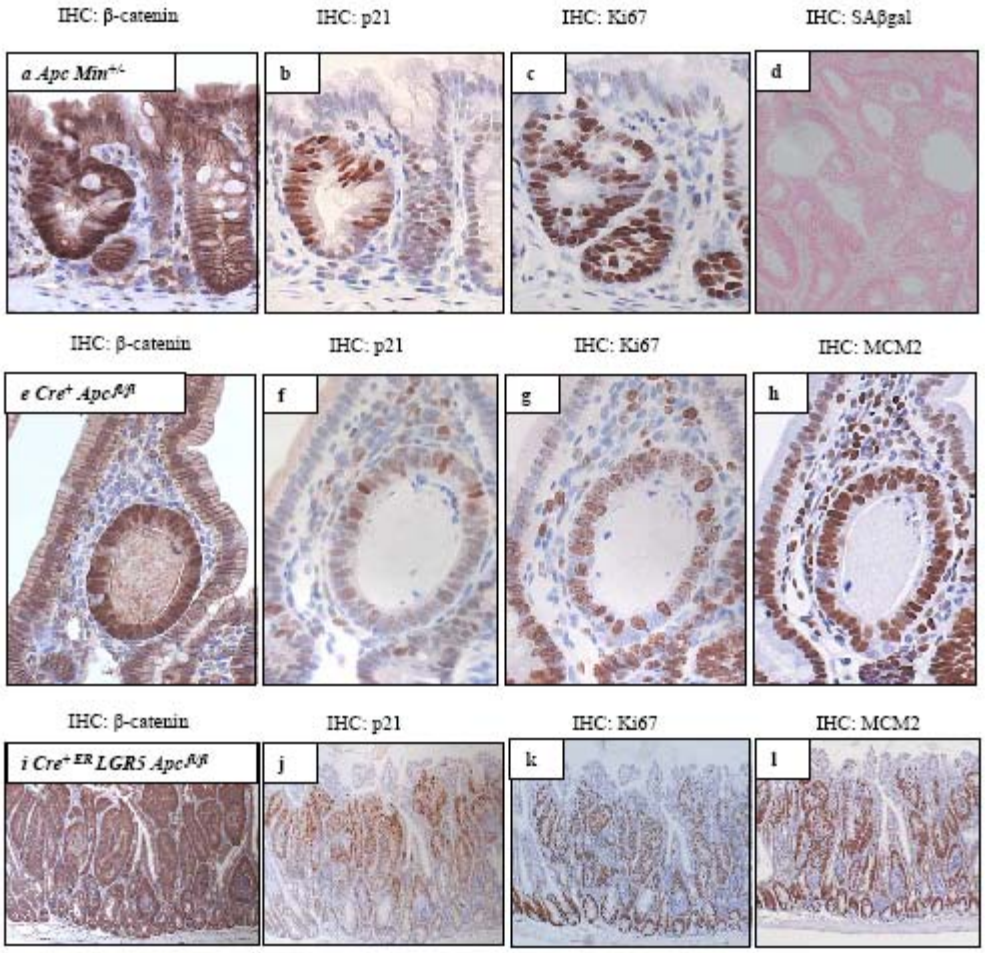


Figure 4.8: Apc deletion does not induce OIS within the intestine.

a-d) small intestinal adenoma's arising from $Apc^{min/+}$ mice at 75 days. IHC in intestinal adenomas from $Apc^{min/+}$ mice showing high levels of β-catenin **(a)**, p21 **(b)** and Ki-67 **(c)**. **d)** intestinal adenomas from $Apc^{min/+}$ mice lack expression of senescence marker Saβgal. Apc deficient intestinal lesions that grow out from a non-stem cell hit (0.5 mg/kg oral gavage), display high levels of β-catenin **(e)**, with some levels of p21**(f)** and high levels of proliferative markers Ki-67**(g)** and MCM2**(h)**. **i-l)** *Lgr5-EGFP*$^{+}$ *-Cre-ER/* $Apc^{fl/fl}$ mice are treated with a single intreaperitineal injection of tamoxifen to induce recombination in the intestinal stem cell, resutling in intestinal adenoma formation at 24 days. **i)** β-catenin IHC showing high levels of expression in intestinal adenoma. **j)** p21 IHC showing upregulation in intestinal adenomas. **k-l)** IHC showing high levels of proliferation as illustrated by Ki67 **(k)** and MCM2 IHC **(l)**.

Finally as p21 was expressed in a subset of cells within the adenoma we tested whether p21 functionally modulated Apc mediated tumourigenesis in the intestine. Thus 20 $AhCre^{+}$ $Apc^{fl/+}$ $p21^{+/+}$ and $AhCre^{+}$ $Apc^{fl/+}$ $p21^{-/-}$ were induced at weaning with β-napthoflavone and aged until they developed intestinal tumourigenesis as scored by hunching, paling of feet and weight loss. No significant differences was seen in either age of death, tumour burden or progression in the $AhCre^{+}$ $Apc^{fl/+}$ $p21^{-/-}$ compared to the $AhCre^{+}$ $Apc^{fl/+}$ $p21^{+/+}$ (Figure 4.9 a-c Mann-Whitney U test , (b) p=0.3118, (c) p=0.1346). To further test if sensescence was playing a role in the intestinal epithelium we also examined whether *INK4A* deficiency could accelerate intestinal tumourigenesis. Given the relatively longevity of $AhCre^{+}Apc^{fl/+}$ mice this made this experiment difficult as $INK4A^{-/-}$ mice develop spontaneous tumourigenesis from 200 days of age. Therefore we crossed $INK4A^{-/-}$ mice to the $Apc^{Min/+}$ model of tumourigenesis, where mice develop intestinal adenomas more rapidly (by 100 days). Once again no difference in time to intestinal ill health or tumour burden was observed between $Apc^{Min/+}$ $INK4A^{+/+}$ or $Apc^{Min/+}$ $INK4A^{-/-}$ animals (Figure 4.9 d-f, Mann-Whitney U test, (e) p= 0.7405, (f) = 0.1601).

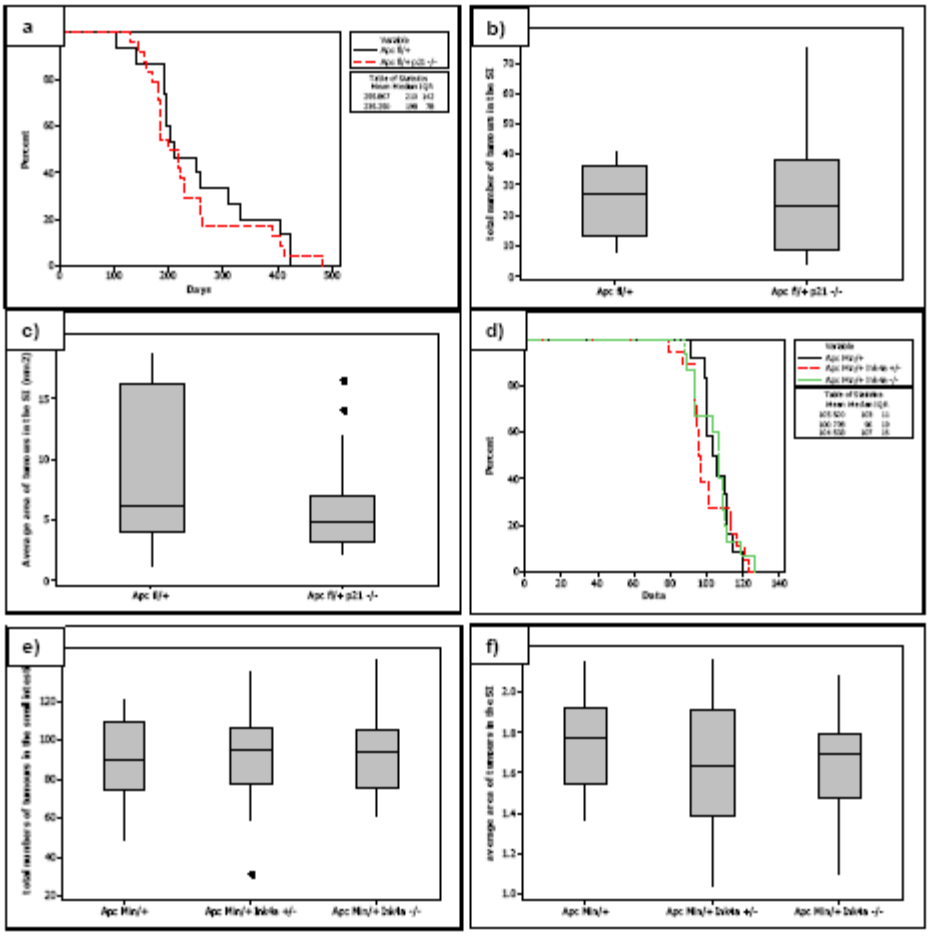


Figure 4.9 :Deletion of p21 or Ink4a does not affect intestinal tumourigenesis

Kaplan Meier survival graph showing no acceleration of tumourigenesis between Apc (*Cre*$^{+}$ *Apc*$^{fl/+}$, n=15) and Apc p21 (*Cre*$^{+}$ *Apc*$^{fl/+}$ *p21*$^{-/-}$, n=24) mice (Log rank, p=.476). No significant differences were observed either in total tumour number (Mann-Whitney, p=0.1893) (**b**), average tumour size (Mann-Whitney U test, p=0.3118), or average tumour area (Mann-Whitney U test, p=0.1346) (**c**). **d)** Survival graph showing no acceleration of tumourigenesis between Min (*Apc*$^{Min/+}$, n=20) and Min Ink4a (*Apc*$^{Min/+}$ *Ink4a*$^{-/-}$, n=25) mice (Log rank, p=0.825). No significant differences were observed either in total tumour number (Mann-Whitney p=0.7405) (**e**) or average tumour size (Mann Whitney U test, p=0.1601)(**f**).

4.1.4 Tissue specific C-Myc suppression of p21

Within the intestinal epithelium, we have previously shown that loss of c-Myc strongly suppresses the phenotypes of Apc loss (Sansom et al., 2007). One of the key functions of c-Myc is to repress p21 through its interaction with MIZ1 and we have previously shown p21 is markedly derepressed when Apc and c-Myc are co-deleted within the intestinal epithelium. However in the renal epithelium we observed a clear induction of p21 coincidentally with the accumulation of β-catenin and thus argues in this context c-Myc expression was not sufficient to repress p21. In other tissues such as the skin, the repression of p21 by c-Myc is crucial for tumourigenesis phenotype (Oskarsson et al., 2006). Given increased c-Myc is not sufficient to repress p21 within the kidney following Apc loss this suggested tumourigenesis in Apc p21 double knockout may proceed in a c-Myc independent fashion. To test this we generated triple knockout Apc Myc p21($AhCre^{+}$ $Apc^{fl/fl}$ $Myc^{fl/fl}$ $p21^{-/-}$) mice and assessed renal tumour formation in these mice. As can be seen in Figure 4.10 a, renal tumourigenesis still proceeded rapidly in these mice (equivalent to the double Apc p21 knockout mice) despite the absence of c-Myc. Apc Myc p21 renal tumours are histologically identical to Apc p21 mice (Figure 4.10b). Similary, Apc Myc p21 tumours display a continued lack of Saβgal (Figure 4.10 c), whilst continuing to express high levels of β-catenin (Figure 4.10 d) and Ki-67 (Figure 4.10g). Immunohistochemical staining for c-Myc shows complete ablation of protein in Apc Myc p21 tumours (Figure 4.10e) and a high upregulation in Apc p21 tumours(Figure 4.10f). As like the renal carcinomas from the Apc p21 mice, Apc Myc p21 tumours are highly proliferative as seen through MCM2 IHC (Figure 4.10h). This is again in complete contrast to Apc deficient intestinal epithelium which absolutely

requires the correct levels of c-Myc for the phenotypes of Apc loss and tumour formation (Sansom et al., 2007).

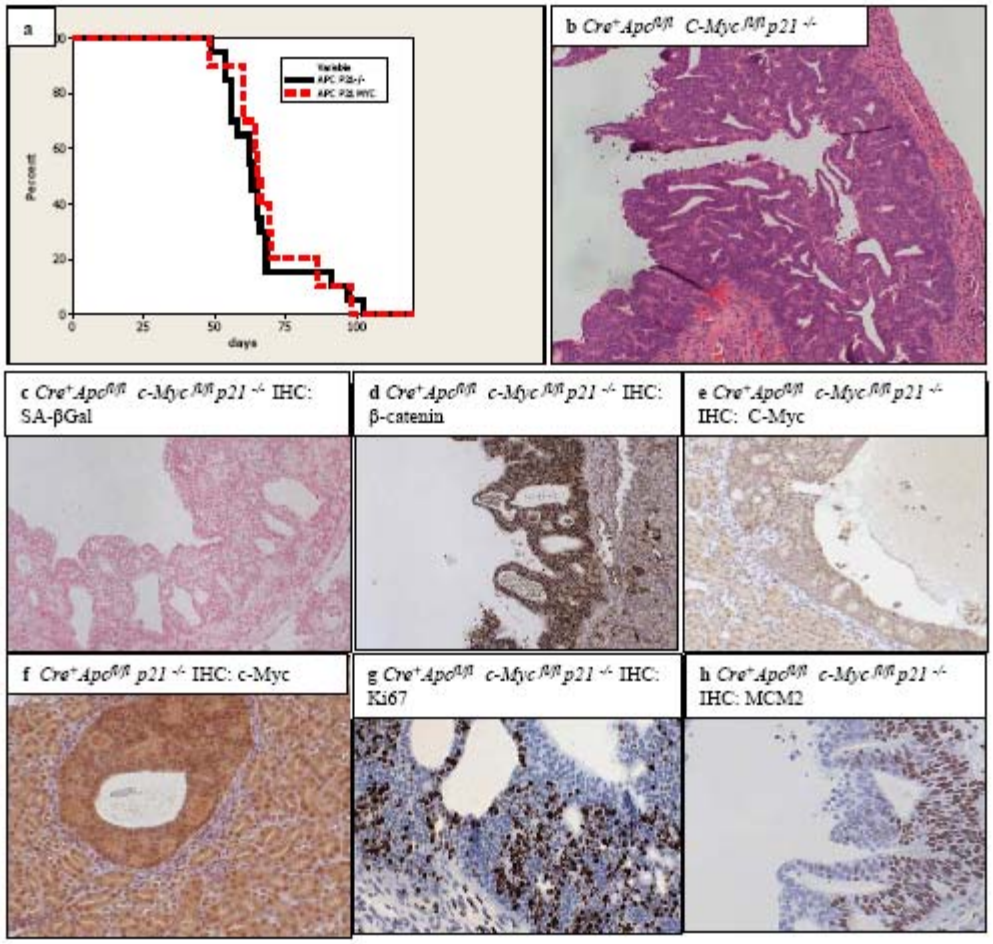


Figure 4.10: c-Myc deletion does not affect onset of tumour development.

a) Kaplen Meier survival graph showing no difference in survival between Apc p21 deficient mice (*Cre*$^{+}$ *Apc*$^{fl/fl}$ *p21*$^{-/-}$, n=31*)* and Apc c-Myc p21 deficient mice (*Cre*$^{+}$ *Apc*$^{fl/fl}$ *c-Myc*^{fl} *p21*$^{-/-}$, n=20) (Log rank p= 0.571), illustrating that *c-Myc* deletion does not affect onset of renal tumourigenesis. **b)** H&E showing Apc c-Myc p21 deficient renal tumours. **c)** Absence of SA β-gal staining in Apc c-Myc p21 deficient renal tumours. **d)** β-catenin IHC in Apc c-Myc p21 deficient renal tumours shows Wnt activation. **e)** c-Myc IHC shows lack of c-Myc expression in Apc c-Myc p21 deficient renal tumours. **f)** c-Myc IHC showing upregulation of c-Myc in Apc p21 deficient renal tumours, indicating that c-Myc is not important for renal tumour development. **(g-h)** Strong upregulation of proliferative markers Ki67 **(g)** and MCM2 **(h)** showing that Apc c-Myc p21 deficient tumours are highly proliferative.

4.2 Discussion:

Taken together our data shows that Apc loss within the renal epithelium provokes a strong disadvantage with cells upregulating p21 and undergoing senescence. It should be noted that it is hard to exclude from all the *in vivo* studies that this may reflect a long growth arrest as proving a cell will never proliferate is impossible using current methodologies. Given recent data on the immune clearance of senescent cells, it is tempting to speculate that the reduction/loss of the majority of Apc deficient cells within the kidney is due to this process. Given this is a stochastic process, it is difficult to delineate whether this is truely the case or instead reflects a selective disadvantage to the Apc deficient cells in development, causing the kidney to be composed of mainly 'wild type unrecombined' cells. Future studies to investigate different immunocompromised strains to the Apc deficient mice will delineate these two hypotheses. These results therefore serve to explain why Apc mutations are not initiating events in the kidney. It also highlights the reason why FAP patients and $Apc^{Min/+}$ mice do not develop renal carcinoma as loss of Apc alone would lead to a senescence and selective disadvantage. Instead this data predicts strong cooperation of the Wnt pathway in renal carcinogenesis once a driver mutation has occured that will block the senescence pathway. To this end we have previously shown that KRAS activation in the kidney cooperates with Apc loss to drive renal carcinoma (Sansom et al., 2006). Given the recent data that VHL protein loss may increase Wnt signalling and Apc is methylated in renal carcinoma, this may be relevant to human carcinogenesis (Zhou et al., 2005, Dulaimi et al., 2004)

Whilst in the intestine Apc loss drives a strong proliferative (and apoptotic) program. Consistent with this, CRC appears to be one of the few cancers where the *INK4A* locus is not deleted and indeed high levels of p16 correlate with a bad prognosis and the neighbouring gene MTAP (which is often co-deleted with the INK4A locus) is overexpressed (Wassermann et al., 2009, Bataille et al., 2004). Taken together this data shows completely different cooperating oncogene and tumour suppressor mutations between the intestine and the kidney. This functional data again reinforces the notion that Wnt signalling deregulation following Apc loss is sufficient to drive a proliferative fate in the intestine and thus mutations key for tumour progression in other tissues to allow from an escape from a growth/senescence are not required within the intestinal epithelium. This is particularly relevant for INK4A loss as this is mutated at very high levels in pancreatic cancer and melanoma, both which have been associated with senescent premalignant lesions. In addition, studies from this lab have shown that p21 gene knockout strongly cooperates with $KRAS^{G12D}$ to drive pancreatic cancer in the mouse and is downregulated in approximately 40% of human pancreatic ductal adenocarcinoma (Morton et al submitted).

This study thus provides crucial insights into the context specific outcome of Wnt signalling and raises questions on how important senescence is as a barrier to tumourigenesis in CRC. Several studies have seen SAβgal postive cells in colorectal cancer patients and the DNA damage response activated. More recent data argued that the inflammatory mediators required for senescence like IL-6 were constraining proliferation of premalignant intestinal lesions (Becker et al., 2005),(Grivennikov et al., 2009). One

potential explanation could be is that other oncogenic/tumour suppressor mutations could have initiated these lesions and driven senescence, for example, BRAF or KRAS. However so far $Kras^{v12}$ and $Kras^{D12}$, LKB1 and PTEN mutation have all been investigated in the murine intestinal epithelial and none induce senescence, indeed most induce proliferation (Sansom et al., 2006, Marsh et al., 2008, Shorning et al., 2009). Therefore the only remaining oncogenic driver of colorectal cancer not studied so far that could induce senescence is $BRAF^{V600E}$ and studies to elucidate whether the impact of this mutation *in vivo* will be of great interest.

Chapter 5: The upregulation of p21 following c-Myc deletion drives differentiation but does not block proliferation of Apc deficient intestinal enterocytes

5.0 Introduction

The c-Myc proto oncogene has been postulated to play a key role in colorectal cancer where Wnt signalling is activated. It has been shown to be overexpressed in 70% of colorectal tumours (Arango et al., 2003) and is a direct Wnt target gene. Moreover c-Myc overexpression has been shown to restore proliferation to colorectal cancer cells where β-catenin has been deleted and most importantly genetic deletion of c-Myc rescues the phenotypes of Apc loss in the murine intestinal epithelium (Sansom et al., 2007). c-Myc is thought to function as an oncogene predominantly through its role as a transcription factor where it has been shown to both transcriptionally activate and repress genes. Following Apc loss, genetic deletion of c-Myc significantly reduces the expression of the majority of the Wnt target genes but also causes a significant increase in the levels of cyclin dependent kinase inhibitor p21 (Sansom et al., 2007). It is now important to delineate the key transcriptional targets of c-Myc that are crucial for the phenotypes of Apc deficiency *in vivo*.

The precise mechanism of how c-Myc transcriptionally represses p21 is relatively well understood as Myc is recruited to the p21 promoter by Miz-1 and inhibits p21 activation (Seoane et al., 2002). Micro-array analysis has confirmed this, implicating p21 as one of the major targets of Myc repression (Gartel and Radhakrishnan, 2005). In normal intestinal epithelium, c-Myc deletion does not cause p21 derepression suggesting that this pathway is more important following either an oncogenic event or following stress (Muncan et al., 2006b). Consistent with this, a marked upregulation of p21 was observed in double knockout Apc c-Myc intestinal enterocytes. A similar phenomenon was

observed within the skin where c-Myc deletion had no or only a mild effect on skin homeostasis and the response to TPA. However c-Myc was essential for tumourigenesis induced by DMBA/TPA treatment and in this instance the repression of p21 was the essential c-Myc target as tumourigenesis was restored in double knockout c-Myc p21 keratinocytes (Oskarsson et al., 2006).

p21 $^{WAF1/CIP1}$ is a cyclin dependent kinase (CDK) inhibitor that belongs to the cip/kip family of CDK inhibitors, and whose main function is to inhibit cyclin/cdk 2 complexes and inhibit cell cycle progression (Gartel and Radhakrishnan, 2005). *In vivo* studies have shown that p21 null mice are viable but develop spontaneous tumours at 16 months of age (Martin-Caballero et al., 2001). In accordance with these findings, previous studies have also shown that deletion of p21 results in an increase in tumourigenesis following irradiation (Jackson et al., 2003). Deletion of p21 has been shown to increase tumourigenesis in Apc 1638 $^{+/-}$ mice (Yang et al., 2001a) , and has also been shown to be essential for the mitotic arrest and inhibition of Apc-initiated tumour formation by sulindac in Apc 1638 $^{+/-}$ mice (Yang et al., 2001b). Expression of p21 is down regulated in human colon tumours, linking defective expression of p21 with shorter survival rates for patients with colorectal cancer (Zirbes et al., 2000). P21 deficiency also decreases the number of mature goblet cells, which is significant as the loss of this lineage and mucin secretion is characteristic of early, preneoplastic aberrant crypt foci in patients who are at risk for developing colon cancer (Zirbes et al., 2000).

In this study, we have investigated whether the upregulation of p21 is crucial for the crypt progenitor cell like phenotype of Apc deficiency. Surprisingly loss of p21 did not restore proliferation to double knockout Apc Myc deficient intestinal enterocytes, though it repressed the ability of double knockout intestinal enterocytes to migrate up the crypt-villus axis and to form villi.

5.1 Results

5.1.1 P21 deletion does not restore the Apc crypt progenitor cell phenotype to doubly mutant Apc c-Myc enterocytes

Previous studies have shown that deletion of Apc *in vivo* leads to a crypt progenitor phenotype, characterized by hyperproliferation and perturbed migration and differentiation (Sansom et al., 2004). To investigate the pattern of p21 localisation, we performed IHC for p21 on wild type, Apc, and Apc Myc deficient intestinal enterocytes. In wild type crypts weak p21 staining is observed in cells at the top of the crypt as they differentiate into villus (Figure 5.1a). Following Apc loss, p21 is upregulated more strongly but again only in a small subset of Apc deficient cells. The p21 positive cells are in the top 1/3 of Apc deficient crypts in cells which often have an increased size (Figure 5.1b). It is possible these cells had originally exited the crypt before Apc loss [it takes 3 days to see nuclear β-catenin within the intestinal crypt presumably due to the time required to induce Cre recombinase expression and turn over the Apc protein (Sansom et al., 2004)]. However within double knockout Apc Myc deficient intestinal enterocytes high p21 expression is now observed through out the crypts (Figure 5.1c). Previous *in vivo* studies have shown that deletion of c-Myc alone is not sufficient to upregulate p21 (Muncan et al., 2006b). Our staining here is consistent with these previous studies. Given these findings and that p21 was now upregulated in almost every crypt cell of doubly mutant Apc Myc mice, we hypothesized that the repression of p21 by c-Myc might be key to the Apc 'hyper- proliferative' phenotype. In order to examine this we generated Cre^{+} $Apc^{fl/fl}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice. To do this we used Cre loxP technology as previously

described and crossed *Cre*$^{+}$ *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ mice to *p21*$^{-/-}$ mice (Ireland et al., 2004, Sansom et al., 2007). Mice were injected intraperitoneally with 3 injections of the inducer β-napthoflavone within one day, which yields nearly 100% recombination within the small intestine, and mice were examined at day 4 post injection.

Figure 5.2 (c-d) shows no gross morphological changes between *Cre*$^{+}$ *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ and *Cre*$^{+}$ *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ *p21* $^{-/-}$ mice. *Cre*$^{+}$ *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ *p21* $^{-/-}$ mice continue to display small crypts like those of *wild type* and *Cre*$^{+}$ *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ mice and do not resemble those of *Cre*$^{+}$ *Apc* $^{fl/fl}$ mice. This illustrates that deletion of p21 does not result in the reversion to the morphological appearance of the crypt progenitor phenotype. In order to determine if *Cre*$^{+}$ *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ *p21* $^{-/-}$ mice continued to express high levels of Wnt signalling, we performed immunohistochemical staining for nuclear β-catenin. Importantly, Figure 5.2e shows a continued upregulation of β-catenin in the crypts of these mice, whilst immunhistochemistry for c-Myc (Figure 5.2f) shows complete protein loss.

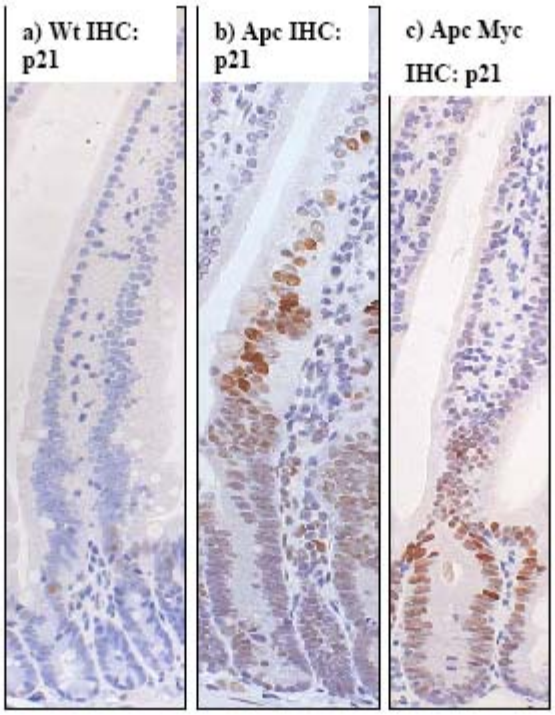


Figure 5.1: p21 is expressed in the crypts of *Cre*$^{+}$ *Apc*$^{fl/fl}$ *Myc*$^{fl/fl}$ mice

(a) Immunohistochemical staining for p21 showing very little expression in wild type (*Wt*) crypts **(b)** Following Apc loss p21 is now upregulated in a subset of cells in the villus.**(c)** Following combined Apc and C-Myc loss, p21 is now highly upregulated throughout the crypt.

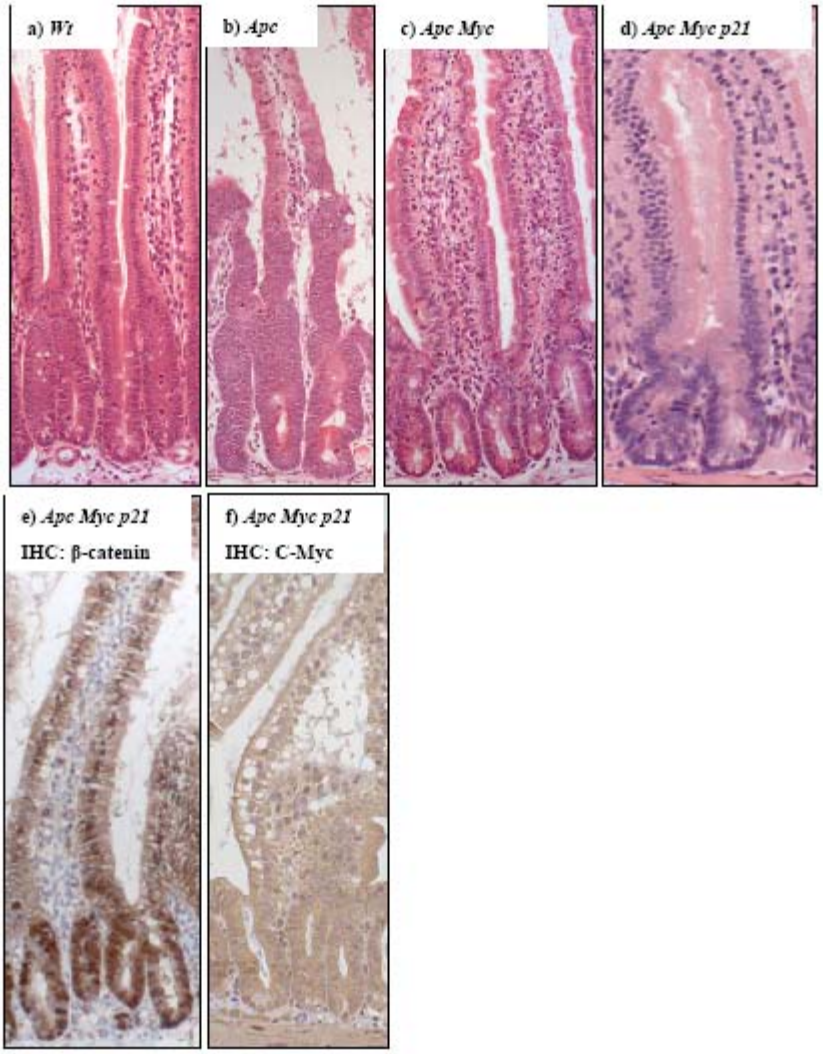


Figure 5.2: p21 deletion following combined c-Myc and Apc loss does not result in any gross morphological changes.

(a) H&E showing small crypts in wt mice, whilst Apc deletion results in a 'crypt-progenitor' phenotype with increased proliferation and the loss of the single cell crypt-villus axis**(b)**. **(c)** Combined C-Myc and Apc loss rescues this phenotype with the reversion to morphologically 'wt' looking crypt. **(d)** crypt from Apc Myc p21 is morphologically indistinguishable from Apc Myc mice.**e)** Immunohistochemical staining for β-catenin showing that nuclear expression is restricted to the base of the crypt. **f)** Immunohistochemical staining for c-Myc confirms a complete ablation of protein

To examine the levels of proliferation more carefully, we investigated BrdU (5-bromo-2-deoxyuridine) incorporation in TKO mice, 2 hour prior to euthanasia. Results showed an increase in the number of BrdU positive cells per crypt in Cre^{+} $Apc^{fl/fl}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice compared to Cre^{+} $Apc^{fl/fl}$ $Myc^{fl/fl}$ *mice* (Figure 5.3a, Mann-Whitney U test, p=0.04). Compensating for this increase in proliferation was an increase in apoptotic levels, as scored through H&E analysis (Figure 5.3b), resulting in a crypt size (total cells per crypt) similar to those of Cre^{+} $Apc^{fl/fl}$ $Myc^{fl/fl}$ mice (Figure 5.3c, Mann-Whitney U test p< 0.04). Histological analysis revealed no significance in the number of mitotic figures between double knockout and TKO mice (Figure 5.3d, Mann–Whitney U test; P>0.04). To investigate if this increased apoptosis and BrdU incorporation resulted in similar levels of proliferation over the longer term, we next pulsed mice with BrdU for 24 hours.. However at 24 hours, no differences in proliferation levels were observed between Cre^{+} $Apc^{fl/fl}$ $Myc^{fl/fl}$ and Cre^{+} $Apc^{fl/fl}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice (Figure 5.4, Mann-Whitney U test; p= 1.00).

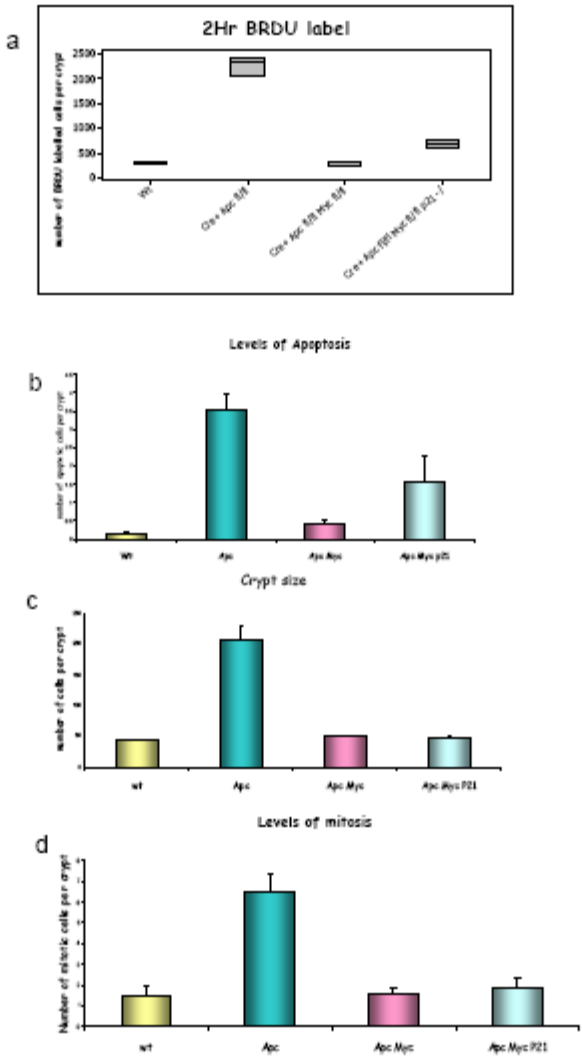


Figure 5.3: Apc Myc p21 mice exhibit higher levels of proliferation and apoptosis resulting in similar crypt sizes to Apc Myc mice.

(a) Box plot showing an increase in proliferation as scored through BrdU incorporation(2 hours post injection) in Apc Myc p21 mice (Mann-Whitney U test $p<0.04$). **(b)** Graph showing an increase in the number of apoptotic cells per crypt in Apc Myc p21 mice compared to Apc Myc mice(Mann-Whitney U test $p<0.04$). **(c)** Graph showing Apc Myc p21 mice exhibit no change in crypt size compared to Apc Myc mice (Mann-Whitney U test $p>0.04$). **(d)** Graph showing that p21 deletion following combined Apc and C-Myc loss does not result in any significant changes in mitosis(Mann-Whitney U test $p>0.04$).

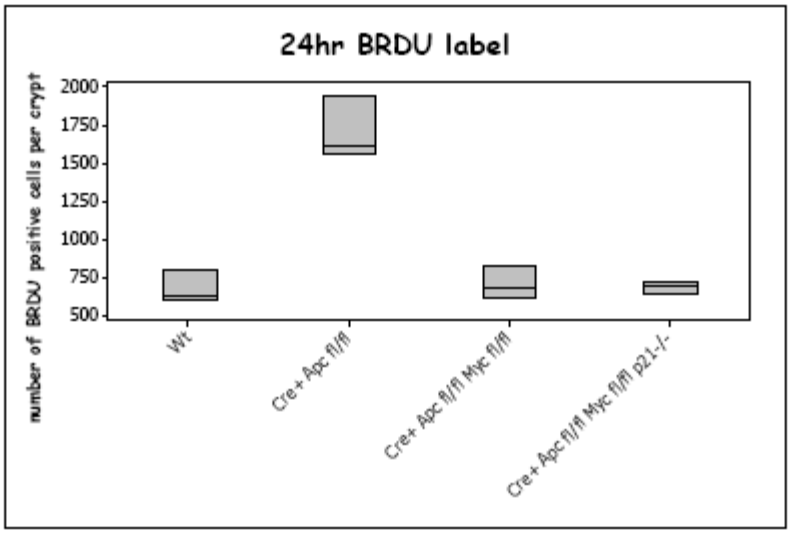


Figure 5.4: ***Cre*** $^{+}$ ***Apc*** $^{fl/+}$ ***Myc*** $^{fl/fl}$ ***p21*** $^{-/-}$ **mice do not display an increase in proliferation at 24 hours** (Mann-Whitney U test; P= 1.00)

In addition to examining proliferation, BrdU pulsing also allow levels of enterocytes movement up the crypt villus axis to be assessed. This is because BrdU is only bioavailable for 1 hour and thus after incorporation cells will divide and move up the crypt-villus axis. Therefore we next scored the capacity of triple mutant cells to migrate along the crypt villus axis by comparing the position of BrdU positive cells at 2 and 24 hours after exposure. No difference in position of BrdU positivity was observed between Apc Myc and TKO mice at 2 hours following BrdU injection (Figure 5.5a). However at 24 hours a clear difference was observed with enterocytes from, Cre^{+} *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ *p21* $^{-/-}$ showing a significant lower labeling position than Apc Myc enterocytes, consistent with a lack of any movement. (Figure 5.5b). This finding was confirmed through β-catenin IHC which shows that in Cre^{+} *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ *mice*, expression of nuclear β-catenin extends from the crypt all the way up the crypt villus axis, however in Cre^{+} *Apc* $^{fl/fl}$ *Myc* $^{fl/fl}$ *p21* $^{-/-}$ mice, expression of nuclear β-catenin is limited to the crypt region only (Figure 5.5c-d). Furthermore, mice were intercrossed to the Rosa 26R LacZ reporter strain to confirm if recombined cells were no longer moving up the crypt villus axis. Staining for β-galactosidase showed that in TKO intestines, only the crypts were positive, with white villi composed of non-recombined 'white cells' (Figure 5.6a-b). However Apc Myc intestines showed positive lacZ expression extending from the crypt up along the villus axis (Figure 5.6c-d). These results demonstrate that in triple mutant mice, p21 loss prevents the movement and subsequent differentiation of cells into villus.

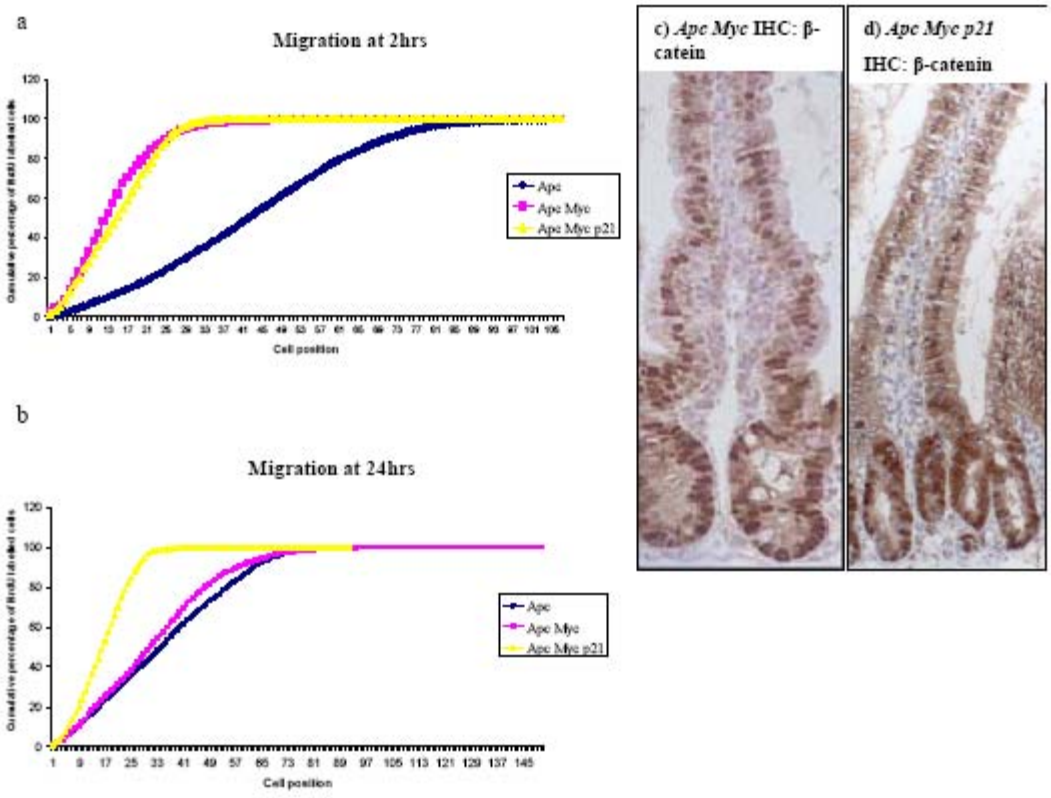


Figure 5.5: Apc Myc p21 mice exhibit a migrational defect seen at 24 hours post BrdU injection.

(a) Cumulative frequency graph (which illustrates the percentage of BrdU positive cells per crypt position) shows no changes in migration at 2 hours post BrdU injection in Apc Myc p21 mice compared to Apc Myc mice. **(b)** Cumulative frequency graph now showing a migration defect in Apc Myc p21 at 24 hours post BrdU injection, with cells unable to move up the crypt villus axis. **(c)** Immunohistochemical staining for β-catenin showing β-catenin positive cells extending from the crypt all the way up the crypt villus axis in Apc Myc mice. **(d)** Immunohistochemical staining for β-catenin showing a migration defects in Apc Myc p21 mice with cells unable to migrate up the villus axis.

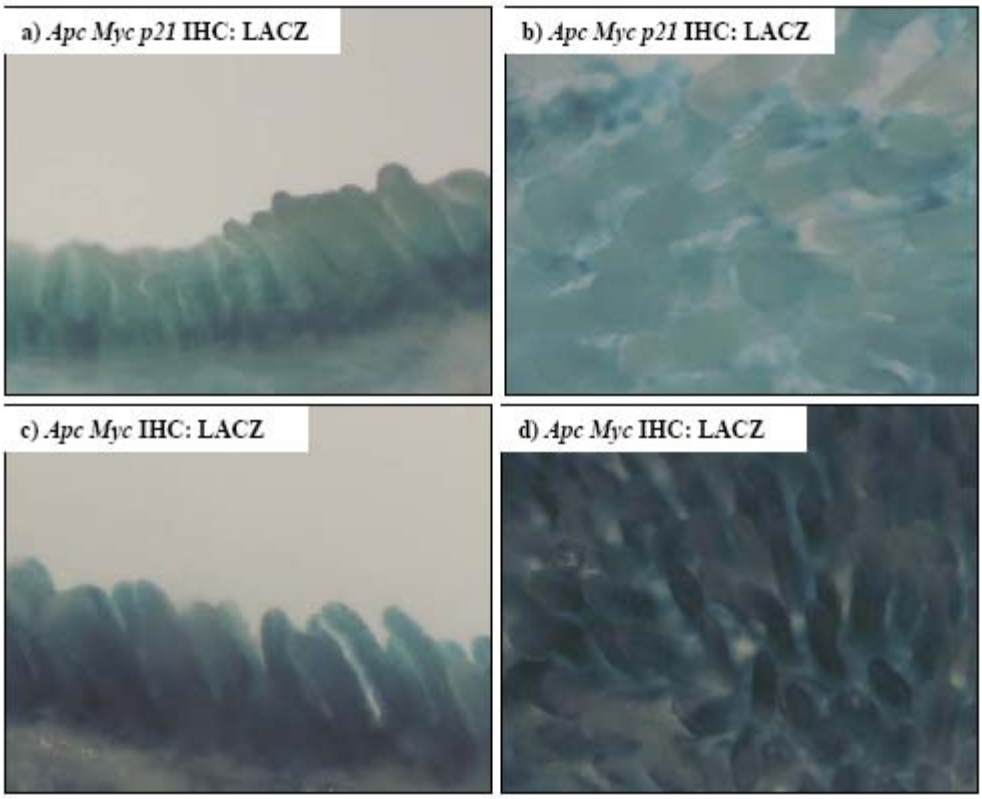


Figure 5.6: Staining for the Rosa26 LacZ reporter gene shows a migrational defect of recombined cells in Apc Myc p21 mice.
(a-b) Full length view of crypt-villus axis **(a)** and overhead view of villi **(b)** showing LacZ staining in Apc Myc p21 mice. Note recombined (blue) cells are unable to migrate past the crypt-villus junction **(a)**, resulting in white (unrecombined) cells) in the villus **(b)**. **(c-d)** Full length view of crypt-villus axis **(c)** and overhead view of villi **(d)** showing LacZ staining in Apc Myc mice. LacZ staining in Apc Myc mice showing normal migration of recombined (blue) cells continuing up the crypt villus axis **(c)**, resulting in blue cells in the villus **(d)**.

Previous *in vivo* studies have shown that deletion of c-Myc within the intestinal crypts stops polyp formation (Muncan et al., 2006b). Indeed there is a clear haploinsufficiency phenotype for c-Myc as Myc heterozygotes are also strongly resistant to tumourigenesis caused by Apc loss (Athineos et al. 2009 submitted). Given the fact that TKO cells was now retained in the crypt, we wanted to investigate whether this would be sufficient to restore tumourigenesis in the absence of c-Myc. To do this we induced $Cre^{+}Apc^{fl/+}$, Cre^{+} $Apc^{fl/+}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice at 6 weeks of age and aged mice until they showed signs of intestinal disease (paling of feet, weight loss of over 20%, hunching). Adenomas in this model are formed following the loss of the remaining Apc allele and show high nuclear levels of β-catenin and c-Myc. Consistent with our previous studies Cre^{+} $Apc^{fl/+}$ mice developed multiple intestinal adenomas (Figure 5.7a average age of death 220 days). In contrast , Cre^{+} $Apc^{fl/+}$ $Myc^{fl/fl}$ $p21^{-/-}$ *mice* survived past 400 days (Figure 5.7 a average age of death 391 days).These Cre^{+} $Apc^{fl/+}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice exhibited a strong reduction in total tumour number as the average tumour number was 37 Cre^{+} $Apc^{fl/+}$ mice, compared to an average tumour number of 5 in Cre^{+} $Apc^{fl/+}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice (Figure 5.7 b). Most importantly, immunohistochemical analysis of these mice revealed that the few tumours that formed in the Cre^{+} $Apc^{fl/+}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice all stained were c-Myc proficient (Figure 5.8e). As expected all of the tumours exhibited high levels of nuclear β-catenin (Figure 5.8d). These results clearly illustrate that despite the absence of p21, adenomas cannot form in the absence of c-Myc.

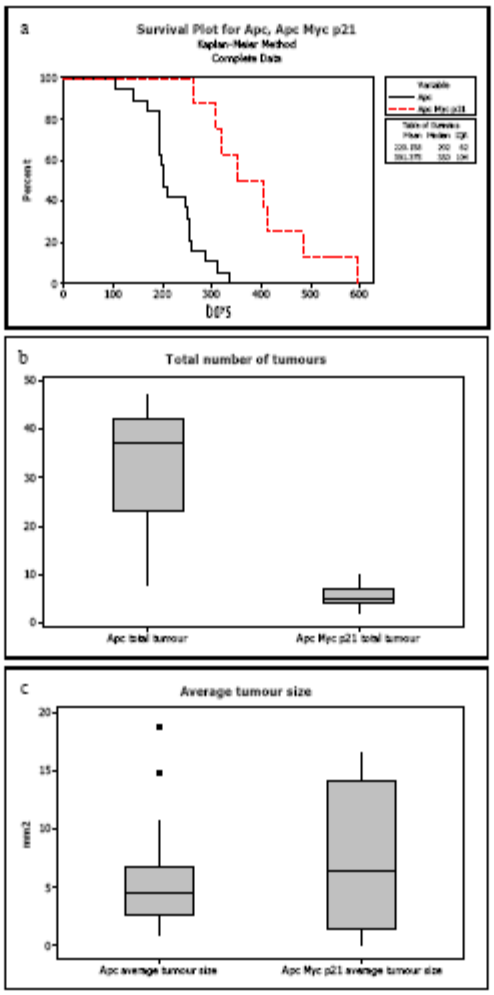


Figure 5.7: Deletion of p21 does not allow double mutant Apc Myc mutant cells to form tumours.

(a) Kaplan Meier survival graph showing significant increase in survival in *Cre*$^{+}$ *Apc*$^{fl/+}$ *Myc*$^{fl/fl}$ *p21*$^{-/-}$ mice compared to *Cre*$^{+}$ *Apc*$^{fl/+}$ mice (Log rank test, p<0.0001) **(b)** Box plot showing significantly more tumours in *Cre*$^{+}$ *Apc*$^{fl/+}$ mice compared to *Cre*$^{+}$ *Apc*$^{fl/+}$ *Myc*$^{fl/fl}$ *p21*$^{-/-}$ mice(Mann-Whitney U test, p=0.000). **(c)** Box plot showing no difference in average tumour size between *Cre*$^{+}$ *Apc*$^{fl/+}$ and *Cre*$^{+}$ *Apc*$^{fl/+}$ *Myc*$^{fl/fl}$ *p21*$^{-/-}$ mice (Mann-Whitney U test, p> 0.04), suggesting that the few tumours that do grow out in *Cre*$^{+}$ *Apc*$^{fl/+}$ *Myc*$^{fl/fl}$ *p21*$^{-/-}$ mice are able to grow for long enough to cause large blockages.

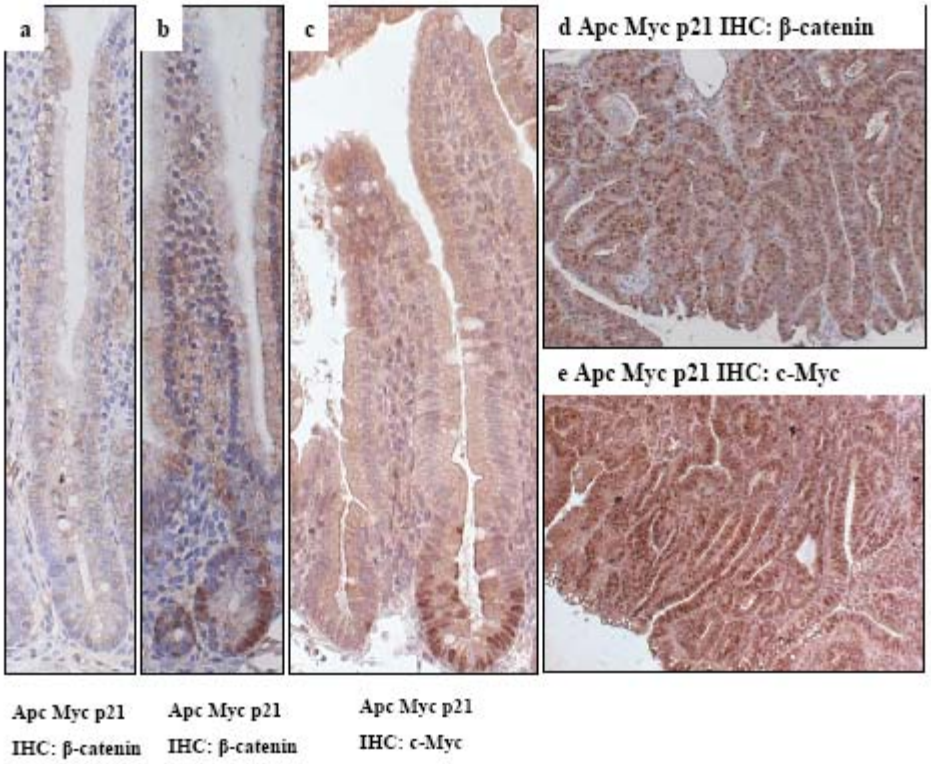


Figure 5.8: Tumours from *Cre*$^{+}$ *Apc*$^{fl/+}$ *Myc*$^{fl/fl}$ *p21*$^{-/-}$ mice are c-Myc proficient
(a) Immunohistochemistry for β-catenin showing that the majority of crypts in *Cre*$^{+}$ *Apc*$^{fl/+}$ *Myc*$^{fl/fl}$ *p21*$^{-/-}$ mice have regenerated from surrounding Wt crypts and no longer express active Wnt signalling. **(b)** IHC for β-catenin showing that some crypts remain recombined and continue to express high levels of β-catenin **(b)** with corresponding high levels of c-Myc **(c)**, illustrating that c-Myc is required for the maintenance of Apc deficient cells. **(d)** All tumours that arise in *Cre*$^{+}$ *Apc*$^{fl/+}$ *Myc*$^{fl/fl}$ *p21*$^{-/-}$ mice have high levels of β-catenin **(d)** and c-Myc **(e)**, illustrating that c-Myc is required for efficient tumour formation.

5.2 Discussion

Previous studies have shown that c-Myc is required for both the phenotypes of acute Apc loss and intestinal tumourigenesis. One of the hallmarks of Apc loss *in vivo* is a marked hyperproliferation which is strongly suppressed by c-Myc deletion (Sansom et al., 2007). Given the upregulation of p21 in these cells and previous studies suggesting that p21 repression by c-Myc is an essential tumour promoting function of c-Myc, we hypothesised TKO intestines would resemble single Apc deficient intestines. However here we show that p21 deficiency does not restore hyperproliferation to Apc Myc doubly knockout mice. Therefore this suggests that rather than transcription repression of cell cycle inhibitors, it is rather the transcriptional activation of cell cycle activators that drive the proliferation following Apc loss. Consistent with this hypothesis I have previously shown that both CDK4 and Cyclin D2 are upregulated following Apc deletion in the murine small intestine (Chapter 6). Indeed more recently we and others have shown that c-Myc heterozygosity suppresses intestinal tumourigenesis in Apc heterozygous mice and reduces hyperproliferation following Apc deletion (Athineos et al. 2009 submitted) Interestingly in these mice although there was a clear reduction in a number of the genes transcriptionally activated following Apc loss, there was not a clear upregulation of p21. This data therefore suggest that targeting the proproliferative targets of Wnt/Myc signalling might have some efficacy for chemoprevention following Apc gene deletion.

Moreover these data suggest that suppressing proliferation might be more important for chemoprevention strategies than trying to reestablish migration/differentiation to Apc deficient cells. In this study, we show that p21 deficiency perturbed the movement of Apc

c-Myc deficient cells and the differentiation of these cells into villus, however these cells were still unable to initate tumourigenesis *in vivo.*

The data presented here once again highlights the need to functionally study tumourigenesis in the correct cellular context *in vivo.* Previous studies have shown the central function of c-Myc in skin carcinogenesis induced by DMBA/TPA is to repress p21 as Myc p21 double knockout keratinocytes form tumours (Oskarsson et al., 2006). Given the strong *in vitro* evidence suggesting p21 repression was key in colorectal cancer cells (van de Wetering et al., 2002) and the marked upregulation of p21 in double Apc c-Myc deficient cells (Sansom et al., 2007), these studies all clearly predicted that p21 repression would be a very important requirement. A similar scenario in the liver following Apc loss has also been observed, where despite the fact that c-Myc is upregulated, c-Myc deficiency has no impact on the phenotypes of Apc loss (Sansom et al., 2007). Indeed in our study we present here, although p21 had no impact on the phenotypes of Apc loss in the intestine, $AhCre^{+}$ $Apc^{fl/fl}$ $p21^{-/-}$ and $AhCre^{+}Apc^{fl/fl}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice rapidly developed renal carcinoma due to expression of the AhCre in the kidney. Thus in this instance, Apc loss induced p21 which was crucial to prevent tumourigenesis and this could occur in the absence of the c-Myc protein (Chapter 4).

Although p21 deficiency was unable to restore tumourigenesis, it was enough to suppress differentiation and epithelial cell migration. At present it is difficult to uncouple these two processes, the movement of epithelial cells up the crypt villus axis could be dependent on them differentiating into villus. However, it is clear that p21 is required for

this process and it is one of the clearest examples *in vivo* of a cell cycle independent phenotypes of p21.

Previous *in vivo* studies have shown that p21 nullizygosity leads to an increase in tumour susceptibility with a tumour onset of 16 months, with the majority of tumours arising from hematopoietic, endothelial, and epithelial origins (Martin-Caballero et al., 2001). Studies have also demonstrated a direct correlation between p21 expression and increased survival rates in colorectal cancer patients (Zirbes et al., 2000) and a strong association between a lack or reduction in expression of p21 and metastasis and associated death (Bukholm and Nesland, 2000). These results may seem at odds with these findings presented here and elsewhere where p21 deficiency makes little differences to the phenotypes of Apc loss or tumourigenesis in Apc heterozygous mice [reviewed in Chapter 4, *Athineos et al*. 2009 submitted). However our studies are examining the function of p21 at the earliest stages of the carcinogenesis process (transformation and adenoma formation) and thus p21 could have later roles in suppressing metastasis. Future work examining p21 deficiency in more aggressive models of colorectal cancer should allow this to be delineated.

Taken together, our results show that the central function of c-Myc is not to transcriptionally repress p21 following Apc loss. However we have elucidated for the first time that p21 can drive differentiation/migration in these cells and this is likely to be independent of its role as a cycle cell inhibitor. Further studies to examine the importance

of other c-Myc target genes following Apc loss is therefore critical to delineate novel targets for colorectal cancer therapy.

Chapter 6: Cyclin D2 is upregulated immediately following Apc loss and is required for efficient intestinal tumourigenesis

6.0 Introduction

Previous studies have shown that acute loss of the Apc gene within the murine intestinal epithelium *in vivo* leads to a 'crypt progenitor cell-like' phenotype (Sansom et al., 2004, Andreu et al., 2005). Here there is a marked increased proliferation of intestinal enterocytes with reduced intestinal differentiation and migration. Coincident with the onset of this 'crypt-progenitor cell like phenotype' is the accumulation of nuclear β-catenin and the transcription of Wnt target genes such as c-Myc and CD44. The upregulation of c-Myc following Apc loss is critical to all the phenotypes observed, with double Apc c-Myc knockout intestines now proliferating to equivalent levels as wild type intestines (Sansom et al., 2007).

Previous studies have shown that CyclinD/CDK4/6 complexes may be essential downstream mediators of c-Myc dependent proliferation (Grandori and Eisenman, 1997). Consistent with this hypothesis, depending on cellular context CDK4, Cyclin D1 and Cyclin D2 have all been proposed to be potential transcriptional targets of c-Myc (Haas et al., 1997). Therefore these studies suggested that Apc deficient cells may be dependent on high levels of Cyclin D/CDK4-6 complexes. Even more pertinently Cyclin D1 has been proposed to be a canonical Wnt target gene and thus would provide a ready mechanism to elevate Cyclin D1 levels following Apc loss. However we have previously shown that Cyclin D1 is not upregulated immediately following Apc loss in the murine intestine and genetic deletion of Cyclin D1 makes no impact the immediate phenotype (including levels of proliferation) following Apc loss. Cyclin D1 is however upregulated

at later stages following Apc loss and is required for efficient adenoma formation (Sansom et al., 2005b).

In this study we focus on the role of Cyclin D2 following Apc loss in promoting proliferation and tumour formation in the intestinal epithelium. Cyclin D2 is normally expressed at the base of the intestinal crypt, where there are the highest levels of Wnt signalling (Yang et al., 2006). Studies have shown that Cyclin D2 is overexpressed in 53% of colon tumours and that over expression of Cyclin D2 may be related to a higher TMN stage of tumour (Mermelshtein et al., 2005). Mechanistically it has been suggested that Cyclin D2 is a direct c-Myc target gene, implying that Cyclin D2 levels should be deregulated following Apc loss. In this study we show that Cyclin D2 is rapidly deregulated following Apc loss where it plays a functional role to promote proliferation and tumourigenesis.

6.1 Results

6.1.1 Deletion of Apc leads to an upregulation of Cyclin D2

First we wished to assess whether Cyclin D2 was upregulated following Apc loss *in vivo*. We have previously deleted *Apc* in the intestinal epithelium using Cre-lox technology. Here mice carrying an inducible knockout *Apc* allele Apc^{580S} (from here on referred to as Apc^{fl}) are intercrossed to mice carrying the *AhCre* transgene which yields near constitutive inducible Cre recombinase expression within the intestinal epithelium following exposure to β-napthoflavone. Fours days following Cre induction these mice develop a robust phenotype, crypts become hyperplastic and β-catenin accumulates within the nucleus. Our previous microarray analysis suggested that Cyclin D2 is immediately upregulated following Apc loss *in vivo* (Sansom et al., 2004). To confirm this we performed QRT-PCR and ISH on wild type and Apc deficient intestine 4 days following Apc gene deletion. In both cases a clear upregulation of Cyclin D2 mRNA was observed suggesting that Cyclin D2 is transcriptionally activated (Figure 6.1a,b). To investigate proteins levels we performed immunohistochemistry and immunoblotting for Cyclin D2 and found it to be clearly deregulated in both cases (Figure 6.2 c-d, g). Indeed IHC analysis showed high Cyclin D2 in every cell with nuclear β-catenin in the Apc deficient intestines, which is in contrast to Cyclin D1 which only marks a few cells at this stage (Figure 6.2f). In addition to Cyclin D2 upregulation, previous microarray analysis also suggested that CDK4 was upregulated. This was confirmed by QRT-PCR , which shows a significant upregulation in CDK4 levels following Apc loss (Figure 6.2h)

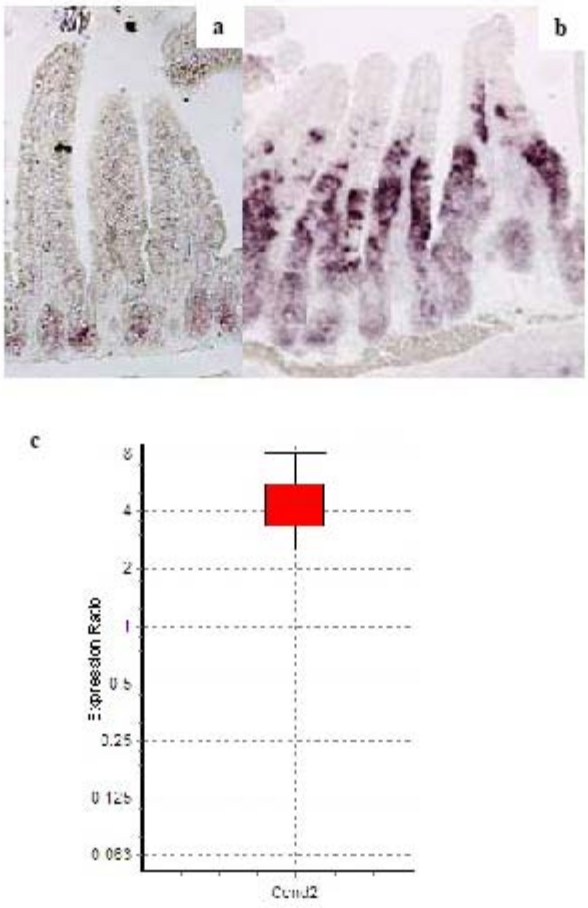


Figure 6.1: Cyclin D2 is upregulated immediately following loss of Apc

ISH for Cyclin D2 on wild type crypts **(a)** and *AhCre*$^{+}$ *Apc* $^{fl/fl}$ crypts **(b)** at day 4 post induction. Note strong upregulation of Cyclin D2 in *AhCre*$^{+}$ *Apc* $^{fl/fl}$ crypts. **c)** QRT-PCR confirming significant upregulation of Cyclin D2 mRNA in *AhCre*$^{+}$ *Apc* $^{fl/fl}$ crypts mice at day 4 post induction, compared to wild type crypts (4.292, p=0.004)

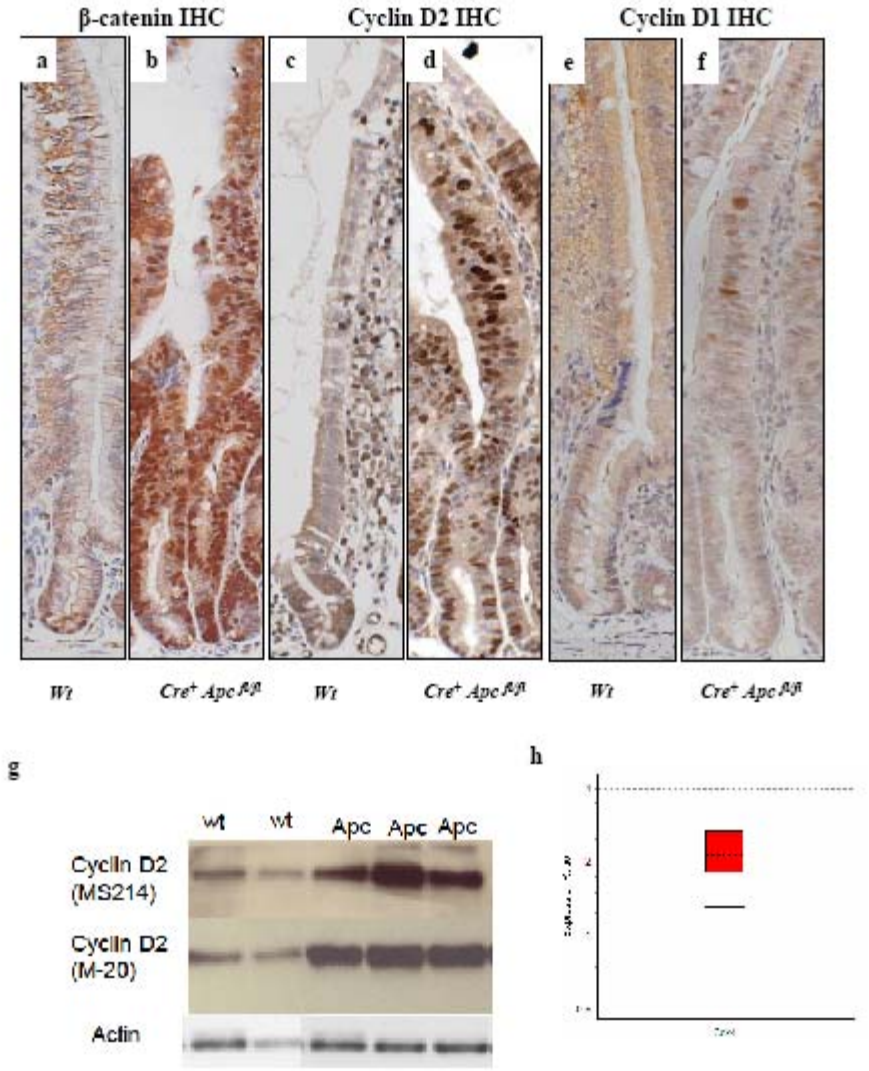


Figure 6.2: Cyclin D2 and CDK4 are upregulated immediately following loss of Apc

a-b) IHC for β-catenin showing low levels of nuclear expression in wild type *(Wt)* crypts **(a)** compared to strong nuclear upregulation throughout the hyperproliferative crypt-villus axis in *AhCre+ Apcfl/fl* crypts **(b). (c)(d)** IHC for Cyclin D2 showing showing high Cyclin D2 in every cell with nuclear β-catenin in *AhCre+ Apcfl/fl* crypts **(d). (e)(f)** IHC for Cyclin D1 showing that following Apc loss, unlike Cyclin D2, Cyclin D1 is only upregulated in a subset of cells at the crypt villus axis **(f). (g)** immunoblotting for Cyclin D2 shows strong upregulation of protein from intestinal epithelial cells extracts of *AhCre+ Apcfl/fl* mice compared to wild type. h) QRT-PCR showing a significant upregulation of CDK4 following loss of Apc, compared to wild type crypts (2.194, p= 0.005)

As Cyclin D2 has previously been shown to be transcriptionally activated by one of the key Wnt targets genes c-Myc, we next investigated whether the overexpression of Cyclin D2 required the presence of c-Myc. Therefore we examined levels of Cyclin D2 four days following combined Apc and c-Myc deficiency (Sansom et al., 2007). Fitting with the fact that c-Myc deletion rescues the proliferation following Apc loss, Cyclin D2 levels were significantly reduced in *AhCre*$^{+}$ *Apc* $^{fl/fl}$ *C-Myc*$^{fl/fl}$ mice. Analysis by QRT-PCR showed significant reduction in Cyclin D2 mRNA levels between *AhCre*$^{+}$ *Apc* $^{fl/fl}$ mice and *AhCre*$^{+}$ *Apc* $^{fl/fl}$ *C-Myc*$^{fl/fl}$ mice (Figure 6.3b). Furthermore, QRT-PCR showed significant reduction in CDK4 mRNA levels between *AhCre*$^{+}$ *Apc* $^{fl/fl}$ mice and *AhCre*$^{+}$ *Apc* $^{fl/fl}$ *C-Myc*$^{fl/fl}$ mice (Figure 6.3b). These results therefore indicate that following Wnt activation within the intestine, the upregulation of Cyclin D2 and CDK4 are C-Myc dependent, arguing that the upregulation of CDK4/Cyclin D2 complexes act as key c-Myc targets in driving proliferation following loss of Apc.

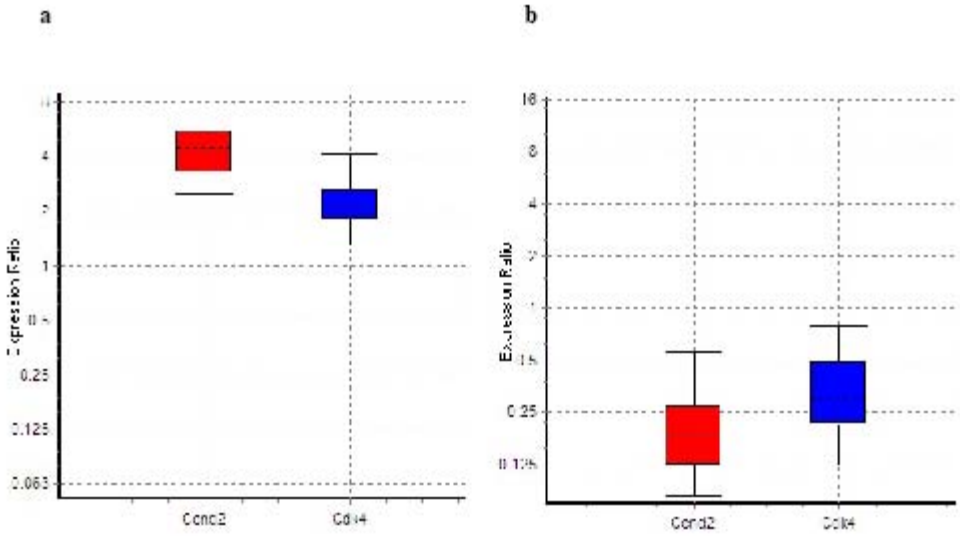


Figure 6.3:The upregulation of Cyclin D2 and CDK4 following loss of Apc are c-Myc dependent

a) QRT-PCR showing the upregulation of Cyclin D2 (4.292, p= 0.004) and CDK4 (2.194, p=0.005) following loss of Apc. **b)** QRT-PCR showing a decrease in expression of both Cyclin D2 (0.193, p= 0.01) and CDK4 (0.32, p= 0.004) in *AhCre*$^{+}$ *Apc*$^{fl/fl}$ *Myc*$^{fl/fl}$, illustrating that the upregulation of both Cyclin D2 and CDK4 are c-Myc dependent.

6.1.2 Cyclin D2 deficiency reduces crypt size and proliferation in Apc deficient intestinal crypts.

We next wanted to test the functional importance of Cyclin D2 upregulation following Apc loss and assess if Cyclin D2 is required for the hyperproliferation we observe. In order to investigate this, we intercrossed *Cyclin D2*$^{-/-}$ mice to *AhCre*$^{+}$ *Apc*$^{fl/fl}$ mice, and analysed the intestinal phenotype 4 days following Apc deletion. Although Cyclin D2 deficiency had no impact on crypt size in mice that are wild type for Apc (*AhCre*$^{+}$ *Apc*$^{+/+}$ Cyclin D2$^{-/-}$) (Figure 6.4a, Mann-Whitney U test, p=1.00), Cyclin D2 deficiency significantly reduced the crypt size of *AhCre*$^{+}$ *Apc*$^{fl/fl}$ *CyclinD2*$^{-/-}$ mice when compared to *AhCre*$^{+}$ *Apc*$^{fl/fl}$ *CyclinD2*$^{+/+}$ mice (Figure 6.4b, Mann- Whitney U test, p=0.01). To investigate whether this was due to less proliferation, we next examined BrdU incorporation and found that *AhCre*$^{+}$ *Apc*$^{fl/fl}$ *CyclinD2*$^{-/-}$ mice had significantly reduced proliferation compared to A*hCre*$^{+}$ *Apc*$^{fl/fl}$ *CyclinD2*$^{+/+}$ mice (Figure 6.5, Mann-Whitney U test, p=0.04). Once again, no impact was seen in Cyclin D2 knockout mice (A*hCre*$^{+}$ *Apc*$^{+/+}$ *CyclinD2*$^{-/-}$*)* (Mann-Whitney U test, Figure 6.5, p=0.1914). We also examined the other phenotypes associated with loss of Apc, namely increased apoptosis, and found no differences between A*hCre*$^{+}$ *Apc*$^{fl/fl}$ and A*hCre*$^{+}$ *Apc*$^{fl/fl}$ *CyclinD2*$^{-/-}$ mice (Data not shown). Cyclin D2 deficiency also did not affect β-catenin accumulation or localisation following Apc loss, highlighting that Cyclin D2 is downstream of Wnt signalling (Figure 6.6c)

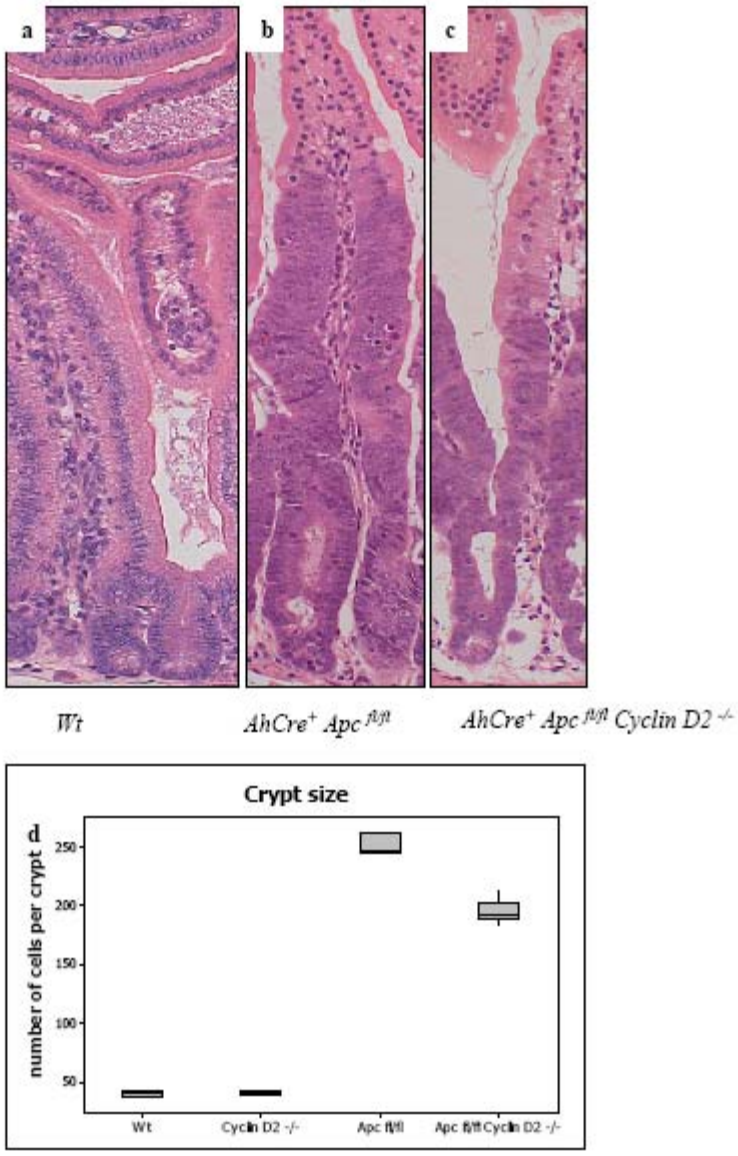


Figure 6.4: Deletion of Cyclin D2 following loss of Apc significantly reduces crypt size

a) H&E showing small crypts with a single cell crypt-villus axis in *wild type* mice. **b-c)** H&E showing a reduction in crypt size in *AhCre+ Apc fl/fl Cyclin D2 -/-* **(c)** compared to *AhCre+ Apc fl/fl* mice**(b). (d)** Box plot showing Cyclin D2 deficiency does not affect crypt size in wild type crypts (Mann-Whitney U test, p= 1.00). However following Apc loss, Cyclin D2 deficiency does significantly decrease crypt size (Mann-Whitney U test, p= 0.01).

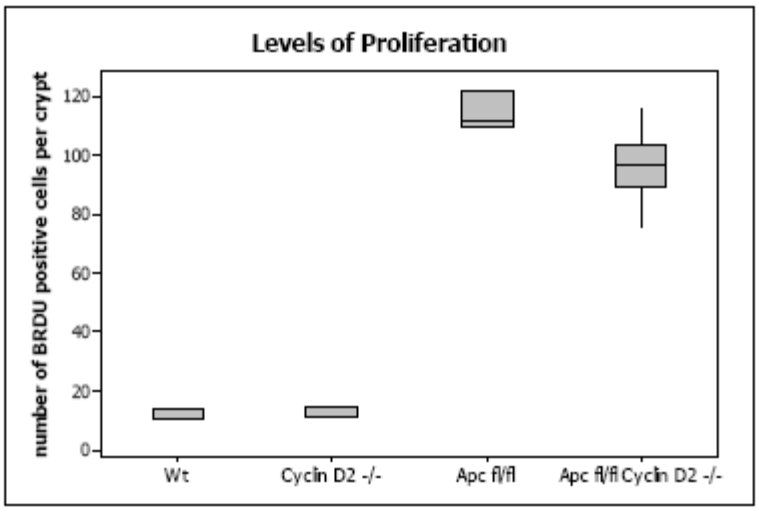


Figure 6.5: Deletion of Cyclin D2 following loss of Apc significantly reduces proliferation

Box plot showing Cyclin D2 deficiency does not affect proliferation in wild type crypts (Mann-Whitney U test, p= 0.1914). However following Apc loss, deletion of Cyclin D2 does significantly decrease proliferation levels (Mann-Whitney U test, p= 0.04).

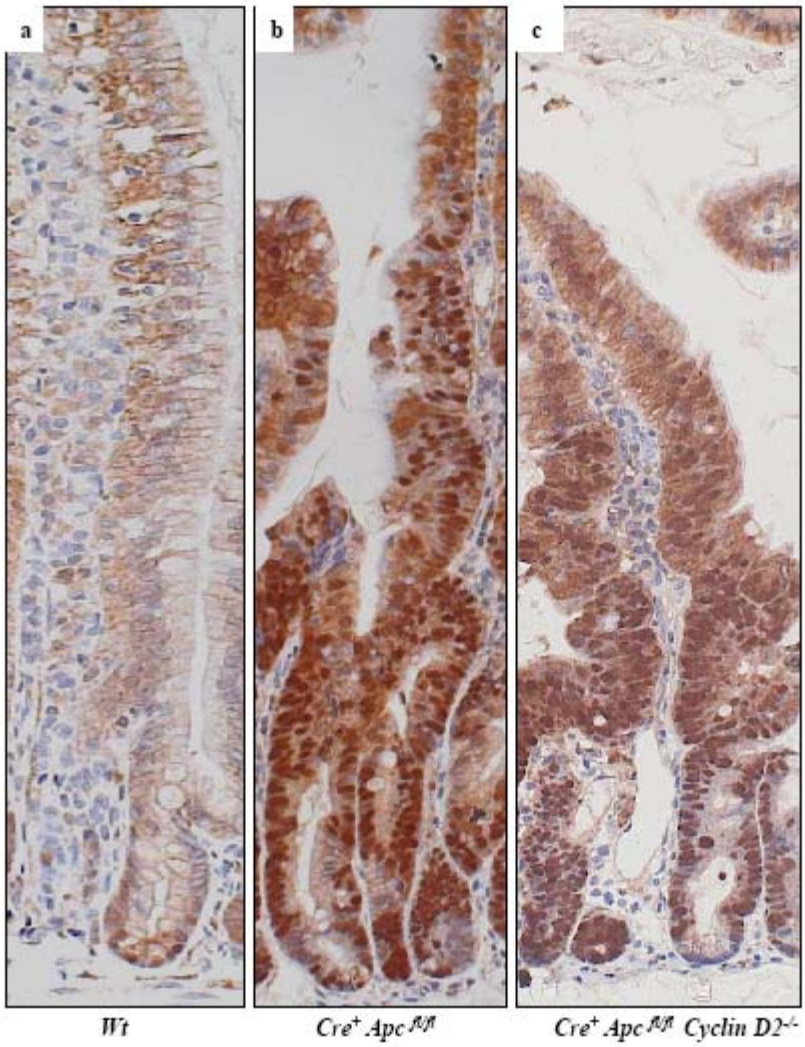


Figure 6.6: Cyclin D2 is downstream of Wnt activation

a-c) IHC for β-catenin showing low levels of nuclear β-catenin in the base of wild type crypts **(a). b-c)** IHC for β-catenin showing that accumulation of nuclear β-catenin remains unchanged in *Cre+ Apc fl/fl Cyclin D2-/-*, illustrating that Cyclin D2 is downstream of Wnt signalling.

6.1.3 Cyclin D2 deficiency leads to a dramatic reduction of tumour burden and increased survival within the Apc $^{Min/+}$ mouse

Given the reduced crypt size and proliferation observed in *AhCre*$^{+}$ *Apc*$^{fl/fl}$ *CyclinD2*$^{-/-}$ mice, we next wished to assess if this was functionally relevant for tumour formation. This was especially interesting given Cyclin D2 deficiency was only affecting proliferation following Apc loss and would allow us to evaluate if reducing only one of the multitude of phenotypes could modify tumourigenesis. To investigate the effect of Cyclin D2 on tumour formation and survival, we crossed *Cyclin D2* $^{-/-}$ mice to the *Apc*$^{Min/+}$ mouse. Two studies were performed. First, in order to determine the effect of Cyclin D2 on survival, a cohort containing *Apc*$^{Min/+}$ (n=29), *Apc*$^{Min/+}$ *Cyclin D2* $^{+/-}$ (n=26) and *Apc*$^{Min/+}$ *Cyclin D2*$^{-/-}$ (n=21) were aged until showing signs of intestinal illness. This included paling feet, starry coats and hunching. Figure 6.7a shows that deletion of Cyclin D2 significantly increased the survival of these *Apc*$^{Min/+}$ *Cyclin D2*$^{-/-}$ when compared to *Apc*$^{Min/+}$ *Cyclin D2*$^{+/+}$ mice (Log rank test, $p<0.001$). Heterozygous deletion of Cyclin D2 did not significantly affect survival in these mice (Log rank test, $p=0.2516$). At death, tumour burden of *Apc*$^{Min/+}$ *Cyclin D2*$^{-/-}$ mice (Figure 6.7b, Mann- Whitney U test, $p= 0.08$) as well as average tumour size (Figure 6.7c, Mann-Whitney U test, $p= 0.0894$), was similar to those in *Apc*$^{Min/+}$ *Cyclin D2*$^{+/+}$ mice, confirming that mice were being euthanized at the same stage of disease.

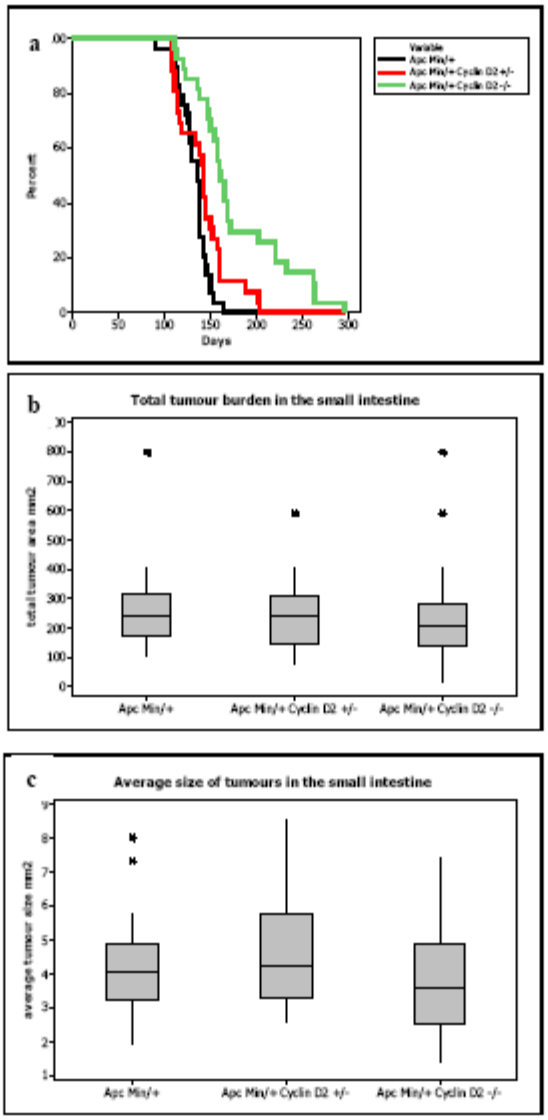


Figure 6.7:Deletion of Cyclin D2 significantly prolongs survival of *Apc* $^{Min/+}$ mice

a) Survival plot shows that *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice survive significantly longer than *Apc* $^{Min/+}$ mice (Log rank test, $p<0.001$). Heterozygous deletion for Cyclin D2 did not affect survival compared to *Apc* $^{Min/+}$ mice (Log rank test, $p=0.2516$). **b)** At death, *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice have a similar total tumour burden (Mann Whitney U test, $p=0.08$), as well as similar average tumour size (Mann-Whitney U test, $p=0.0894$) to *Apc* $^{Min/+}$ mice, illustrating that mice were being sacrificed at the same stage of disease **(c).**

To ensure mice had increased lifespan due to delayed tumourignesis we aged a second cohort which were euthanized at a timepoint of 110 days to examine levels of tumourigenesis (*Apc*$^{Min/+}$ n=30, *Apc*$^{Min/+}$ *Cyclin D2* $^{+/-}$ n=30, *Apc*$^{Min/+}$ *Cyclin D2* $^{-/-}$ n=23). Once again, *Apc*$^{Min/+}$ *Cyclin D2* $^{-/-}$ displayed a significant reduction in both total tumour burden (Figure 6.8a, Mann-Whitney U test, p‹ 0.0001), as well as average tumour size (Figure 6.8b, Mann- Whitney U test, p< 0.0001). As in the ageing cohort, heterozygous deletion of Cyclin D2 did not affect total tumour burden (Figure 6.8a, Mann-Whitney U test, p= 0.2451) or average tumour size (Figure 6.8b, Mann-Whitney, p=0.1452)

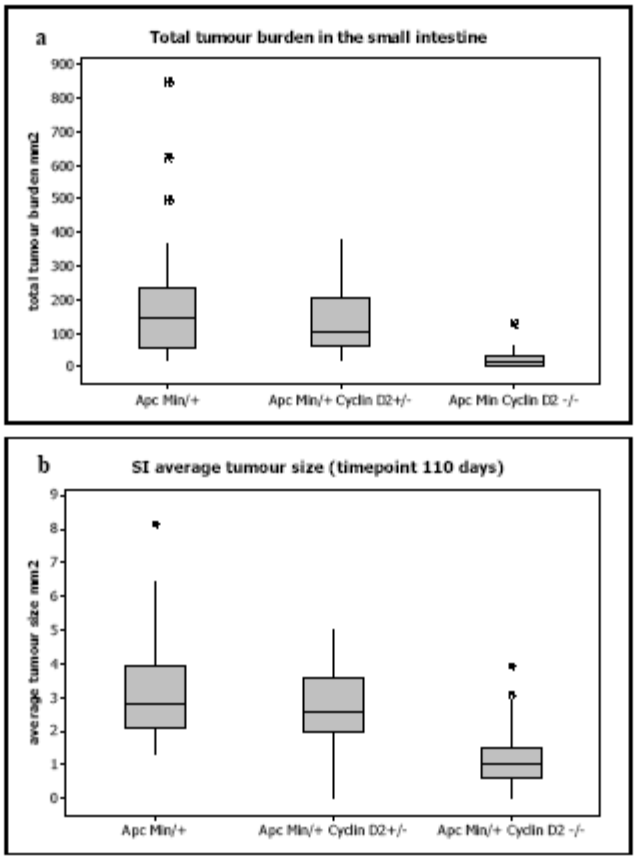


Figure 6.8: Deletion of Cyclin D2 significantly decreases tumour burden and average tumour size in *Apc* $^{Min/+}$ mice

a) Box plot showing that *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice have significantly less tumours in the small intestine than *Apc* $^{Min/+}$ mice (Mann-Whitney U test, $p<0.001$). Heterozygous deletion for Cyclin D2 did not affect total tumour burden compared to *Apc* $^{Min/+}$ mice (Mann-Whitney U test, $p=0.2451$). **b)** Box plot showing *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice have a significantly smaller average tumour size than *Apc* $^{Min/+}$ mice (Mann-Whitney U test, $p< 0.001$). Similarly to tumour burden, heterozygous deletion for Cyclin D2 did not affect average tumour size compared to those of *Apc* $^{Min/+}$ mice ($p=0.1452$)

Figure 6.9 shows immunohistochemical staining for β-catenin, showing strong nuclear staining within the polyps of both *Apc*$^{Min/+}$ (Figure 6.9a) and *Apc*$^{Min/+}$ *Cyclin D2*$^{-/-}$ (Figure 6.9b) mice. Importantly, immunohistochemical staining for Cyclin D2 shows a complete ablation of protein from both the colonic epithelium and polyps from *Apc*$^{Min/+}$ *Cyclin D2*$^{-/-}$ mice (Figure 6.9 c). Moreover, Figure 6.10 shows BrdU IHC in polyps from both sets of mice. Polyps from *Apc*$^{Min/+}$ *Cyclin D2*$^{-/-}$ mice are significantly less proliferative than *Apc*$^{Min/+}$ mice as scored through total number of BrdU positive cells per 500 tumour cells (Figure 6.10, Mann-Whitney U test, p= 0.04).

Taken together, these results show that Cyclin D2 is key for intestinal tumourigenesis following activation of the Wnt pathway. Therefore deletion of Cyclin D2 results in a significant increase in survival in the *Apc* $^{Min/+}$ mouse model, which is due to a reduction in the ability of Apc deficient cells to proliferate.

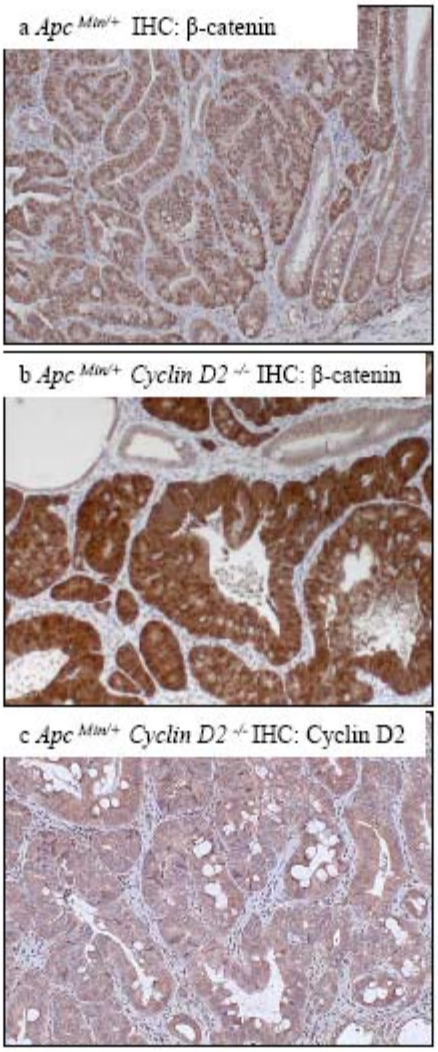


Figure 6.9: Adenomas from *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice continue to display high levels of β-catenin

a-c) IHC for β-catenin showing high levels of nuclear β-catenin in adenomas from both *Apc* $^{Min/+}$ **(a)** and *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ **(b)** mice. **c)** IHC for Cyclin D2 showing complete ablation of protein expression in adenomas from *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice

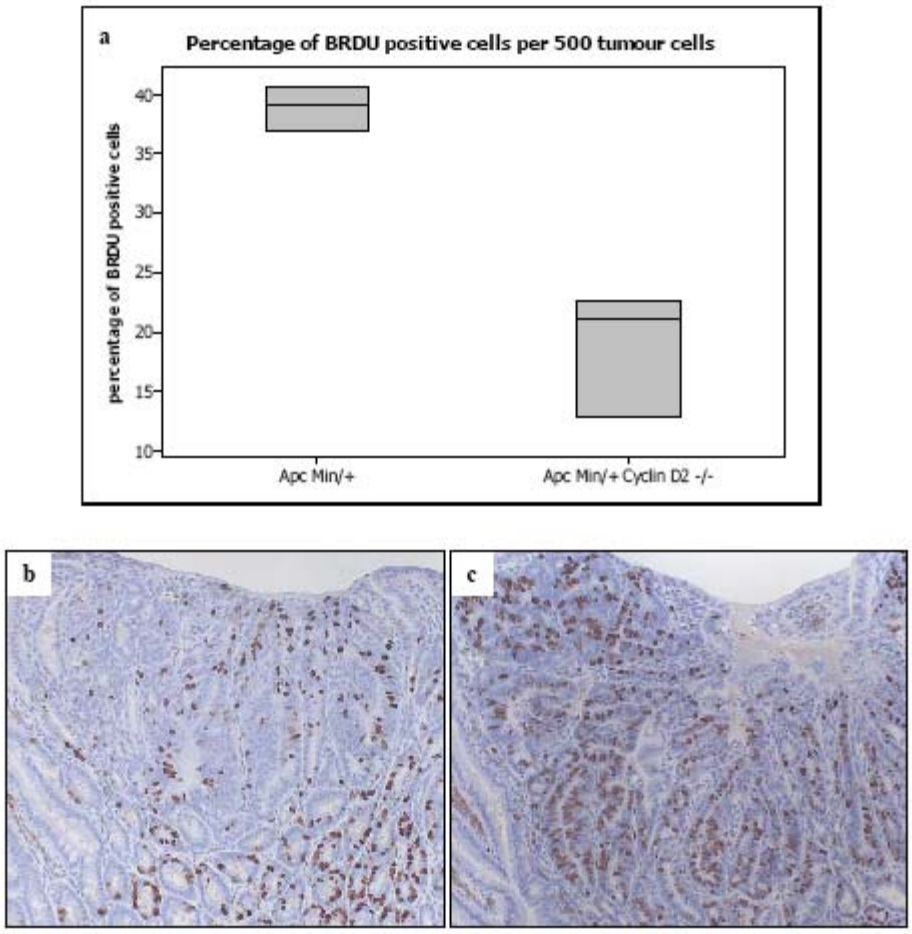


Figure 6. 10: Adenomas from *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice are significantly less proliferative than those from *Apc* $^{Min/+}$ mice

a) Box plot showing a significant reduction in proliferation in tumours arising from *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ compared to *Apc* $^{Min/+}$ mice (Mann-Whitney U test, p=0.04). **b)** IHC for BrdU showing less BrdU positive cells within tumours from *Apc* $^{Min/+}$ *Cyclin D2* $^{-/-}$ mice, compared to those of *Apc* $^{Min/+}$ mice **c).**

6.2 Discussion

In this study we have shown that Cyclin D2 is required for efficient proliferation and tumourigenesis following Apc loss. This finding is particular pertinent to human colorectal cancer as previous studies have shown that overexpression of Cyclin D2 has been reported to be the most considerable aberration among G1-phase regulators in human colonic polyps (Bartkova et al., 2001). Moreover, a positive relation has also been reported between overexpression of Cyclin D2 and higher TNM stage of tumour, suggesting that overexpression of Cyclin D2 correlates to a high metastatic degree of tumour (Mermelshtein et al., 2005).

There has been much debate over the role of Cyclin D1 as a Wnt target gene in colorectal cancer. Our previous studies have shown that Cyclin D1 is not immediately upregulated following Apc loss in the murine intestinal epithelium and our data shown here suggests Cyclin D2 is more important at these earliest stages of intestinal neoplasia. However, it has been clearly shown that Cyclin D1 is upregulated in adenomas of $Apc^{Min/+}$ mouse and Cyclin D1 deficiency can suppress tumourigenesis (Sansom et al., 2005b). Thus our finding that Cyclin D2 loss can also suppress tumourigenesis is even more impressive, as there are still high levels of Cyclin D1 within these adenomas which cannot completely compensate for the lack of Cyclin D2. This is in contrast to normal intestinal epithelium which shows no defect in proliferation in the absence of Cyclin D2, highlighting that Apc deficient intestinal enterocytes are somewhat dependent on high Cyclin D2 levels. Previous *in vivo* studies have generated mice which express knockout deletions of Cyclin D1 and Cyclin D2 [reviewed in (Santamaria and Ortega, 2006)]. In both cases, mice are

completely viable with observable defects only in specific cell types. For example, deletion of Cyclin D1 results in neurological abnormalities as well as defects in mammary cell proliferation during pregnancy (Fantl et al., 1995, Sicinski et al., 1995). Whilst, Cyclin D2 deletion majorly effect pancreatic beta cell, granulosa cell and B-lymphocytes proliferation (Georgia and Bhushan, 2004, Sicinski et al., 1996b). Given the key role that these D-type Cyclins play in controlling the cell cycle, these relatively minor defects in specific cell type proliferation can be attributed to the compensatory effect of D-type Cyclins. For example, combined deletion of Cyclin D1 and D2 results in death in the first three weeks of life due to hypoplastic cerebellum (Santamaria and Ortega, 2006). It is therefore tempting to speculate that Apc deficient epithelium would be unable to proliferate in the absence of both Cyclin D1 and Cyclin D2. However, due to the reduced survival of double Cyclin D1/D2 knockouts, studies examining the combined effects of Apc, Cyclin D1 and Cyclin D2 would need to be done *in utero*, and may prove difficult.

Apc deletion within the intestinal epithelium, leads to a 'crypt progenitor cell like phenotype' of hyperproliferation, failed differentiation and failed migration. In this study, we show that reduction of just one of these phenotypes, hyperproliferation can strongly suppress tumourigenesis. This raises the possibility that other strategies such as inhibiting CDK4/6 (which we have shown is upregulated following Apc loss) may also suppress tumourigenesis and unlike Cyclin D2 there are small molecule kinase inhibitors to these molecules currently under preclinical development and in Phase 1 trials. Pyridopyrmidine (PD -0332991 from Phizer), which selectively inhibits CDK4 and CDK6, is currently in phase 1 clinical trials for mantle cell lymphoma and phase 2-3 clinical trials for multiple

myeloma and hormone receptor-positive advanced breast cancer. Of interest to this study, Pyridopyrmidine (PD -0332991) was shown to cause regression in mice with a human colon carcinoma xenograft. However despite these finding Pyridopyrmidine (PD -0332991) has not yet begun clinical trial testing for colon cancer [reviewed in (Lapenna and Giordano, 2009)]. Our previous studies have shown that c-Myc deletion rescues all the phenotypes following Apc loss and thus blocks tumour formation and indeed heterozygosity for c-Myc also strongly suppresses tumourigenesis. Although this makes c-Myc an excellent target, given the difficulty in targeting a transcription factor such as c-Myc, it may be a more efficacious strategy to inhibit in combination a number of pathways downstream of Apc loss where drugs are already available. It is interesting to note in this study that expression of Cyclin D2 and CDK4 following Apc loss were c-Myc dependent, illustrating that the upregulation of these Cyclin D2/CDK4 complexes is key for c-Myc dependent proliferation following Apc loss.

Taken together, these results indicate the important proliferative role that CyclinD/CDK4-6 complexes plays following Wnt activation that drive tumour formation. Moreover, raises the possibility that inhibition of Cyclin D/CDK4-6 may be useful in those individuals with high risk of colorectal cancer.

Chapter 7: Summary

This section gives a brief overview of the main conclusions of this thesis.

One of the key aims of this thesis was to examine the role of c-Myc in signalling apoptosis following DNA damage *in vivo.* To study this I have conditionally deleted *c-Myc* from the adult murine intestine and investigated the apoptotic response of intestinal enterocytes. Remarkably, c-Myc deletion completely abrogated the immediate wave of apoptosis following both ionizing irradiation and cisplatin, recapitulating the phenotype of p53 deficiency in the intestine. Consistent with this finding, c-Myc deficient intestinal enterocytes did not upregulate p53. Mechanistically this was linked to an upregulation of the E3 Ubiquitin ligase MDM2, which targets p53 for degradation in c-Myc deficient intestinal enterocytes. This was confirmed with treatment of c-Myc deficient intestinal enterocytes with the MDM2 inhibitor Nutlin, which restored p53 function and apoptosis.

Therefore, I have elucidated for the first time *in vivo* an essential role for endogenous c-Myc in signalling DNA damage induced apoptosis through the control of the p53 tumour suppressor protein.

Secondly, another aim of this thesis was to examine the underlying tissue specific nature of *Apc* gene deletion in cancer. To do this I investigated the consequences of *Apc* gene deletion within the renal epithelium.

I have shown that *Apc* gene loss within the renal epithelium leads to an upregulation of the senesence markers p21, p16 and senescence associated beta-galactosidase. Apc deficient cells are cleared and very rarely initiate tumourigenesis. However combined *Apc* and *p21* gene deletion rapidly initiated tumourigenesis with all mice developing renal carcinoma by 2 months of age. I have also shown that in the context of the renal epithelium, following Apc loss, c-Myc is unable to repress p21, and therefore renal tumourigenesis in Apc p21 double knockout mice proceeds in a c-Myc independent fashion.

In contrast, I have shown that *Apc* loss within the intestinal epithelium only induces p21 in a small subset of cells. All Apc deficient cells in the intestinal epithelium were Ki-67 positive and no evidence of senescence was observed. Combined *Apc* and *p21* loss had no impact on either the short term phenotypes of *Apc* loss or tumourigenesis.

Taken together these results show that for the first time that *Apc* loss *in vivo* can invoke a senescence program but in a context dependent fashion. This implies escape from senescence is not a crucial pathway to overcome in colorectal cancers that are initated by Apc loss. This study also provides the first genetic evidence that p21 can act as potent tumour suppressor *in vivo* downstream of a senescence pathway. This finding is in accordance with other data from this lab which has shown that p21 gene knockout

strongly cooperates with $Kras^{G12D}$ to drive pancreatic cancer in the mouse, and p21 expression is downregulated in approximately 40% of human pancreatic ductal adenocarcinoma (Morton et al submitted).

Thirdly, in order to determine the functional importance of repression of p21 by c-Myc in Apc deficient cells, I have generated triple knockout $AhCre^{+}Apc^{fl/fl}$ $Myc^{fl/fl}$ $p21^{-/-}$ mice. Results from these experiments showed that intestinal crypts from Apc Myc p21 mice were morphologically identical to those of double mutant $AhCre^{+}Apc^{fl/fl}$ $Myc^{fl/fl}$ mice, with levels of proliferation remaining unchanged between both sets of mice. Importantly, intestines from triple knockout mice displayed significantly lower levels of proliferation compared to those of $AhCre^{+}Apc^{fl/fl}$ mice. However intestinal enterocytes from triple knockout mice were unable to move up the crypt villus axis and differentiate into villus. I have also shown in a tumourigenic study that despite p21 deletion, triple knockout mice are unable to form tumours in the absence of c-Myc. Taken together these results illustrate for the first time *in vivo* that following combined Apc and c-Myc loss, p21 is able to drive differentiation, and remarkably this function is independent of its role as a cell cycle inhibitor. Furthermore, these studies show that the repression of p21 by c-Myc is not essential for the enhanced proliferation that is observed immediately following Wnt activation in the intestine, nor is this repression key for tumourigenic progression.

Lastly, in this study I investigated the expression and functional relevance of Cyclin D2 upregulation following Apc loss in the intestinal epithelium. I have shown that Cyclin D2 and CDK4 are upregulated immediately following Apc loss and knockout of Cyclin D2 reduced enterocyte proliferation and crypt size within Apc deficient intestinal epithelium. Importantly however, Cyclin D2 deficiency did not affect proliferation of normal enterocytes. Moreover, Cyclin D2 deficiency dramatically reduced tumour growth and development in $Apc^{Min/+}$ mice. Therefore, I have shown that Cyclin D2 is required for efficient proliferation and tumourigenesis following Apc loss, which is mechanistically due to the direct upregulation of Cyclin D2 by c-Myc. Taken together, these studies suggest that the inhibition of Cyclin D/CDK4/6 complexes may prove effective treatment for patients with high risk of colon cancer.

In conclusion, the key aim of this thesis was to investigate the downstream mechanisms by which c-Myc induces and controls proliferation following loss of Apc. Taken together results from this study have elucidated that the repression of p21 by c-Myc is not essential for the tumour promoting function of c-Myc following loss of Apc. Given that Cyclin D2 and CDK4 are immediately unregulated following loss of Apc, this suggests that rather than transcription repression of cell cycle inhibitors, it is rather the transcriptional activation of cell cycle activators that drive the proliferation following Apc loss. Given the broad reach of Myc as a transcription factor, actual drug targeting of Myc *in vivo*, may prove to be very difficult. Therefore, it would be more effective to target downstream effectors of Myc such as Cyclin D/CDK4-6 complexes, especially given the fact that CDK 4-6 inhibitors are already in clinical trials for treatment against

other cancers such as lymphoma, myeloma and breast cancer. It would be of great interest to examine the effect of such CDK 4-6 inhibitors in the $Apc^{Min/+}$ model of tumourigenesis.

Lastly, the other key aim of this thesis was to question the role of colorectal cancer in senescence. In this study, I have shown that *Apc* loss within the renal epithelium is strongly disadvantageous, with cells upregulating p21 and undergoing senescence. This provides a clear rationale to explain why *Apc* mutations are not intiating oncogenic events in the kidney. In the intestine, where *Apc* loss drives carcinogenesis, *Apc* mutation drives a strong proliferative program. Therefore, this study yields crucial insights into the context specific outcome of Wnt signalling and suggests that senescence is not a key barrier to tumourigenesis in CRC that has been initiated with an *APC* mutation. Given that other oncogenic drivers of senescence such as $Kras^{v12}$, $Kras^{D12}$, LKB1 and PTEN have all been shown to induce proliferation rather than senescence within the intestinal epithelium, it would be of great interest to investigate the outcome of $BRAF^{V600e}$ mutations, as it is the last remaining oncogenic driver of colorectal cancer possible of inducing senescence.

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