Investigating the roles of RKIP and p53 in colorectal carcinoma

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Author 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.

Abstract

Raf Kinase Inhibitor Protein (RKIP) was originally described as an inhibitor of the Ras-Raf-MEK-ERK pathway, exerting its action by the physical inhibition of the interaction of Raf with MEK. It has subsequently been shown to play important roles in a number of other signalling pathways, including the NFκB pathway and in the stability of the mitotic spindle. Not surprisingly given that it impacts on many important signalling pathways RKIP levels have been shown to be important in the progression of a number of different cancers. RKIP expression is lost or decreased in a number of common human cancers and decreased still further in tumour metastases.

One of the tumours in which RKIP is downregulated is colorectal cancer (CRC). Importantly it has been shown that not only is RKIP depleted in tumour tissue when compared with normal tissue but that the level of RKIP within a tumour is inversely correlated with the likelihood of metastatic relapse and with patient prognosis. Although we already have a number of very good prognostic indicators in CRC, one group of patients for whom new prognostic indicators would be useful are patients with Dukes B CRC. These are patients with locally advanced but non-metastatic disease and at present there is no firm consensus on their correct post-operative management. Therefore we set out to examine whether RKIP is a useful prognosticator in this particular group using a tissue microarray (TMA) with samples from over 200 patients with Dukes B CRC. The analysis revealed a strong inverse correlation between RKIP levels and disease specific survival. Moreover, in a multivariate analysis RKIP emerged as an independent prognostic indicator along with lympho-vascular invasion and peritoneal invasion,

two well-known and powerful prognosticators. This allowed for the generation of a simple prognostic index, using information from the different independent indicators, allowing for improved patient risk stratification.

This led us to examine whether RKIP could also function as a predictive marker in CRC. To do this we again used a TMA, this time consisting of a much larger cohort of patients across the whole range of tumour stages. The results confirmed the prognostic utility of RKIP and indicated that patients whose tumours have low levels of RKIP may derive a greater benefit from chemotherapy than those patients whose tumours have high levels, although this result did not reach statistical significance.

In the second part of the thesis I have examined the effect of RKIP in previously characterised mouse models of CRC. To do this I have used a germline RKIP knockout mouse and in the first instance crossed it to the APC^{580S} mouse. In this mouse APC is lost conditionally within the intestine and liver. RKIP knockout did not have any effect on the rate of tumourigenesis or on the invasiveness of tumours in this model. However, in the setting of acute homozygous deletion of APC, RKIP knockout resulted in a decrease in apoptoses in the small intestine and an increase in aberrant mitotic activity in the liver. To follow this up I have examined the effect of RKIP knockout in a mouse model of superficially invasive CRC, specifically to see if RKIP knockout can promote invasive and metastatic behaviour. In this model the APC^{580S} mouse is crossed to mice which conditionally express oncogenic KRas. Although RKIP knockout did not result in an increase in invasive tumours in this model there was a shift in tumour location from the small intestine to the colon. This shift appeared to be due, at least in part to an increase in chromosomal instability in the tumours.

The final aim of the thesis was to develop a mouse model of CRC which more closely recapitulates the late stages of the human disease, specifically invasion and metastasis. To do this we have crossed the APC^{580S} mouse with either a conditional p53 knockout or with a mouse that conditionally expresses a point mutation of p53 ($p53^{R172H}$). In human tumours the majority of abnormalities of p53 are point mutations that result in the production of mutant protein that accumulates in tumour cells. There is evidence that this mutant protein may have oncogenic properties beyond the simple loss of normal p53 protein function. Therefore we have also used this model to study the differing effects of p53 loss and point mutation in CRC. We found that mice homozygous for p53 deletion ($p53^{fl/fl}$) and those expressing a single copy of the mutant allele with loss of the second copy ($p53^{R172H/fl}$) developed invasive tumours with nearly 100% penetrance and indeed metastasis was observed. Remarkably, although mice that were heterozygous for p53 deletion ($p53^{fl/+}$) only rarely developed invasive tumours almost 100% of mice expressing a single copy of the mutant allele ($p53^{R172H/+}$) developed invasive tumours. We went on to show that the increase in invasion seen in this model is related to an increase in Wnt signalling, which is associated with increased expression of pro-invasive Wnt targets such as fascin. We also showed a novel pro-invasive role for ARF in this process. This is also an excellent model of Dukes B CRC and therefore the ideal model to test the effect of RKIP deletion on invasion and metastasis.

These studies led us to examine the differences in effect between knockout and mutant p53 in another tumour model. In this we used a novel model of the aggressive tumour pleomorphic rhabdomyosarcoma to demonstrate that mutant p53 can both promote both tumourigenesis and metastasis more potently than p53 knockout.

These studies have demonstrated the value of RKIP in the clinically important Dukes B CRC population and shown its possible utility as a predictive marker in this group. Although we have not seen an effect of RKIP knockout in traditional mouse models of CRC we have developed a novel model which closely recapitulates Dukes B CRC and may be useful in elucidating the effect of RKIP knockout. We have also used this model to gain novel insights into the invasive process, in particular into the role played by mutant p53.

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List of Abbreviations

APC: Adenomatous polyposis coli (*gene*/protein)

ARF: Alternative reading frame

ATM: Ataxia-Telangectasia Mutated (ATM)

ATR: Ataxia-Telangectasia and Rad3 related

BRDU: Bromodeoxyuridine

Bub1: Budding uninhibited by benzimidazoles

CD: Crohn's disease

CIMP: CpG island methylator phenotype

CIN: Chromosomal instability

CK1α: Casein kinase 1α

CRC: Colorectal Cancer

EMT: Epithelial to mesenchymal transition

ERK: Extra-cellular signal related kinase

FAP: Familial adenomatous polyposis

GFP: Green fluorescent protein

GIST: Gastrointestinal stromal tumour

GPCR: G-protein coupled receptor

GRK2: G-protein coupled receptor kinase-2

GSK: Glycogen synthase kinase

HCC: Hepatocellular carcinoma

H&E: Haematoxylin and eosin

HGD: High grade dysplasia

HNPCC: Hereditary non-polyposis colorectal cancer

HPRT1: hypoxanthine phosphoribosyltransferase 1

IHC: Immunohistochemistry

IKK: IκB Kinase

ISH: In-situ hybridisation

LEF-1: Lymphoid enhancer factor-1

LGR5: Leucine-rich repeat-containing G-protein coupled receptor 5

LOH: Loss of heterozygosity

LVI: Lympho-vascular invasion

MAPK: Mitogen activated protein kinase

MDM2: Murine double minute

MEK: MAPK/ERK Kinase

Min: Multiple intestinal neoplasias

MSI: Microsatellite instable

MSP: Methylation specific polymerase chain reaction

MSS: Microsatellite stable

NBF: Neutral buffered formalin

PCR: Polymerase chain reaction

PEBP: Phosphatidylethanolamine-binding protein

pERK: Phosphorylated extra-cellular signal related kinase

PI-3Kinase: Phosphoinositide 3-kinase

PKC: Protein kinase C

PTEN: phosphatase and tensin homolog (*gene*/protein)

Rb: Retinoblastoma (*gene*/protein)

RKIP: Raf Kinase Inhibitor Protein (*gene*/protein)

RTK: Receptor tyrosine kinase

rtPCR: Reverse transcriptase polymerase chain reaction

SAMP repeats: Serine-Alanine-Methionine-Proline repeats

Sox9: Sry-related HMG box-9

TCF: T-Cell factor

TGF-β: Transforming growth factor-β

TMA: Tissue microarray

UC: Ulcerative Colitis

ZEB-1: Zinc finger E-box-binding homeobox-1

Chapter 1 Introduction

In this introduction I will firstly discuss the role played by Raf Kinase Inhibitor protein (RKIP) in cancer and then go on to discuss the role it plays in signal transduction and in other relevant pathways. I will then introduce the RKIP knockout mouse.

The second part of the introduction will cover colorectal cancer (CRC) and will discuss some of the genes that are important in the genesis and progression of this disease, in particular *APC* and *p53*.

In the final part I will discuss mouse models of CRC, as an introduction to our work in which we have developed a novel paradigm of this disease and used it to study some of the mechanisms underlying invasion in CRC.

1.1 Introduction to RKIP

1.1.1 An overview of RKIP

RKIP is a member of the phosphatidylethanolamine-binding protein (PEBP) family. The family of proteins displays evolutionary conservation and is defined by the presence of a shared ligand binding domain (Schoentgen and Jolles 1995). The *RKIP* gene is located on chromosome 12 and encodes a 21 kDa protein, comprised of 187 amino acids. The crystal structure for the human protein has been solved and was published in 1998 (Figure 1.1) (Banfield, Barker et al. 1998). RKIP is expressed in the majority of normal mammalian tissues, including the intestine, but is expressed at its highest levels in the brain, testis, liver and kidney (Frayne, McMillen et al. 1998; Frayne, Ingram et al. 1999). RKIP has been shown to play a number of important roles in mammalian physiology (Keller, Fu et al. 2004), in particular in neural development, where it is thought to play a role in the development of the myelin sheath (Moore, Perry et al. 1996) and in spermatogenesis, where high levels of the protein are found in the maturing sperm (Perry, Hall et al. 1994).

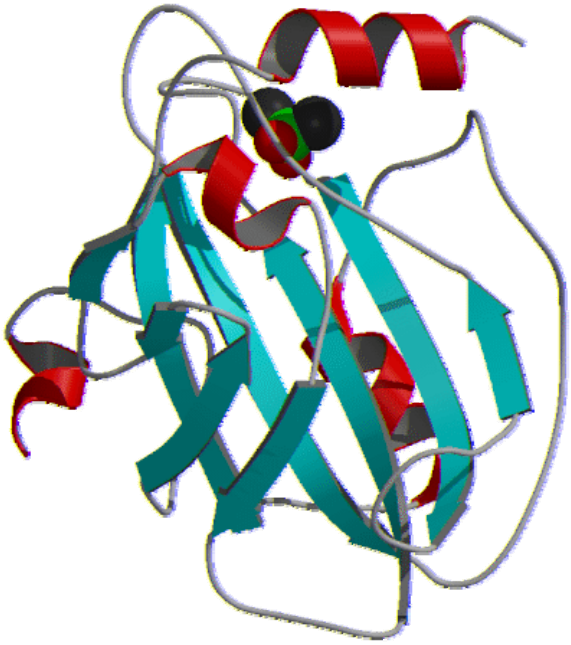


Figure 1.1: Crystal structure of the human RKIP protein

Taken from Banfield et al 1998.

1.1.2 Role of RKIP in cancer

RKIP deregulation has been shown to occur in a number of different tumour types including CRC, breast, prostate and hepatocellular carcinoma (HCC) as well as melanoma and a number of rarer tumours. In breast cancer it has been shown that RKIP levels are higher in primary tumours than in lymph node metastases (Hagan, Al-Mulla et al. 2005). Similarly in prostate cancer RKIP levels are lower in metastases than in primary tumours, RKIP levels are also decreased in tumours of higher Gleason grade (Fu, Smith et al. 2003). Similar results have been demonstrated in HCC and melanoma (Schuierer, Bataille et al. 2004; Schuierer, Bataille et al. 2006) and also in rarer tumours such as gastrointestinal stromal tumour (GIST) and pituitary adenomas (Fougner, Bollerslev et al. 2008; Martinho, Gouveia et al. 2009). Therefore, there is a general trend that RKIP expression is decreased in cancer tissue compared to normal tissue and moreover that it is lower still in metastases. In combination these results have lead to the conclusion that in cancer RKIP functions predominantly as a metastasis suppressor.

In vitro and *in vivo* studies have also helped to elucidate the role of RKIP in tumour development. Low RKIP levels have been shown to increase the motility and invasive potential of HCC and melanoma cells (Schuierer, Bataille et al. 2004; Lee, Tian et al. 2006). The idea of RKIP as a metastasis suppressor has been further strengthened by studies using orthotopic models of prostate and breast cancer (Fu, Smith et al. 2003; Li, Gao et al. 2009). Fu *et al* used two prostate cancer cell lines, C4-2B and LNCaP. The C4-2B cells are derived from the parental LNCaP cell line but demonstrate a greater degree of invasive and metastatic potential. C4-2B cells show lower levels of RKIP expression, than the

parental LNCaP cells. When the C4-2B cells were transfected with an RKIP overexpression vector they demonstrated decreased invasion as measured by *in vitro* assays. Furthermore, when these cells were injected into mouse prostates the RKIP overexpressing cells developed significantly fewer metastases compared with the vector controls. Interestingly, this phenotype occurred without any change in the primary tumour size and indeed the level of RKIP in the cells appeared to make no difference in proliferation or in colony formation assays (Fu, Smith et al. 2003). These results demonstrate that RKIP is a metastasis suppressor gene, which has been defined as one which interferes with metastasis, without effecting the primary tumour (Steeg, Bevilacqua et al. 1988).

1.1.2.1 Role of RKIP in prognosis and prediction

Not only are RKIP levels decreased in tumours but it has been shown that in certain cancers the levels of RKIP protein expression in primary tumours show a strong inverse correlation with survival (i.e. patients with low levels of RKIP protein have a worse outcome than those patients with higher levels of RKIP). This has been shown in prostate cancer (Fu, Kitagawa et al. 2006), GIST (Martinho, Gouveia et al. 2009) and, as will be discussed in more detail below, in CRC. The ability of RKIP expression levels to predict prognosis in a number of different tumour types clearly gives it the potential to be extremely useful in clinical practice. However, it would be of even greater value if RKIP expression levels were able to predict response to therapy (i.e. act as a predictive marker). There is some evidence that RKIP may function as a predictive marker as well as being a prognostic marker. It has been shown that in the case of pituitary adenomas the level of RKIP expression can predict the response to octreotide

therapy (Fougner, Bollerslev et al. 2008). There are also *in vitro* studies showing that RKIP can sensitise cells to chemotherapy induced apoptosis (Chatterjee, Bai et al. 2004; Jazirehi, Vega et al. 2004). Taken together, these studies suggest that RKIP may have the ability to function in this manner and therefore, it would be useful to determine if RKIP could act as a "bone fide" predictive marker in CRC.

1.1.2.2 RKIP in CRC

The important role played by RKIP in CRC was first elucidated in a study performed in our laboratory. It was noted upon examination of RKIP expression levels in a wide variety of tissues that they differed significantly between normal colonic epithelium and CRC (Al-Mulla, Hagan et al. 2006). Moreover, RKIP expression was decreased in lymph node metastases, when compared to primary tumours. Of most importance was the observation that the expression level of RKIP in the primary tumour was significantly inversely correlated with overall 5-year survival and that that this relationship was independent of other important prognostic indicators that are widely used, including lympho-vascular invasion (LVI) and Dukes stage. The authors went on to confirm these findings in a second smaller cohort of early stage (Dukes A&B) CRC and were able to reproduce the earlier findings and furthermore demonstrate that low RKIP expression correlated with an increased risk of metastatic relapse. Interestingly, while the effect of RKIP on prognosis was independent of standard prognostic markers there was a positive correlation between RKIP expression and apoptotic index (Al-Mulla, Hagan et al. 2006). This was not surprising as RKIP has been implicated in controlling apoptosis (discussed later) (Chatterjee, Bai et al. 2004;

Jazirehi, Vega et al. 2004) and provides a potential mechanism for the effect of RKIP on prognosis.

Further studies have confirmed RKIP as a prognostic marker in CRC. Minoo *et al* demonstrated in a very large cohort of over 1000 CRC patients that RKIP expression levels correlate with both prognosis and with the presence of metastases in both mismatch repair (MMR) proficient and deficient tumours (Minoo, Zlobec et al. 2007). Zlobec *et al* then went on to show that RKIP was an independent marker of metastasis in both univariate and multivariate analysis. This was also the first study to attempt to combine the RKIP status with another independent prognostic indicator in order to provide more prognostic information than either alone. They were able to demonstrate that patients whose tumours had high RKIP expression, no LVI and negative lymph nodes carried the lowest risk of metastatic relapse while those tumours with low RKIP expression, LVI and lymph node metastases carried the highest risk (Zlobec, Baker et al. 2008).

From the above it can be seen that RKIP has been established as a prognostic marker in a number of different tumours. However in order to understand how it is exerting this effect it is important to understand the role that RKIP plays within the cell. This will be discussed in the next section.

1.1.3 The role of RKIP in signal transduction

RKIP was originally described as an inhibitor of the Ras/Raf/MEK/ERK signalling cascade (this is the prototypical MAP Kinase (MAPK) cascade and will be referred to as MAPK throughout) (Yeung, Seitz et al. 1999). The MAPK signalling cascade is a highly conserved pathway which conveys mitogenic and differentiation signals from the cell membrane to the nucleus (Yeung, Janosch et al. 2000). It plays an important role in a wide variety of cellular processes, including proliferation, differentiation, apoptosis, angiogenesis and metastasis (Beeram, Patnaik et al. 2005). Therefore, disruption of this pathway has the potential to lead to wide ranging consequences and it is not surprising that this pathway is found to be deranged in a number of human disease states, including cancer. RKIP inhibits this pathway at the level of Raf. In the original study it was demonstrated that RKIP inhibits Raf-1 (Yeung, Seitz et al. 1999), it has since been shown that RKIP can also inhibit B-Raf (Park, Yeung et al. 2005) although this finding has not been replicated by others (Trakul, Menard et al. 2005). The mechanism of inhibition is a physical disruption of the binding of Raf-1 to MEK, thus preventing phosphorylation of MEK. This is achieved by the fact that although RKIP will bind both to Raf-1 and to MEK, it cannot bind to both simultaneously as the binding sites for each protein on RKIP overlap (Yeung, Janosch et al. 2000). Binding of RKIP to Raf blocks two key Raf activation sites, namely Ser388 and Tyr341, which are the sites phosphorylated by p-21 Associated Kinase (PKA) and Src family kinases respectively (Trakul, Menard et al. 2005). When the cell is in a quiescent state RKIP is found bound to Raf, thus preventing activation of MEK (Yeung, Seitz et al. 1999; Yeung, Janosch et al. 2000). Upon stimulation of the cell by growth factors RKIP is released from Raf following a phosphorylation event on Ser153 of RKIP by Protein Kinase C (PKC)

(Corbit, Trakul et al. 2003) (discussed in more detail below). This release of RKIP allows for phosphorylation of MEK by Raf and the subsequent downstream activation of the pathway. Decrease in the mitogenic signal then allows for the re-association of Raf and RKIP (Figure 1.2).

As mentioned above the phosphorylation of RKIP by PKC leads to its dissociation from Raf, freeing Raf to activate signalling via MEK. This is part of a complimentary role played by RKIP in the modulation of signal transduction in the cell. G-protein coupled receptors (GPCR) are a large family of transmembrane receptors. Upon ligand binding to GPCR PKC is activated and phosphorylates RKIP on Ser153. This phosphorylation leads not only to the dissociation of RKIP from Raf but also to the subsequent binding of RKIP to G-protein coupled receptor kinase-2 (GRK2), resulting in its inhibition (Lorenz, Lohse et al. 2003). GRK2 plays an important regulatory role over GPCR signalling, leading to internalisation of the receptor and degradation of the signal (Krupnick and Benovic 1998; Ribas, Penela et al. 2007). Thus by inhibiting GRK2 RKIP functions to promote the GPCR generated signal. Therefore, it can be seen that as well as inhibiting mitogenic signals in the quiescent cell RKIP plays an important role in reinforcing these signals in the presence of growth factors or other stimuli (Figure 1.2).

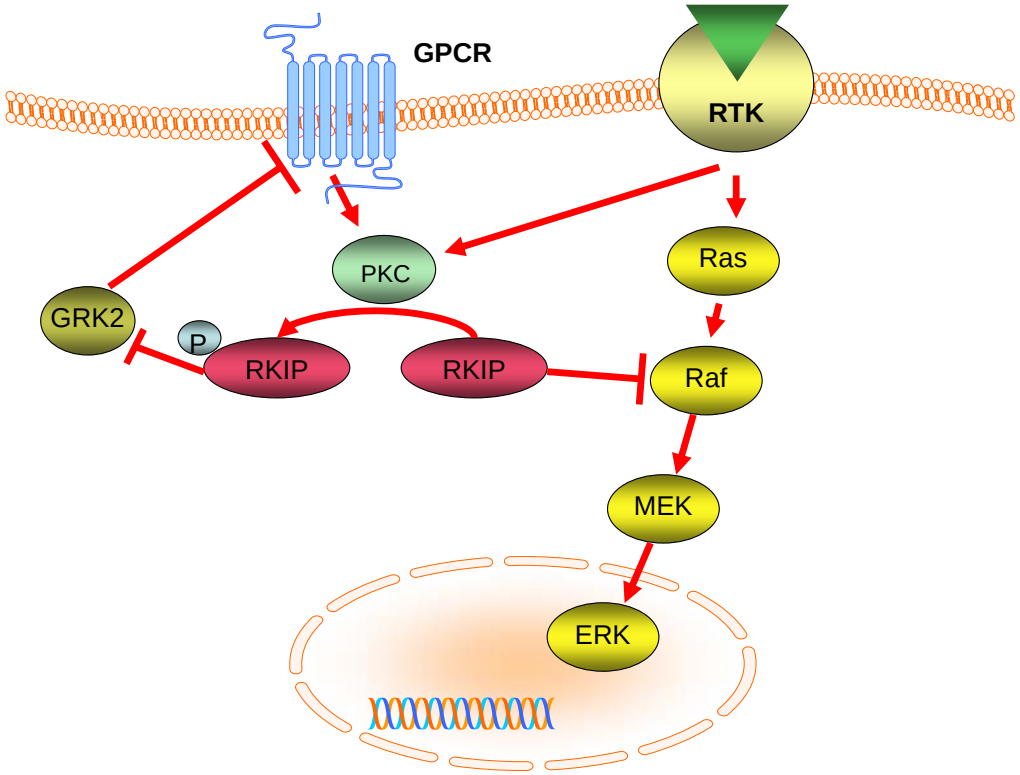


Figure 1.2: The role of RKIP in MAPK and GPCR signalling

In quiescent cells RKIP prevents Ras/Raf/MEK/ERK signalling by the inhibition of Raf. Upon mitogenic stimulation RKIP can be phosphorylated by Protein Kinase C (PKC). This results in cessation of the blockade of Raf and instead RKIP inhibits G-protein coupled receptor kinase 2 (GRK2) an inhibitor of G-protein coupled receptor (GPCR) signalling. (RTK: Receptor Tyrosine Kinase) (Adapted from Zeng et al 2008).

RKIP is now known to play an important role in other pathways such as the NFκB pathway (Yeung, Rose et al. 2001). It performs this function by its inhibition of the IκB Kinase (IKK) complex. In the un-stimulated cell, NFκB is held in the cytoplasm by binding to IκB. When cells undergo stimulation by TNF IκB may be phosphorylated by IKK (DiDonato, Hayakawa et al. 1997; Mercurio, Zhu et al. 1997). The phosphorylated form of IκB is recognised by ubiquitin ligases and the ubiquitinated form is then targeted for degradation by the proteosome (Brown, Gerstberger et al. 1995; Lin, Brown et al. 1995). This allows the now unbound NFκB to translocate to the nucleus, where it performs its function as a transcription factor. Yeung *et al* demonstrated that RKIP can physically interact with and block the action of IKK (Yeung, Rose et al. 2001). RKIP therefore prevents the phosphorylation of IκB; this in turn prevents the degradation of IκB which remains bound to NFκB, thus maintaining its location within the cytoplasm, where it cannot exert its effect as a transcription factor (Figure 1.3). This inhibition of a pathway, known to be important in cell survival implies a potential regulatory role for RKIP in this process. This will be discussed in more detail below.

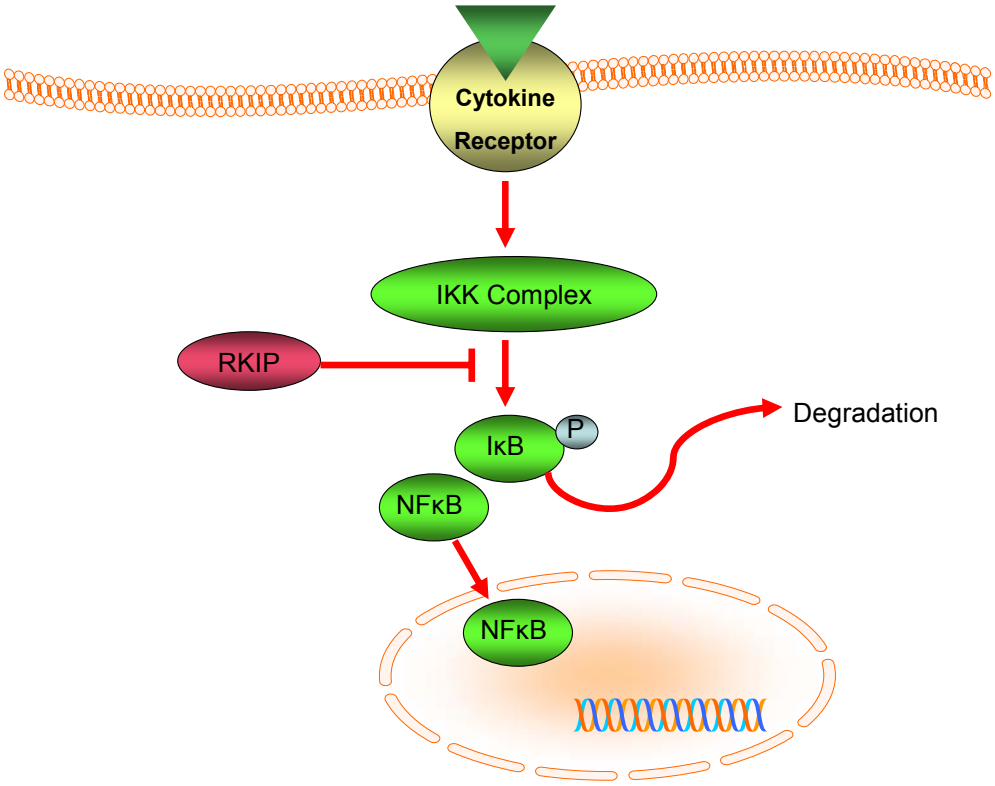


Figure 1.3: The role of RKIP in NFκB signalling

In quiescent cells RKIP prevents NFκB signalling by the inhibition of the IKK complex. Upon mitogenic stimulation RKIP can be phosphorylated by Protein Kinase C (PKC). This results in cessation of the blockade IKK, allowing phosphorylation and degradation of IκB, thus allowing NFκB to translocate to the nucleus. (RTK: Receptor Tyrosine Kinase) (Adapted from Zeng et al 2008).

1.1.3.1 The role of RKIP in cell cycle checkpoint integrity and in chromosomal stability

More recently RKIP has been shown to play a role in the regulation of the spindle checkpoint (Eves, Shapiro et al. 2006). The cell cycle contains a number of checkpoints, which have evolved to maintain an ordered progression through the cell cycle. The checkpoints are designed to sense any irregularities in the progression through the cycle and arrest the progression, until the irregularities can be corrected. The spindle checkpoint is a means by which the cell maintains fidelity of the chromosomal number and order during mitosis and the observation that phosphorylated RKIP binds to the kinetochore of dividing cells suggested a role for RKIP in the process (Eves, Shapiro et al. 2006). In this study the authors also demonstrated that RKIP depleted cells show a more rapid transition through mitosis and loss of the normal spindle checkpoint response to taxol treatment. RKIP was shown to exert this effect through Aurora B kinase. This kinase plays a role in both chromosomal alignment and in regulation of the spindle checkpoint and is particularly active when spindle tension is reduced in response to taxol treatment (Eves, Shapiro et al. 2006). Depletion of RKIP in cells leads to decreased levels of Aurora B kinase activity and decreased localisation of phosphorylated Aurora B at the kinetochore. The authors further showed that the phenotype of RKIP loss could be mimicked by hyperactivity of the MAPK pathway and that inhibition of the pathway rescued the effects of RKIP loss, therefore strongly implicating a role for MAPK signalling in this phenotype (Eves, Shapiro et al. 2006).

This work leads to the possibility that RKIP depletion in cancer cells could lead to an increase in chromosomal instability. This has been tested in human

tumours by Al Mulla *et al*, using both comparative genomic hybridisation (CGH) and loss of hetozygosity (LOH) analyses. They were able to demonstrate that low levels of RKIP expression correlate with an increase in genomic instability in microsatellite stable colorectal cancer (Al-Mulla, Hagan et al. 2008). Although this is the only published study examining this question it supports the role for RKIP in the maintenance of the spindle checkpoint and therefore in the maintenance of chromosomal stability.

1.1.3.2 RKIP in other cellular processes

RKIP has also been shown to play a role in other cellular processes which are known to be important in the development of cancer, in particular apoptosis, differentiation and cell migration.

Apoptosis or programmed cell death is an important physiological process involving the removal of cells which are useless or harmful to the organism (Letai 2008). The evidence that RKIP can regulate the apoptotic machinery of the cell comes from a number of studies. Initially there was the observation of a positive correlation between apoptotic index and RKIP expression in CRC (Al-Mulla, Hagan et al. 2006). It also emerged that high levels of RKIP could sensitise cancer cells to drug induced apoptosis (Chatterjee, Bai et al. 2004; Jazirehi, Vega et al. 2004). In prostate and breast cancer cell lines Chaterjee *et al* demonstrated that RKIP levels are induced in treatment-sensitive DU145 cells following the administration of DNA damaging drugs. This induction of RKIP sensitised the cells to undergo apoptosis. Inhibition of RKIP induction by siRNA protected these cells from the apoptosis induced by DNA damaging agents. In the DNA-damaging agent insensitive RC-1 cell line no such induction of RKIP

occurred in response to treatment and these cells did not undergo apoptosis. However, it could be shown that overexpression of exogenous RKIP now rendered these cells drug-sensitive. The authors showed that the raised level of RKIP was a response to DNA damage. Furthermore, it was shown that the mechanism of RKIP induced apoptosis was through inhibition of the NFκB and MAPK pathways (Chatterjee, Bai et al. 2004). Subsequently, a similar result was shown in non-Hodgkin's B-cell lymphoma cells, where treatment of the cells with Rituximab (a monoclonal antibody to CD20, used in the treatment of B-cell lymphoma) led to an upregulation of RKIP expression and subsequent sensitisation of the cells to chemotherapy induced apoptosis. The authors also demonstrated that RKIP was exerting its effects through its action on the MAPK and NFκB pathways. Inhibition of these pathways leads to a decrease in activity of the AP-1 and NFκB transcription factors, leading to downregulation of the anti-apoptotic Bcl-XL gene (Jazirehi, Vega et al. 2004).

Cellular differentiation is an important physiological process by which cells leave the cell cycle and express genes and proteins appropriate for their function and location. RKIP has been shown to be involved in this process in a number of systems. Mature human keratinocytes express higher levels of RKIP than do cells in the immature basal layer and keratinocytes in culture can be induced to differentiate following the overexpression of RKIP (Yamazaki, Nakano et al. 2004). Similarly immature human macrophages exposed to high levels of RKIP have been shown to express markers of differentiation such as CD11c and CD36. In macrophages this effect of RKIP was shown to be due, at least in part, to its effect on NFκB signalling (Schuierer, Heilmeier et al. 2006). Cellular differentiation is an important element in cancer development.

The ability to move is required by many different types of cell, both in physiological and in disease states. In cancer cells motility is particularly important in the process of invasion and metastasis. A number of studies have reported a role for RKIP in cell motility. However, to date there appears contradictory evidence as to whether RKIP functions to enhance or inhibit cell motility. Lee *et al* demonstrated that in HCC cells with low levels of RKIP expression, the expression of exogenous RKIP resulted in a decrease in cellular motility (Lee, Tian et al. 2006). Similarly, in melanoma cell lines restoration of RKIP levels was shown to decrease cell migration and also invasion in *in vitro* assays (Schuierer, Bataille et al. 2004). Fu *et al* also showed that low levels of RKIP was associated with an increase in invasive capacity in prostate cancer cell lines (Fu, Smith et al. 2003).

Other investigators have reported different results with regard to cell motility. Both Mc Henry *et al* and Zhu *et al* reported a pro-migratory role for RKIP (Zhu, Mc Henry et al. 2005; Mc Henry, Montesano et al. 2008). In addition the former study showed that raised RKIP levels are associated with a decrease in cell-cell interactions, although there was a strengthening of cell-substratum interaction (Mc Henry, Montesano et al. 2008). There is not enough data to fully reconcile these results at present. It is likely that the differences may be due, at least in part, to cell and context specific activities of RKIP. In both of the studies citing RKIP as a pro-migratory force the majority of the experiments were carried out in Madin-Darby Canine Kidney (MDCK) cells and less dramatic results were obtained when the authors shifted to the breast cancer MCF7 cell line. In the studies indicating an anti-migratory role for RKIP the experiments were preformed in highly invasive, metastatic cell lines. Taken in combination this may indicate that the anti-migratory role of RKIP is cancer cell specific. It should

also be pointed out that although cell motility is important for tumour cell invasion the two processes are distinct. Given that studies have consistently shown an anti-invasive function for RKIP it may be the case that other factors play a role in the increased invasion seen in cells with low levels of RKIP. RKIP has been shown to modulate levels of matrix metaloproteinases (MMP) (Xu, Peng et al. 2007; Delassus, Cho et al. 2008); this offers a possible explanation for the effect on invasion.

1.1.4 Regulation of RKIP expression

Although the exact mechanisms of the regulation of RKIP expression are not known a number of possible binding sites for transcription factors such as AP-1 have been identified (Odabaei, Chatterjee et al. 2004). This is logical, given that AP-1 levels are upregulated upon activation of the MAPK pathway and therefore it controls its own activation by activating transcription of a Raf inhibitor, thereby creating a negative feedback loop.

What is also controversial is the mechanism by which RKIP is down-regulated in cancer cells. It has been shown that RKIP mRNA and protein levels correlate well, indicating that RKIP loss occurs at the levels of RNA production or stability (Beach, Tang et al. 2008). To date no mutation in the *RKIP* gene has been reported and therefore other mechanisms of RKIP downregulation have been investigated, including epigenetic mechanisms. The *RKIP* promoter contains a number of CpG repeats. In general these CpG dinucleotides are under-represented in the genome as a whole but are common in the promoter regions of many genes. CpG rich regions are termed CpG islands and methylation of these has been associated with silencing of the corresponding genes both in a physiological setting (McGhee and Ginder 1979) and in cancer cells, where methylation has been shown to silence a number of tumour suppressor genes, including *Rb* (Ohtani-Fujita, Dryja et al. 1997) and *MIh-1* (Herman, Umar et al. 1998; Veigl, Kasturi et al. 1998). This raised the possibility that the *RKIP* promoter may be methylated.

A number of studies have investigated this possibility and yielded conflicting results. Al Mulla *et al* used methylation specific PCR (MSP) to examine the RKIP

promoter in 82 cases of CRC. They reported that over 72% of the cases with low or absent RKIP expression demonstrated *RKIP* promoter methylation (Al-Mulla, Hagan et al. 2008). However, Minoo *et al* also examined cases of CRC using MSP and were unable to show methylation of the *RKIP* promoter in any of the 14 cases that they examined (Minoo, Zlobec et al. 2007). Supporting this later view was the study by Scuierer *et al* in which they showed that treatment of melanoma cells which had low levels of RKIP expression with the demethylating agent 5-azacytidine did not result in any change in the RKIP expression level (Schuierer, Bataille et al. 2004). Given the controversy over the role of methylation in *RKIP* silencing it has been suggested that there is a need to apply a quantitative methylation analysis approach, such as pyrosequencing, to the problem (Al-Mulla, Hagan et al. 2008).

Another epigenetic mechanism of *RKIP* silencing has been explored in prostate carcinoma. Beach *et al* noted that RKIP expression levels in prostate cancer cells could not be altered by the administration of 5-azacytidine and therefore looked for alternative mechanisms of RKIP regulation. They observed that the levels of RKIP expression closely correlated with those of E-cadherin (Beach, Tang et al. 2008). Snail has been shown to be an important regulator of E-cadherin expression, acting as a transcriptional repressor (Batlle, Sancho et al. 2000; Cano, Perez-Moreno et al. 2000) and given that the *RKIP* promoter contains a Snail recognition E-Box motif the authors investigated whether Snail can also affect RKIP transcription. They were able to demonstrate by both overexpression and knockdown of Snail that it was indeed directly affecting RKIP levels, both at the RNA and protein levels. Chromatin immunoprecipitation (CHIP) assays demonstrated a direct interaction between Snail and the RKIP promoter (Beach,

Tang et al. 2008). The potential role for Snail in the control of RKIP expression in other tumour types has not been tested.

1.1.5 Introduction to the RKIP knockout mouse

In the mouse RKIP is expressed in nearly all tissues, with the highest levels seen in the brain and testis (Theroux, Pereira et al. 2007). The mouse genome also contains a number of RKIP family members (RKIP2-5). RKIP2 is expressed in the testes, but has not been detected in other tissues (Hickox, Gibbs et al. 2002; Theroux, Pereira et al. 2007), while expression of RKIP3-5 have not been detected in any tissue in the mouse, indicating that these three family members are silent pseudogenes (Theroux, Pereira et al. 2007).

The *RKIP* knockout mouse was developed using the gene-trap technology. This is a high throughput technology, which allows for the knockout of a wide variety of genes at random, but has the advantage of incorporating a reporter which allows for easy identification of the gene which has been knocked out. Briefly, the technique involves the insertion of the "gene trap" into the genome of a mouse embryonic stem (ES) cell using either electroporation or a retrovirus (Stanford, Cohn et al. 2001). The gene trap consists of a promoterless sequence containing a LacZ reporter and a neomycin resistance gene (this allows for selection of cells expressing the gene trap). The LacZ and neomycin resistance elements may also be combined. In addition there is a splice acceptor site upstream of the LacZ reporter (Figure 1.4). The gene trap inserts into an intron and therefore upon activation of the gene results in the generation of a fusion mRNA, consisting of the upstream exons of the targeted gene and the LacZ reporter. This results in the production of a mutant, loss of function protein.

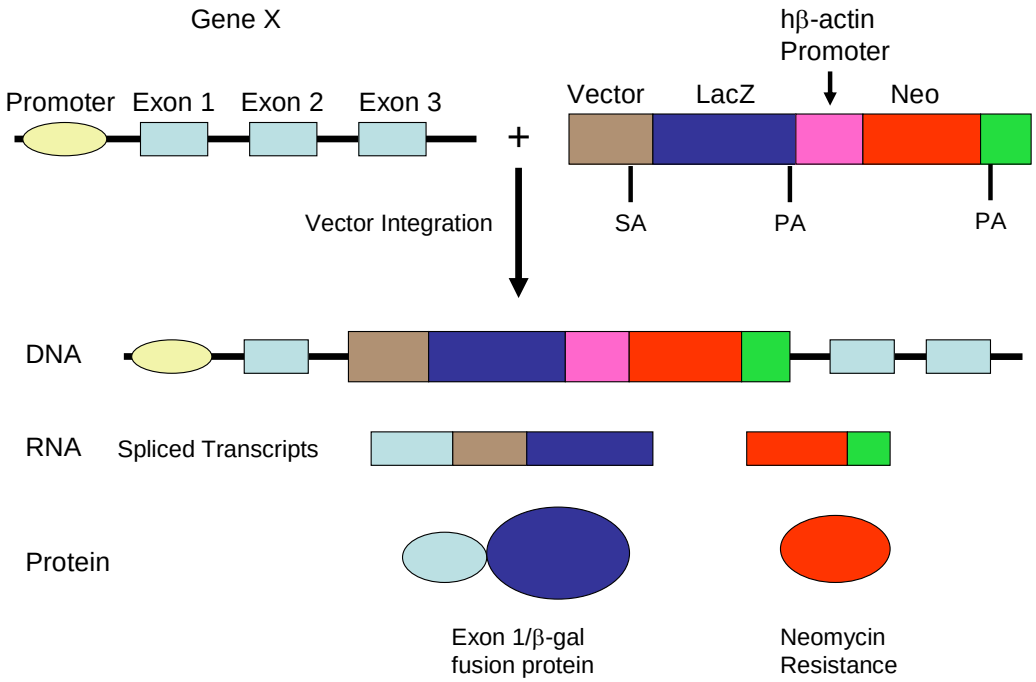


Figure 1.4: Gene-trap strategy

The “trap” consists of a vector which contains a splice acceptor site (SA), LacZ and a neomycin resistance gene (Neo), which is under the control of an autonomous promoter (in this case β-actin), this allows for neomycin selection of the embryonic stem cells in which vector integration has occurred. In the example above the trap has inserted into exon 1 of gene X. The presence of the splice acceptor in the vector leads to the production of a fusion transcript consisting of exon 1 of gene X and LacZ, which is translated to a non-functional fusion protein. The neomycin resistance gene, being under the control of an autonomous promoter is transcribed and translated separately (Adapted from Stanford et al, 2001).

In the case of the *RKIP* knockout mouse the gene trap is inserted into intron 1 of the *RKIP* gene (Figure 1.5). The *RKIP* knockout mouse is viable, breeds normally and shows transmission of the *RKIP* mutation in the expected Mendelian ratios. The mouse shows only subtle phenotypes with a mild defect in olfactory function and in spermatogenesis (Moffit, Boekelheide et al. 2007; Theroux, Pereira et al. 2007).

The *RKIP* knockout mouse has not been associated with an increased risk of tumour development. At first glance this may raise questions about the presumed anti-cancer role of RKIP. However when one looks at the evidence for the role that RKIP plays in tumour development this should not come as a surprise, given that RKIP has been shown to function as a metastasis suppressor rather than a tumour suppressor. As pointed out above metastasis suppressor genes do not necessarily play a role in the initiation of tumourigenesis, but instead exert their effects at the later stages of tumour progression. Therefore, it is most appropriate to test the effect of RKIP loss in the mouse by examining its effect in an established model of tumourigenesis.

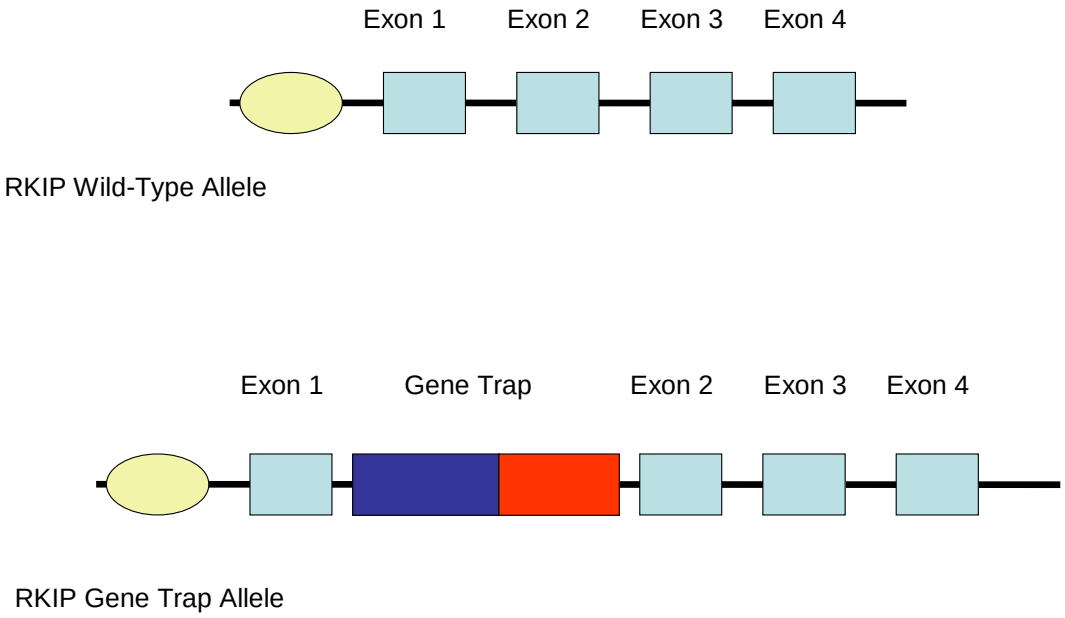


Figure 1.5: Diagrammatic representation of the *RKIP* Gene Trap knockout

In the case of the *RKIP* knockout mouse the *LacZ* and *Neo* genes are combined as a β-geo element, which is inserted into intron 1 of the mouse *RKIP* gene, on Chromosome 5. This results in the production of a non-functional, truncated fusion protein, which only retains exon 1 of the native *RKIP* (Adapted from Theroux et al, 2007). *Neo*: Neomycin Resistance Gene.

1.2 Introduction to colorectal carcinoma

Although RKIP has been shown to play an important role in a number of different cancer types some of the strongest data linking RKIP to prognosis has been in CRC. Therefore, we have decided to focus our studies on the role of RKIP in this disease. The next section will introduce some of the important clinical aspects and also the underlying biology of CRC.

1.2.1 Colorectal carcinoma epidemiology

CRC is the second most common cancer and the second leading cause of cancer deaths in the Western world (Fernandez, La Vecchia et al. 2005; Ferlay, Autier et al. 2007). In the UK alone approximately 360,000 new cases are diagnosed every year. The incidence of the disease is the same in men and women up to the age of 50. However, there is a significant divergence in older individuals with the disease more common in men in this age group. Over the past 35 years the incidence of the disease has remained almost static (with a slight increase in the incidence seen in men). There has, however, been a steady decrease in mortality from the disease. This has been the result of a number of factors, including improved treatment and earlier detection.

CRC is predominantly a disease of the Western world and historically has been relatively less frequent in Africa and Asia. This observation indicated an environmental trigger for the pathogenesis of the disease. And indeed it has been shown in epidemiological studies that there is an association between a diet high in red meat and low in fibre and CRC (Norat, Bingham et al. 2005). The association with diet has been strengthened by the observation of a recent increase in the rates of CRC seen in Japan. This increase coincides with a move towards a more Western style diet in this country. Other risk factors for the disease include obesity (which is thought to increase CRC risk in men by up to 50%) and low levels of physical exercise (Slattery, Edwards et al. 2003; Moghaddam, Woodward et al. 2007). Similar to other types of cancer it has been shown that alcohol and cigarette smoking are significant risk factors (Moskal, Norat et al. 2007; Botteri, Iodice et al. 2008). There is evidence that type-II diabetes mellitus is associated with an increased risk, furthermore it would

appear that this association is independent of obesity and level of physical activity (Larsson, Orsini et al. 2005).

Other factors have been reported which are associated with a positive impact on CRC risk. These include use of non-steroidal anti-inflammatory (NSAID) medication. In back to back studies published in the New England Journal of Medicine it was shown that aspirin is associated with a lower incidence of CRC (Baron, Cole et al. 2003; Sandler, Halabi et al. 2003). Hormone replacement therapy and the oral contraceptive pill are also thought to reduce risk (Grodstein, Martinez et al. 1998; Fernandez, La Vecchia et al. 2001).

Two important medical conditions which are known to increase the risk of CRC are ulcerative colitis (UC) and Crohn's disease (CD). These two conditions are often put together under the umbrella term of chronic inflammatory bowel disease, but there are important differences between the two, both in terms of pathogenesis, clinical features and risk of CRC. Patients with CD are approximately twice as likely to develop CRC as the general population (Jess, Gamborg et al. 2005). Patients with UC have a further increased risk of developing CRC, with a standardised incidence ratio reported to be 5.7 (Ekbom, Helmick et al. 1990). The risk of CRC in UC is greater with increased disease severity and duration.

Genetics also plays a strong role in the risk of CRC. There is evidence for this in the number of familial CRC syndromes which have been described and also in the fact that CRC risk is approximately doubled by having a first degree relative with the disease. Moreover, the risk increases almost to approximately fourfold if two first degree relatives have had the disease or if one relative has had CRC before

the age of 45 (Johns and Houlston 2001). The genetics and molecular biology of CRC will be discussed in greater detail below.

1.3 Colorectal carcinoma staging and treatment

1.3.1 Normal intestinal anatomy and physiology

Before discussing the staging of CRC it is important to briefly describe the normal anatomy and physiology of the intestine. The intestine consists of three layers. The outer layer consists of 2 bands of smooth muscle at right angles to one another, between the muscle layers lye nerve plexi which help to control the peristaltic function of these muscle layers. Outside the muscle layers there is a layer of adipose and connective tissue of variable thickness, this is covered by the peritoneum for the majority of the length of the intestine. Moving inwards the next layer is the sub-mucosa, which consists of connective tissue, blood and lymphatic vessels. Payer's patches, which are an important part of the immune system, also lie within the sub-mucosa (Kumar 2004). Superficial to the sub-mucosa is the mucosa. This is the layer of cells which carry out most of the varying functions of the intestine. In the small intestine the mucosa is projected into folds and invaginations called villi and crypts respectively, in the colon only crypts are seen and the villi are replaced by flat inter-crypt spaces (Sancho, Batlle et al. 2004). The purpose of this micro-anatomy is to greatly increase the intestinal surface area and therefore increase the absorptive capacity of the intestine. The cells lining the crypts and villi are specialised and play very specific functions. At the base of the crypts are the Paneth cells; these secrete various peptides involved in anti-microbial and other activity (Porter, Bevins et al. 2002). Above the Paneth cells are the intestinal stem cells, which give rise to all of the cell lineages making up the intestinal epithelium. The stem cell divides

to give rise to a population of cells known as the transit amplifying cells. These in turn migrate to the crypt-villus junction, where they can differentiate into one of three cells lineages (Sancho, Batlle et al. 2004). Enterocytes make up the majority of cells in the villus and are predominantly involved in nutrient absorption. Goblet cells produce mucus, which forms a protective layer over the epithelium and finally the enteroendocrine cells produce hormones including Substance P and serotonin (Hocker and Wiedenmann 1998; Sancho, Batlle et al. 2004).

1.3.2 Staging of CRC

As discussed above the outcome for patients with CRC has improved steadily over the last 40 years for a number of reasons. The main factor which predicts outcome in CRC is the stage of disease at diagnosis (Sobin L. 2002). The stage of disease is an indication of the extent of the disease progression i.e. depth of invasion and spread to distant sites.

1.3.3 Dukes Staging of CRC

The Dukes staging system was devised by Cuthbert Dukes in 1932 (Dukes 1932) and remains in use today, with only relatively minor alterations. Dukes staging uses the letters A-D to denote progression of the tumour. A Dukes A tumour is the earliest stage and can have invaded into, but not through the muscularis propria. A Dukes B tumour has extended through the muscularis propria, into the surrounding adventitia but importantly it has not metastasised. Dukes C and D tumours are associated with lymph node and distant metastases respectively (Figure 1.6).

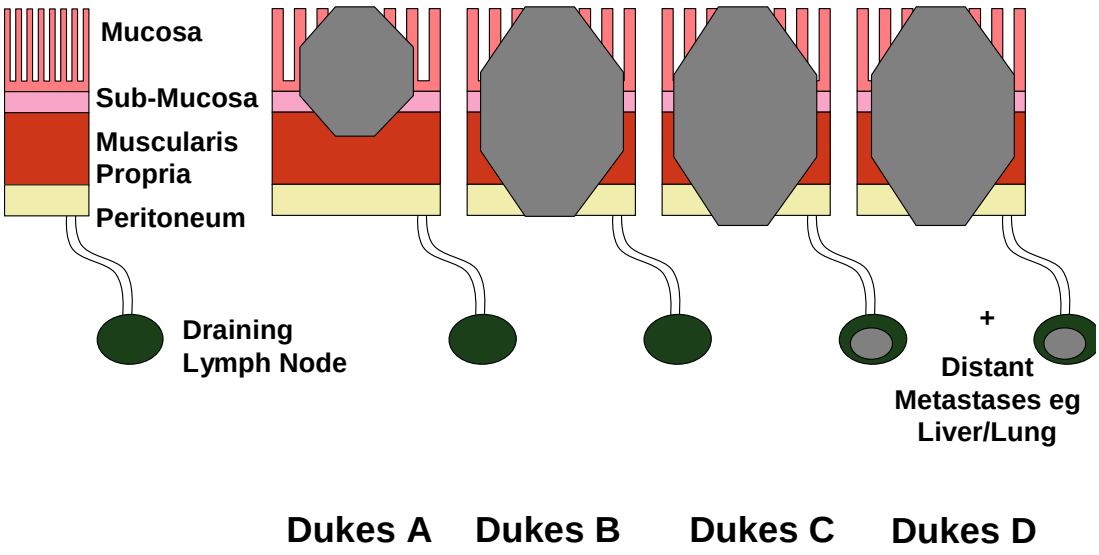


Figure 1.6: Dukes Staging of Colorectal Cancer

A Dukes A tumour can invade into but not beyond the muscularis propria (the bowel wall). A Dukes B tumour has invaded beyond the muscularis propria into the surrounding adipose tissue or through to the peritoneum. When a tumour metastasises to regional lymph nodes it becomes Dukes C. Finally a tumour which metastasises to a distant site such as the liver becomes Dukes D.

The reason that the Dukes staging system for CRC has remained virtually unchanged for so long is the valuable prognostic information that it provides. Patients with a Dukes A tumour have an average 5-year survival of ~85%, compared to ~5% for patients with a Dukes D tumour (Table 1.1). The system also has the advantages of being simple, widely recognised and highly reproducible.

Dukes Stage	5-Year Survival
A	83%
B	64%
C	38%
D	3%

Table 1.1: 5-Year Survival of Patients with Colorectal Cancer, stratified by Dukes Stage

(Adapted from NICE. Improving Outcome in Colorectal Cancers: Manual update 2004)

The most commonly diagnosed stage of CRC is Dukes stage B, followed by C, with the very early and late stages of the disease diagnosed less frequently (Burton, Norman et al. 2006). This however is changing with the advent of CRC screening. CRC screening has been shown to result in earlier diagnosis and improved survival in patient populations. It will therefore result in an increase in the diagnosis of early stage CRC (Dukes A&B) and will focus attention on the management of these early stages of the disease (Towler, Irwig et al. 2000; Lindholm, Brevinge et al. 2008).

Since the time when Dukes first described the system there have been a number of refinements, which provide extra prognostic information and therefore allow for improved patient risk stratification. One of these is the TNM system. TNM

stands for Tumour, lymph Nodes, Metastases and treats tumour staging in much the same way as the Dukes system, but separates the elements (Sobin L. 2002). In CRC the T-stage is an important determinant of prognosis, essentially it further subdivides Dukes A&B tumours (Table 1.2).

Dukes Stage	Description	T-Stage	Description
A	A tumour confined to the Lamina Propria, Sub-mucosa or Muscularis Propria.	**1**	A tumour confined to the Lamina Propria and Sub-mucosa.
		2	A tumour that has invaded into but not through the Muscularis Propria.
B	A tumour that has invaded through the muscularis propria but has not metastasised.	**3**	A tumour that has invaded into but not through the peritoneal fat.
		4	A tumour that has invaded through the peritoneum.

Table 1.2: Comparison of the Dukes and T-Staging systems for CRC.

The importance of this distinction can be seen when one examines the impact of the different T-stage on Dukes B tumours. Patients with T3 tumours have a significantly improved survival over patients with T4 tumours (Shepherd, Baxter et al. 1997; Lennon, Mulcahy et al. 2003). Further observations that prognosis is negatively impacted by the presence of tumour cells in blood or lymphatic vessels (LVI), tumour budding or having a small number of lymph nodes examined histologically have helped to refine patient risk stratification, which aids in treatment decisions (Compton 2003).

1.3.4 Treatment of CRC

The mainstay of CRC treatment is surgery, consisting in the majority cases of removal of the tumour, the adjacent bowel and the draining lymph nodes (This approach may be modified in very early or late stages of the disease). In patients with very early stage disease (Dukes A) this surgery is curative in the vast majority of cases. In patients who are discovered after surgery to have involvement of draining lymph nodes or distant organs there is strong evidence that the use of post-operative (adjuvant) treatment will improve survival (NIH 1990). For patients with advanced local disease in the absence of metastases (Dukes B) the picture is less clear-cut (Figueredo, Coombes et al. 2008).

Five year survival for Dukes B CRC patients is relatively favourable at 74-80% (Eisenberg, Decosse et al. 1982; Petersen, Baxter et al. 2002; O'Connell, Maggard et al. 2004). However, this leaves up to 25% of patients with pathologically negative lymph nodes (pN0) developing recurrent disease (Nicastri, Doucette et al. 2007). This has lead to controversies regarding the use of adjuvant chemotherapy in this group (Sobrero and Köhne 2006). Recent large studies have shown that adjuvant chemotherapy may confer a small survival benefit (Andre, Boni et al. 2004; Kuebler, Wieand et al. 2007; Quasar Collaborative, Gray et al. 2007), and a recent meta-analysis showed an improvement in disease-free survival, although no improvement in overall survival could be shown (Figueredo, Coombes et al. 2008). These authors suggested that adjuvant treatment should be considered in patients with high-risk features (Figueredo, Coombes et al. 2008), as it is only these patients who are likely to benefit from adjuvant treatment. Although this has yet to be demonstrated in a trial (Petersen, Baxter et al. 2002; Figueredo, Coombes et al. 2008), a recent study by Andre *et al*

(Andre, Boni et al. 2009) showed a trend towards a benefit with the addition of oxaliplatin to standard chemotherapy in a high-risk subgroup of Dukes B CRC and have suggested that further trials should be confined to this subgroup. Their high-risk group was broadly defined by featuring at least one of the following: peritoneal invasion, tumour perforation, bowel obstruction, poorly differentiated tumour, LVI, or <10 lymph nodes examined. These data suggest that a refinement of risk assessment could identify the Dukes B group who will benefit most from adjuvant therapy and that the extra prognostic information provided by RKIP could be beneficial.

1.4 The biology of colorectal cancer

Like many human cancers CRC arises following a multi-step progression from dysplastic epithelium, through early and late stage adenomas, to invasive carcinomas and finally metastasis. We have learnt that these steps in the progression are usually associated with characteristic genetic events. This progression through the different stages was first described in CRC by Vogelstein (Vogelstein, Fearon et al. 1988) and has been dubbed the "Vogelgram" (Figure 1.7). In the majority of CRC the earliest event is mutation and subsequent loss of function of the *Adenomatous Polyposis Coli* (*APC*) gene. As is the case with many tumours much of what we know about *APC* and other genes involved in CRC development comes from our study of autosomal familial cancer syndromes.

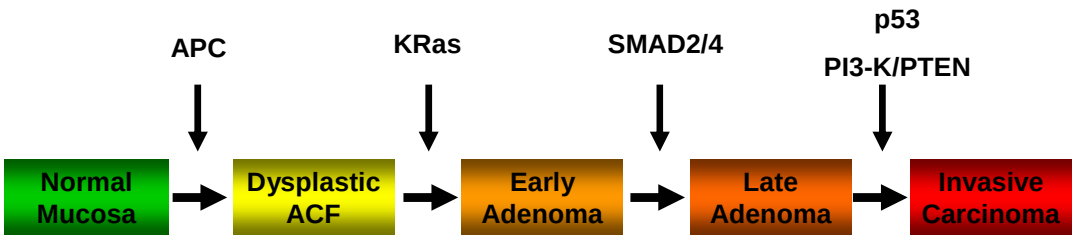


Figure 1.7: The "Vogelgram"

The diagram depicts the step-wise progression of the tumour, from the earliest detectable lesion (ACF) through to invasive and potentially metastatic carcinoma, as it acquires increasing numbers of mutations. While the majority of early lesions will have a mutation in APC, thereafter the mutations that are acquired become more variable, it is likely that this contributes to differences in biological and clinical behaviour between tumours (Adapted from Kumar et al, 2003). ACF: Aberrant Cryt Foci.

1.4.1 Familial CRC syndromes

The two best described familial CRC syndromes are Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC, also known as Lynch syndrome). Other syndromes have also more recently been described such MUTYH (MutY Homologue) associated polyposis (Sieber, Lipton et al. 2003) (also termed MYH associated polyposis or Attenuated polyposis).

1.4.2 Familial Adenomatous Polyposis (FAP)

FAP was first described in described in the late 1940's by Gardner (FAP is also known as Gardner's syndrome) (Gardner 1948). Patients with FAP develop hundreds of colonic polyps from a very early age. One or more of these polyps invariably progress to invasive cancer, so that the rate of CRC in these patients is 100% by the age of 40. Although CRC is the major clinical problem associated with FAP, patients also suffer other manifestations of the disease. These may be enteric, such as duodenal adenomas, gastric fundic gland polyps and tumours of the pancreatico-biliary tree, or they may be extra-enteric, such as osteomas and brain tumours (Kinzler and Vogelstein 1996).

The genetic basis underlying FAP first came to light when it was noted that FAP patients shared deletion of an area on the long arm of chromosome 5 (Herrera, Kakati et al. 1986; Bodmer, Bailey et al. 1987; Solomon, Voss et al. 1987). It was subsequently noted that deletions in this region were found to occur commonly in patients with sporadic as well as familial CRC and the gene was identified as *APC* (Kinzler, Nilbert et al. 1991; Kinzler, Nilbert et al. 1991; Nishisho, Nakamura

et al. 1991). Mutation of the *APC* gene was shown to occur in both sporadic and familial CRC (Nishisho, Nakamura et al. 1991) and it was demonstrated that this loss of both copies of *APC* was the important early event in adenoma formation (Ichii, Horii et al. 1992; Levy, Smith et al. 1994). It was subsequently shown that this was also the case in the APC^{Min} mouse which is a model of FAP (Levy, Smith et al. 1994; Luongo, Moser et al. 1994). Cumulatively these data delivered a molecular explanation behind the phenotype seen in patients with FAP. *APC* is a tumour suppressor gene and patients with FAP inherit a germline mutation in one of the *APC* alleles. However, in keeping with Knudson's "two-hit hypothesis" regarding the behaviour of tumour suppressor genes the remaining wild-type copy of the gene is capable of performing the tumour suppressive function and thus must be lost in order for tumour progression to occur (Knudson 1971).

1.4.3 Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

As the name suggests patients with hereditary non-polyposis colorectal cancer (HNPCC), develop fewer colonic adenomas than do patients with FAP. The syndrome was recognised much later than FAP (Lynch, Lynch et al. 1977). The tumours that arise in HNPCC have distinct clinical and pathological features. Clinically tumours tend to arise later than in FAP, typically around the 5th decade (although there is a wide spread, with age at first diagnosis ranging from 14 to 82 years). The site of the tumours is also specific, with the majority (~70%) arising in the ascending (proximal) colon. This is different to FAP, where tumours arise throughout the entire length of the bowel and to sporadic CRC where only a minority (~30%) of tumours arise in the proximal colon (Lynch, Smyrk et al. 1996). At the histological level CRC associated with HNPCC is more

likely to be poorly differentiated (Jass, Smyrk et al. 1994; Losi, Fante et al. 1995), with a significant percentage composed of a continuous sheet of cells fringed by a prominent lymphocytic reaction (the so-called medullary carcinoma) (Jass, Smyrk et al. 1994). Like FAP, HNPCC is associated with an increased risk of other cancers. In the case of HNPCC these tend to occur in stomach, small intestine, pancreas, upper urologic tract (renal pelvis and ureter), endometrium and ovary (Lynch, Smyrk et al. 1993; Watson and Lynch 1993). Probably the most important clinical feature associated with HNPCC tumours is their favourable prognosis (Sankila, Aaltonen et al. 1996). The exact mechanism for this more indolent behaviour is not known for definite, although it has been shown that HNPCC tumours are more likely to be diploid than sporadic CRC (Kouri, Laasonen et al. 1990).

Mutations of *MIh-1*, *Msh-2*, *PMS1* and *PMS2*, all genes involved in DNA mismatch repair were found to occur in families with HNPCC (Leach, Nicolaides et al. 1993; Bronner, Baker et al. 1994; Nicolaides, Papadopoulos et al. 1994; Papadopoulos, Nicolaides et al. 1994). Mutation of these genes leads to microsatellite instability (MSI), which is the hallmark of these tumours. As has been the case with FAP and *APC* it has now been shown that although HNPCC tumours are rare there is a subset of sporadic CRC which show similar loss of DNA mismatch repair genes and consequent MSI. These tumours form a distinct clinical and pathological subgroup. Like their counterparts in HNPCC sporadic MSI tumours tend to be located in the proximal colon. For reasons that are poorly understood they are more likely to occur in older patients and in women and like HNPCC tumours are associated with an improved prognosis, when compared to CRC as a whole. It has also emerged that the “Vogelgram” for these tumours differs from the classical picture. Firstly, the precursor lesions differ at the

histological level. MSI tumours develop in the main from "sessile serrated adenomas"; this is a pathological entity that has been described relatively recently (Torlakovic and Snover 1996; Goldstein, Bhanot et al. 2003). The mutations and other genetic events in MSI tumours also differ. Mutations in *B-Raf* are more common (Jass, Baker et al. 2006) and mutations in *APC* and *p53* less so (Salahshor, Kressner et al. 1999). Other mutations may occur in genes such Transforming Growth Factor-β (*TGF-β*), a gene which contains a microsatellite region within its coding sequence (Markowitz, Wang et al. 1995). Moreover, these changes generally occur on a background of gene promoter methylation (the CpG Island Methylator Phenotype (CIMP)), which results in the epigenetic silencing of genes (Hawkins, Norrie et al. 2002). For example the DNA mismatch repair genes themselves are often silenced in this way in these tumours. There is now a consensus therefore that this represents a different pathway to tumour development to the more common *APC* initiated pathway.

1.5 Role of APC in CRC

Although from the above discussion it is clear that the molecular classification has moved on and become more complex than the original "Vogelgram" (A classification based on 5 molecular subtypes has been proposed, (Table 1.3)), those tumours arising from mutation of *APC* remain the most common. The *APC* gene was identified by cloning the genetic locus known to be lost in FAP patients in chromosome 5 (Groden, Thliveris et al. 1991; Kinzler, Nilbert et al. 1991). The gene encodes a large (312 kDa) protein which contains a number of domains. The first clues to its role in CRC came to light when it was shown to be a binding partner for β-catenin, thus implicating a role for APC in the Wnt signalling pathway (Rubinfeld, Souza et al. 1993; Su, Vogelstein et al. 1993). The role of APC in this pathway will now be discussed in more detail.

	CIMP Status	Microsatellite Status	Chromosomal Instability	Molecular Events	Precursor Lesion	% Total
1	Negative	MSS	Instable	Mutations of APC, KRas, p53	Adenoma	57%
2	Low	MSS/MSI-Low	Instable	Mutations of APC, KRas. MGMT methylation	Adenoma/ Serrated Polyp	20%
3	High	MSI-High	Stable	Methylation MLH-1, BRAF mutation	Serrated Polyp	12%
4	High	MSI-Low	Stable	Part methylation MLH-1, BRAF mutation	Serrated Polyp	8%
5	Negative	MSI-High	Stable	Mutation of mismatch repair genes (HNPCC)	Serrated Polyp	3%

Table 1.3: Proposal for Molecular Classification of Colorectal Cancer:

The classification is based on the levels of tumour methylation as well as microsatellite and chromosomal stability (Adapted from Jass, 2007). CIMP: CpG Island Methylator Phenotype, MSS: Microsatellite Stable, MSI: Microsatellite Instable.

1.5.1 The Wnt signalling pathway

The *Wnt* gene was originally described in mice and Drosophila and is conserved up to higher organisms (Klaus and Birchmeier 2008). The pathway becomes active when a Wnt ligand binds the Frizzled receptor (Bhanot, Brink et al. 1996; Wang, Macke et al. 1996), this signals through Dishevelled to inactivate Glycogen Synthase Kinase3 (GSK3) (Siegfried, Wilder et al. 1994). GSK3 functions as part of the β-catenin destruction complex. After β-catenin is phosphorylated by Casein Kinase 1α (CK1α) it is then phosphorylated by GSK3, targeting it for destruction by the proteasome (Yost, Torres et al. 1996; Liu, Li et al. 2002). Following the inactivation of GSK3 β-catenin is free to accumulate in the cytoplasm and translocate to the nucleus. Once in the nucleus β-catenin binds T-Cell Factor (TCF) and Lymphoid Enhancer Factor-1 (LEF1) to promote transcription of Wnt target genes (Figure 1.8) (Behrens, von Kries et al. 1996; Huber, Korn et al. 1996; Molenaar, van de Wetering et al. 1996). Wnt targets include *Myc* and *CyclinD1* which, given the role that they play in functions such as proliferation and the progress of the cell cycle have clear roles in tumour formation (He, Sparks et al. 1998; Shtutman, Zhurinsky et al. 1999; Tetsu and McCormick 1999). Therefore, it can be seen that the Wnt signalling pathway is pro-proliferative and pro-cell survival and it is in the best interests of the cell to closely regulate its activity.

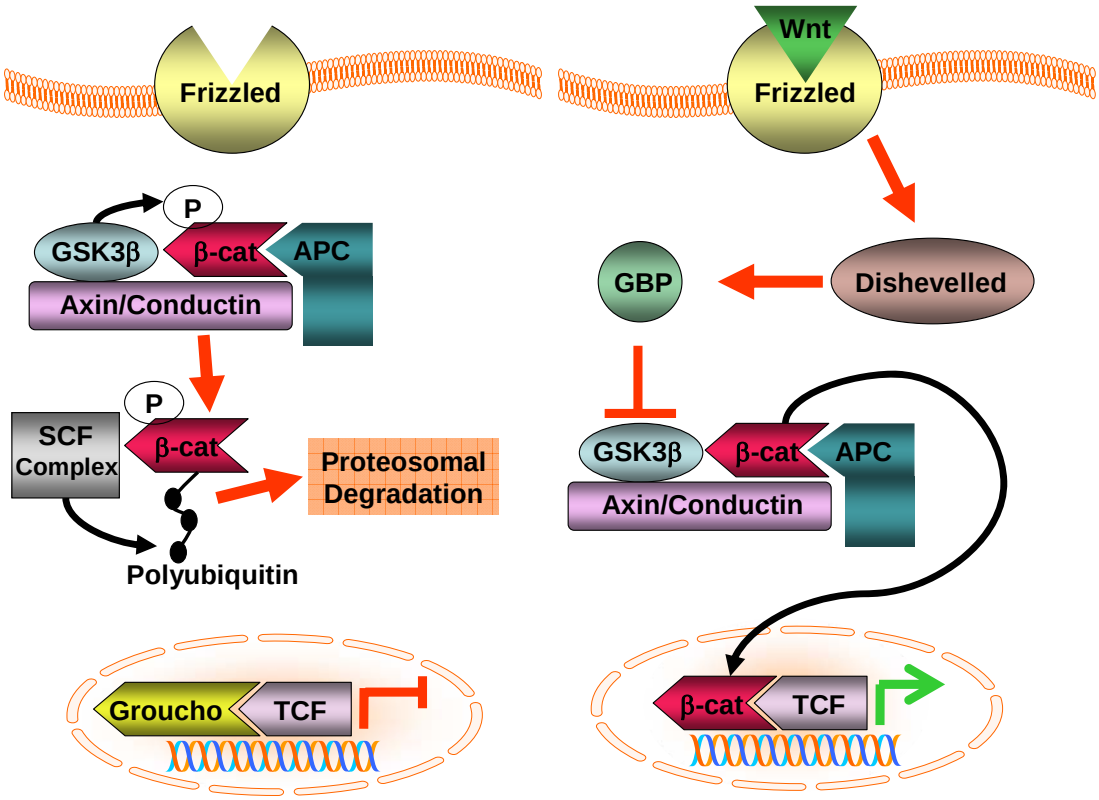


Figure 1.8: The Canonical Wnt Signalling Pathway

In the absence of a Wnt signal (left) β-catenin is bound by the β-catenin destruction complex and phosphorylated by GSK3β. Once phosphorylated β-catenin is then recognised by an SCF complex which mediates the addition of a polyubiquitin chain to the β-catenin molecule. The ubiquitinated β-catenin is then recognised and destroyed by the proteosome. Thus β-catenin cannot translocate to the nucleus and Groucho represses TCF mediated transcription. Upon the binding of a Wnt ligand to the Frizzled receptor (Right) Dishevelled activates GBP, which inhibits the phosphorylation of β-catenin by GSK3β. β-catenin can now translocate to the nucleus and promote the transcription of Wnt target genes. The same process happens when APC is absent as the β-catenin destruction complex can now no longer be formed (Adapted from Frodde et al 2001). SCF Complex: SKP/Cullin/F-Box Complex, GBP: GSK3 Binding Protein.

APC is a key player in the regulation of the pathway. It, along with axin/conductin and GSK3 form the β-catenin destruction complex, which leads to the phosphorylation of β-catenin by GSK3 and its subsequent ubiquitination and destruction by the proteasome (Aberle, Bauer et al. 1997). In the absence of nuclear β-catenin TCF and LEF interact with Groucho proteins and the transcription of Wnt target genes is suppressed (Figure 1.8) (Cavallo, Cox et al. 1998; Roose, Molenaar et al. 1998). As mentioned above the APC protein is large with a multiple domains (Figure 1.9). There are several 15 and 20 amino acid repeats that play a role in the interaction and subsequent downregulation of β-catenin (Hulsken, Birchmeier et al. 1994). Meanwhile the SAMP (Serine-Alanine-Methionine-Proline) repeats which are interspersed among the 20 amino acid repeats mediate the binding of APC to axin/conductin (Behrens, Jerchow et al. 1998; Hart, de los Santos et al. 1998). The importance of the SAMP region is underlined by the finding that mice with APC mutations that retain the SAMP region do not develop polyposis (Smits, Kielman et al. 1999). Mutations of APC generally result in the production of a truncated protein, with loss of some or all of the 20 amino acid repeats, mutations are most common between codons 1286 and 1513, this is referred to as the mutation cluster region (MCR) (Polakis 2000). As a tumour suppressor *APC* obeys Knudson's 2-hit hypothesis so that loss of function of the second *APC* allele is required before tumour initiation can occur. Interestingly, in patients with FAP the exact nature of the second hit depends on the initial germline mutation, specifically the number of remaining 20 amino acid repeats in the mutant protein. Such that if the germline mutant contains no 20 amino acid repeats the second hit is likely to be a mutant form of the protein containing 1-2 20 amino acid repeats. However if the germline mutant contains 1-2 of the repeats the second hit will be either LOH of a mutation with no

remaining 20 amino acid repeats (Fodde, Smits et al. 2001; Pollard, Deheragoda et al. 2009).

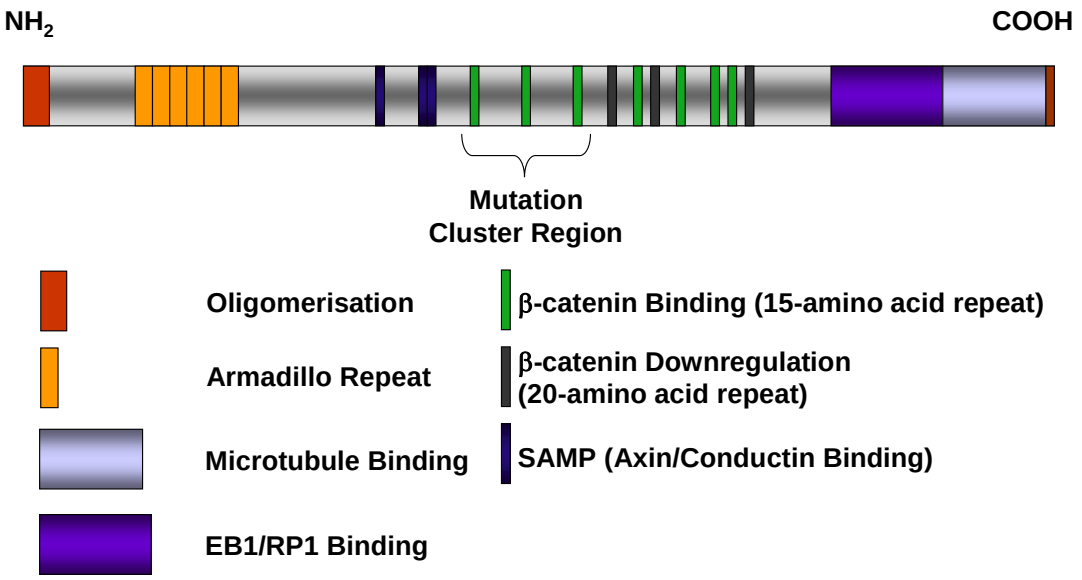


Figure 1.9: Diagrammatic representation of the APC protein

The protein can be seen to contain a number of regions important for its various functions, including binding to microtubules and the microtubule associated protein EB1. Also shown are the 15 and 20 amino acid repeats, which are important for β-catenin binding and regulation. The mutation cluster region occurs among these 15-amino acid repeats (adapted from Fodde et al, 2001). SAMP: Serine-Alanine-Methionine-Proline.

The loss of APC function results in the inability of the destruction complex to function and therefore β-catenin is not ubiquitinated and destroyed (Ahmed, Hayashi et al. 1998). Instead the now stable β-catenin accumulates in the cytoplasm and then translocates to the nucleus where in conjunction with its co-factors TCF/LEF it can promote the transcription of Wnt target genes. One consequence of this is that the presence of β-catenin in the nucleus of a cell functions as a readout of dysfunctional APC activity. In a normal intestine nuclear β-catenin can only be seen in the proliferative cells at the base of the crypt (Sansom, Reed et al. 2004). In a tumour however the de-regulated Wnt signalling pathway (the result of APC loss) can be detected by the presence of nuclear β-catenin.

However, despite the presence of *APC* gene mutations there is controversy regarding the activation of Wnt signalling and the localisation of β-catenin in CRC. Studies on human tumours have reported that nuclear β-catenin can only be detected at the invasive front of tumours (Brabletz, Jung et al. 2001) and cannot be detected in adenomas from patients with FAP (Phelps, Chidester et al. 2009). These data are at odds with our previous studies where we see Wnt targets such as c-Myc, CD44 and Cyclin D2 deregulated immediately following APC loss (Sansom, Meniel et al. 2007) and with microarray studies from human tumors showing increased levels of Wnt target gene expression (Sabates-Bellver, Van der Flier et al. 2007; Van der Flier, Sabates-Bellver et al. 2007). We have also seen nuclear β-catenin staining throughout adenomas in Apc^{Min} mice. This apparent discrepancy between *APC* mutation and a lack of activation of the Wnt signalling pathway has been termed the β-catenin paradox and is yet to be fully explained.

1.5.2 Roles of APC outside the canonical Wnt signalling pathway

There is strong evidence to suggest that the main function of APC loss in early CRC is to promote deregulated Wnt signalling. This evidence comes from studies showing that other mutations that activate the pathway will lead to a very similar phenotype (Harada, Tamai et al. 1999) and that the deletion of key Wnt targets such as *Myc* is capable of rescuing the phenotype induced by *APC* loss (Sansom, Meniel et al. 2007). The rare sporadic human CRCs that are found to display mutations in other genes in the pathway, such as *axin* or *β-catenin* adds further weight to the argument (Liu, Dong et al. 2000; Polakis 2007). However, there is now evidence emerging that loss of APC in tumours may lead to other effects.

Chromosomal Instability (CIN) is an important event in CRC, occurring in the vast majority of tumours outside the MSI pathways (Table 1.3). CIN can take 2 distinct forms; a quantitative defect, with an increase in chromosome number (polyploidy) or a chromosomal rearrangement, resulting from chromosomal fragmentation and reunion. There is now growing evidence that APC loss can play a significant role in the development of CIN. Kaplan *et al* showed that APC can be found at the kinetochore during mitosis and that cells that contain mutant *APC* are deficient in chromosomal segregation. This deficit in chromosomal segregation then leads to an increase in chromosome number (Kaplan, Burds et al. 2001). Fodde *et al* also demonstrated localisation of APC to the kinetochore and the presence of supernumerary chromosomes in *APC* mutant cells. In addition they showed the presence of multi-polar mitotic spindles (Fodde, Kuipers et al. 2001). It has been shown that multi-polar mitosis can

exert multi-directional forces on chromosomes, which can result in chromosomal fragmentation and thus allow for chromosomal structural abnormalities (Doxsey 1998). Thus, it can be seen from these studies that an *APC* mutation results in both types of CIN seen in CRC. The one caveat that has been associated with these studies is that they were performed in mouse embryonic stem cells. In these cells there is a defect in p53 cell cycle control functions (Aladjem, Spike et al. 1998). So it has been suggested that in order to generate CIN in a differentiated cell *APC* mutation must coincide with a loss of p53 function. Studies in human adenomas, designed to assess whether CIN occurs at a very early stage (prior to the development of *p53* or other mutations) have yielded contrasting results (Sieber, Heinimann et al. 2002; Cardoso, Molenaar et al. 2006). Interestingly, in human CRC the tumours that show *APC* mutation and CIN are generally those that are also associated with *p53* mutation. This would suggest a possible cooperation between *APC* and *p53* mutations in promoting CIN, there is evidence in both the mouse and in humans to suggest that this may be the case (Alberici, de Pater et al. 2007).

1.6 Other genes important in CRC

From the above discussion the importance of APC loss in CRC is clear. However, loss of APC occurs as an early event in the development of CRC and other genetic "hits" are required for tumour progression. In human CRC these have been shown to include mutations in *KRas*, the PI3-Kinase (phosphoinositide 3-kinase) pathway and the p53/ARF pathway. We have used mutations in some of these pathways to study the progression of CRC in the mouse, therefore they will be discussed in the next section.

1.6.1 KRas in CRC

As discussed above KRas is an important effector of the MAPK pathway (Figure 1.10). Mutation of *KRas* is found in ~33-43% of human CRC (Bos, Fearon et al. 1987; Lievre, Bachet et al. 2006; Benvenuti, Sartore-Bianchi et al. 2007; Barault, Veyrie et al. 2008). Activation of KRas occurs when it binds GTP at the expense of GDP, mutation of *KRas* commonly maintains the protein in its GTP bound state and therefore renders it constitutively active (Dhillon, Hagan et al. 2007). The majority of these activating mutations occur in codons 12, 13 and 61 (Bos 1989). Mutation of *KRas* is a relatively early event in CRC development, occurring in a significant proportion of large but not small adenomas (Vogelstein, Fearon et al. 1988). The importance of activation of this pathway in CRC can be seen by the fact that activating mutations in the *BRaf* gene occur in ~12-13% of tumours (Benvenuti, Sartore-Bianchi et al. 2007; Barault, Veyrie et al. 2008). BRaf is directly downstream of KRas (Figure 1.2) and mutations that result in constitutive activation will also lead to hyperactivation of the MAPK pathway. It would appear that *KRas* and *BRaf* mutations are mutually exclusive in CRC

(Rajagopalan, Bardelli et al. 2002; Benvenuti, Sartore-Bianchi et al. 2007; Di Nicolantonio, Martini et al. 2008). Tumours which show *BRaf* mutations are also more likely to show mismatch repair deficiencies (Rajagopalan, Bardelli et al. 2002).

As *KRas* is commonly mutated in CRC and occupies an important position along the path of tumour progression, mouse models which utilise *KRas* mutations would be useful. Indeed studies already exist in which mutation of the gatekeeper gene *APC* has been combined with *KRas* mutations (Sansom, Meniel et al. 2006). In this study the authors demonstrated that the addition of the activated *KRas* not only accelerated tumourigenesis but also promoted the formation of invasive tumours (Sansom, Meniel et al. 2006), this makes it an appropriate model to study the potential effects of a metastasis suppressor gene such as *RKIP*.

1.6.2 PI3-Kinase pathway in CRC

Like the MAPK pathway the PI3-Kinase pathway is downstream of Ras (Figure 1.10) and activation of the pathway can promote a wide range of cellular functions, including cell growth, survival and motility (Courtney, Corcoran et al. 2010). Inappropriate activation of this pathway can take the form of activating mutations of *PI3-Kinase* itself or alternatively inactivating mutations of the *PTEN* gene which encodes the phosphatase and tensin homolog protein (PTEN), one of the main inhibitors of the PI3-Kinase pathway. In CRC *PI3-Kinase* mutations are observed in ~13-32% of tumours (Samuels, Wang et al. 2004; Frattini, Signoroni et al. 2005; Velho, Oliveira et al. 2005; Perrone, Lampis et al. 2009). Mutation of *PI3-Kinase* is thought to be a later event in tumour progression, being rare in

adenomas (Samuels, Wang et al. 2004). Mutations of *PTEN* are found in ~10% of CRC (Perrone, Lampis et al. 2009) but loss of PTEN protein expression has been seen in a greater proportion of CRC (Laurent-Puig, Cayre et al. 2009; Perrone, Lampis et al. 2009). As is the case with *KRas* and *BRaf* mutation of *PI3-Kinase* and loss of PTEN expression have been reported to be mutually exclusive (Frattini, Signoroni et al. 2005). However mutations in the PI3-Kinase and KRas/MAPK pathways have been shown to co-exist (Velho, Oliveira et al. 2005).

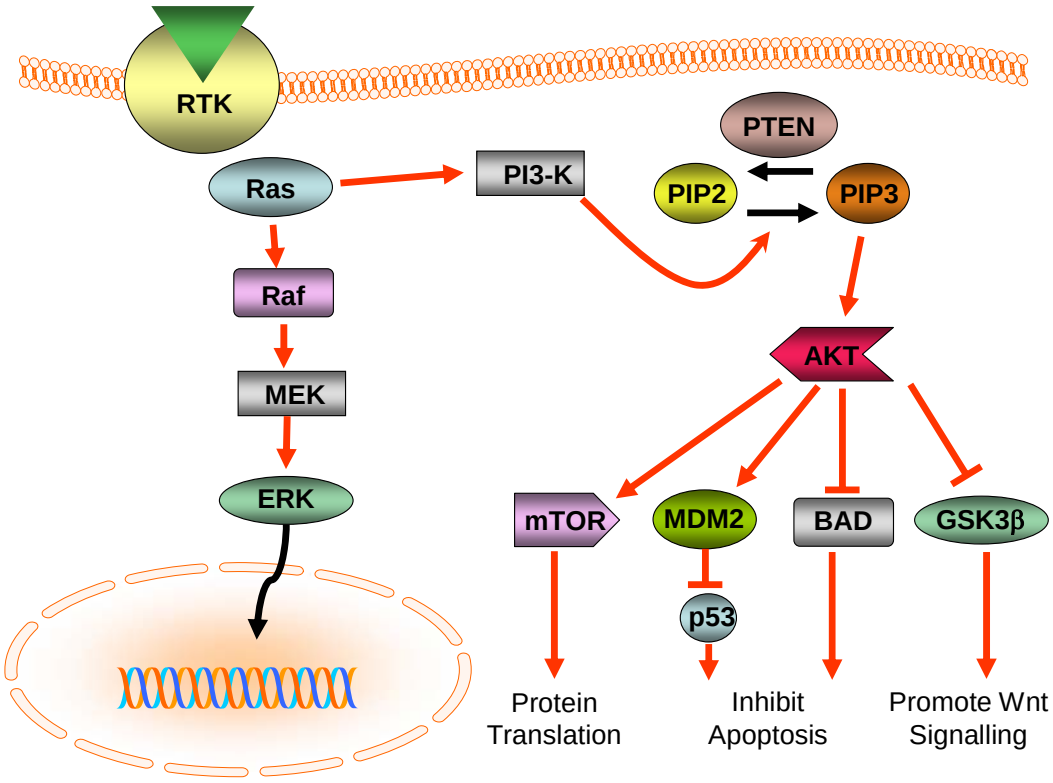


Figure 1.10: MAPK and PI3-Kinase pathways

Binding of ligand to RTK results in activation of Ras, which can activate the classical MAPK pathway but also PI3-Kinase. PI3-Kinase can then phosphorylate PIP2 to PIP3, which activates AKT. The phosphatase PTEN can reverse this reaction and thus inhibit the pathway. Activated AKT can then promote pro-growth and proliferation and anti-apoptotic signals as shown. In CRC this pathway can become constitutively activated by activating mutations in Ras or PI3-Kinase or by inactivation mutations of PTEN (Adapted from Courtney et al, 2010 and Siena et al 2009). RTK: Receptor Tyrosine Kinase, mTOR: Mammalian Target of Rapamycin.

1.7 Role of p53 in CRC

p53 exerts an influence over a wide variety of cellular processes, including cell cycle control and apoptosis. p53 is a key protein in the cell's response to DNA damage, which if left unchecked could lead to DNA replication errors and therefore mutation (Levine and Oren 2009).

As a protein that has been extensively studied much is now known about the pathway of p53 activation and about its regulation. In a normally functioning cell p53 is continuously produced and turned over at the same rate. The result of this is a very low basal level of p53 within the cells and a short half-life of about 20 minutes (Reich, Oren et al. 1983). The reason for this seemingly futile cycle of p53 production and destruction is that it allows the cell to increase the levels of p53 extremely rapidly simply by shutting down its degradation. The main facilitator of p53 degradation is MDM2 (Murine double minute, HDM2 in humans, for simplicity I will refer to MDM2 throughout). MDM2 functions as an E3 ubiquitin ligase, facilitating the ubiquitination and subsequent proteasomal degradation of p53 (Haupt, Maya et al. 1997; Honda, Tanaka et al. 1997; Kubbutat, Jones et al. 1997). MDM2 can also function in a number of other ways to antagonise the activity of p53, including direct binding and therefore blocking the transactivation domain of p53 (Oliner, Pietenpol et al. 1993). Therefore, it can be seen that inhibition of MDM2 will lead to an increase in p53 levels.

p53 is stabilised in response to a number of stimuli, such as genotoxic stress (e.g. irradiation), hypoxia, DNA damage or oncogene activation (Maltzman and Czyzyk 1984; Graeber, Peterson et al. 1994; Hermeking and Eick 1994; Wagner, Kokontis et al. 1994; Serrano, Lin et al. 1997). In response to DNA damage p53 is

stabilised by the activity of Ataxia-Telangectasia Mutated (ATM) and Ataxia-Telangectasia and Rad3 related (ATR). ATM becomes activated in response to DNA breaks and can phosphorylate p53 on Ser15 (Banin, Moyal et al. 1998). Another target of ATM is Chk-2 Kinase, once activated this kinase can phosphorylate p53 on Ser20, which makes up part of the MDM2 binding domain, thus protecting it from MDM2 mediated ubiquitination and degradation (Chehab, Malikzay et al. 2000; Shieh, Ahn et al. 2000). Once activated p53 translocates to the nucleus where it acts as a transcription factor, leading to the transcription of target genes, including such as *p21* and *BAX* (Figure 1.11). Depending on the cellular context, this can lead to a number of different outcomes, including cell cycle arrest (giving the cell an opportunity to repair the damage prior to entry into the cell cycle) (Baker, Markowitz et al. 1990; Diller, Kassel et al. 1990; Mercer, Shields et al. 1990; Michalovitz, Halevy et al. 1990), senescence (after which cells may be cleared by the immune system) (Serrano, Lin et al. 1997; Wang, Blandino et al. 1998) or in the most extreme case apoptosis (Yonish-Rouach, Resnitzky et al. 1991; Shaw, Bovey et al. 1992).

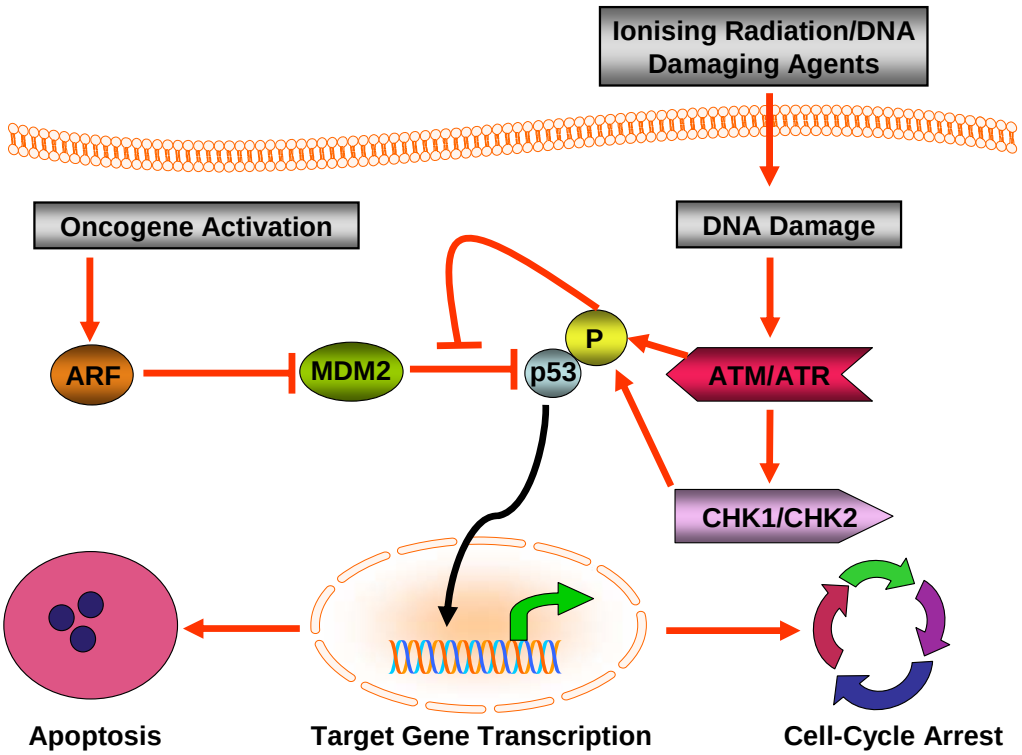


Figure 1.11: Activation of p53

In the absence of stress p53 is constantly turned over as it is ubiquitinated by MDM2 and subsequently degraded in the proteosome. In the presence of DNA damage, ATM and ATR are activated, these in turn activate CHK1/2 kinases which can phosphorylate p53, reducing the ability of MDM2 to ubiquitinated it. Similarly in response to oncogenic stress ARF becomes activated, this binds and sequesters MDM2 away from p53. Once p53 is stabilised it translocates to the nucleus, where it mediates transcription of genes resulting in a number of outcomes, including cell-cycle arrest and apoptosis (Adapted from Sherr 2006).

The cells of the intestine are exquisitely sensitive to apoptosis in response to a number of stimuli, such as ionising radiation (Potten 1992) and cytotoxic chemotherapeutic agents (Ijiri and Potten 1987). In both cases it has been shown that this apoptosis occurs in a p53 dependent manner (Merritt, Potten et al. 1994; Pritchard, Watson et al. 1997). Although it seems that the apoptosis occurs in different cell populations (Pritchard, Watson et al. 1997). In the case of the cytotoxic agent 5-Flouro-Uracil (5FU) apoptosis is seen predominantly in the cells of the transit amplifying zone and is induced via incorporation of the drug into RNA (Pritchard, Watson et al. 1997). The response to irradiation is thought to be mediated predominantly via DNA damage pathways. The observation that the amount of apoptosis in the intestine of irradiated ATM knockout mice is initially reduced compared to wild-type controls but then recovers over longer time points suggests an important immediate role for ATM in this process but that other pathways can then be activated to compensate for the lack of ATM (Gurley and Kemp 2007). Downstream of p53 apoptosis in the intestine is induced following the upregulation of pro-apoptotic p53 targets such as DR5, Bid, PUMA and Noxa (Fei, Bernhard et al. 2002). However the well characterised pro-apoptotic p53 target Bax is not upregulated (Coates, Lorimore et al. 2003) nor required for p53 dependent apoptosis in the intestine (Pritchard, Potten et al. 1999).

Given the role of p53 in the response to DNA damage discussed above it is not surprising that it has been shown to be important in the development of CIN, which is known to occur in the majority of CRC (Issa 2008). It has been shown that in cells that have mutation of p53 there is a marked increase in CIN (Donehower, Godley et al. 1995; Borel, Lohez et al. 2002; Fujiwara, Bandi et al. 2005). In these studies it was shown that in the absence of functioning p53

tumours developed more rapidly in a mouse model of mammary carcinoma and the resulting tumours showed an increase in aneuploidy as well as genetic deletions and amplifications (Donehower, Godley et al. 1995). Mechanistically, it was demonstrated that in the absence of p53 cells can by-pass the G1 tetraploidy checkpoint, following incomplete cytokinesis (Borel, Lohez et al. 2002). The resulting tetraploid cells then show an increase in chromosomal deletions, amplifications and aneuploidy (Fujiwara, Bandi et al. 2005). There is now evidence that CIN can play a direct role in tumour development (Weaver, Silk et al. 2007). This suggests that some of the effect of p53 loss in CRC may be mediated in this way.

It can be seen that p53 plays a vital role in protecting cells against the potential tumourigenic effects of both DNA damage and oncogene activation. This activity of p53 creates a major hurdle for a potential cancer cell to overcome and therefore it is easy to see why the majority of human cancers have been shown to develop mutations of *p53* or in the p53 pathway (Levine and Oren 2009). As has been noted above in the intestine p53 mutation is common in the later stages of CRC but rare in the early, adenomatous stage of the disease (Vogelstein, Fearon et al. 1988). It has also been reported that there are very limited effects of p53 loss in the setting of acute loss of APC (Reed, Meniel et al. 2008). These data would suggest that in the early stages of intestinal tumour development there is no selective pressure to lose function and that this loss of p53 only becomes important in the transition from a benign adenoma to an invasive carcinoma.

1.7.1 p53 mutation in cancer

When originally discovered it was thought that *p53* was an oncogene. This resulted from data demonstrating that p53 isolated from cancer cells was capable of enhancing the oncogenic properties of other cells (Eliyahu, Raz et al. 1984; Jenkins, Rudge et al. 1984; Parada, Land et al. 1984; Wolf, Harris et al. 1984; Eliyahu, Michalovitz et al. 1985). However evidence that *p53* was in fact a tumour suppressor began to emerge when it was shown that *p53* is deleted in a number of different tumour cell lines (Wolf and Rotter 1984; Wolf and Rotter 1985). This created somewhat of a paradox, which was only resolved by the realisation that in the original studies in which the expression of p53 had led to an increase in oncogenic properties, this p53 had been cloned from cancer cells and was in fact a mutant form of the protein.

Unlike many tumour suppressors, the mutations that occur in *p53* tend to be point mutations which result in the expression of a mutant form of the protein, which often accumulates in tumour cells (Bartek, Bartkova et al. 1991; Brosh and Rotter 2009). Indeed, it has been shown that accumulated p53 as detected by immunohistochemistry correlates well with the presence of a *p53* mutation (Soussi and Beroud 2001). The majority of these mutations occur in the so called "hot spot" region, which includes the DNA binding domain (Hollstein, Sidransky et al. 1991). Many of these mutations prevent the binding of p53 to DNA and therefore prevent it from acting as a transcription factor. The mutations may directly prevent DNA binding by altering an amino acid which interacts with the DNA (e.g. R273H), these are termed "DNA contact" mutations. Alternatively the mutation may result in an alteration of the tertiary structure of the protein and prevent DNA binding this way (e.g. R175H), these are termed "conformational"

mutations (Brosh and Rotter 2009). What has become increasingly clear is that these mutant forms of the p53 protein play an enhanced role in oncogenesis, beyond this simple loss of DNA binding function (Sigal and Rotter 2000).

One explanation for this is that mutant p53 has a dominant negative effect and indeed this does seem to occur. When binding to DNA p53 forms a homotetramer. Mutant p53 is capable of binding to molecules of wild-type p53, thus forming a heterotetramer which is now incapable of binding DNA (Milner and Medcalf 1991; Milner, Medcalf et al. 1991). However, the observation that transfection of p53 null cells with a mutant form of p53 enhances the oncogenic properties of the cells has lead to the "gain of function" hypothesis, i.e. that the mutant form of the protein has distinct functions which promote tumourigenesis, unrelated to the function of the wild-type protein (Wolf, Harris et al. 1984; Shaulsky, Goldfinger et al. 1991). A number of studies have supported this hypothesis. Theses include studies showing that although mice expressing a mutant form of p53 have a very similar lifespan to mice in which p53 is deleted, they show a very different tumour spectrum and also an increase in tumour aggressiveness, with an increase in metastasis seen (Lang, Iwakuma et al. 2004; Olive, Tuveson et al. 2004).

1.7.2 Introduction to ARF

As mentioned above p53 activation occurs in response to oncogenic stress, preventing propagation of the oncogenic stimulus and subsequent tumour formation. One of the most important modulators of this response is the protein ARF ($p14^{ARF}$ in humans, $p19^{ARF}$ in mice. For simplicity I will refer to ARF throughout). The ARF protein is encoded from the *CDKN2A* locus, on chromosome 9 in humans and chromosome 4 in mice. This locus codes for 2 gene products, one is the tumour suppressor $p16^{INK4A}$, which inhibits the cyclin dependent kinases CDK4 and CDK6. The coding sequence which gives rise to the ARF mRNA originates from a different exon to that of $p16^{INK4A}$ (Exon 1β) but exons 2 and 3 are common to both although the different initiator codons from the two genes result in an Alternate Reading Frame (hence the name ARF) and the production of a protein which is completely unrelated to $p16^{INK4A}$ (Quelle, Zindy et al. 1995).

1.7.3 The role of ARF in the p53 pathway

Following its discovery at the *CDKN2A* locus it was found that like $p16^{INK4A}$, ARF is also a tumour suppressor (Kamijo, Zindy et al. 1997). ARF is normally undetectable or present at only very low levels in a normal cell, however in the presence of an oncogenic stimulus (such as increased levels of c-Myc) levels of ARF increase within the cell (Zindy, Eischen et al. 1998). ARF then binds directly to the p53 inhibitor MDM2, via an MDM2 binding domain at the N-terminal of the protein (Weber, Taylor et al. 1999; Sherr 2001). Binding of ARF to MDM2 has two effects; firstly this binding inhibits the E3 ubiquitin ligase activity of MDM2 and secondly the ARF/MDM2 complex localises to the nucleolus, thus sequestering

MDM2 away from p53 in the nucleoplasm (Weber, Taylor et al. 1999; Lowe and Sherr 2003). Therefore, upon the detection of an oncogenic stimulus the resulting increased levels of ARF allow for the stabilisation of p53 and subsequent cell-cycle arrest or apoptosis (Figure 1.11) (Quelle, Zindy et al. 1995; Kamijo, Zindy et al. 1997; Kamijo, Weber et al. 1998; Radfar, Unnikrishnan et al. 1998)

The importance of this pathway in terms of tumour surveillance and prevention can be seen in the results of both *in vitro* and *in vivo* studies. MEFs which overexpress c-Myc will normally undergo apoptosis, as a result of ARF induced p53 activation. However, rare immortal clones will emerge and these will commonly show mutations in either *p53* or *ARF* (Zindy, Eischen et al. 1998). Similarly the Eμ-Myc mouse which develops B-cell lymphoma is initially protected from tumourigenesis as the increased levels of proliferation induced by high levels of Myc are counterbalanced by high levels of apoptosis induced by ARF and p53. In the tumours that almost invariably develop in these mice mutations in either *ARF* or *p53* are common (Eischen, Weber et al. 1999; Jacobs, Scheijen et al. 1999; Schmitt, McCurrach et al. 1999). Moreover, crossing these mice to animals which are heterozygous for *ARF* leads to acceleration of tumourogenesis (Eischen, Weber et al. 1999). Overexpression of Ras in primary MEFs results in cell-cycle arrest, rather than apoptosis. Again this effect is mediated through the ARF dependent stabilisation of p53. In contrast, in ARF null MEFs Ras overexpression results in hyperproliferation and transformation (Kamijo, Zindy et al. 1997).

ARF knockout mice are tumour prone, although initially this was not realised as the first *ARF* knockout mouse utilised a knockout of exons 2 and 3 of the *CDKN2A*

locus (the exons that are common to both $p16^{INK4A}$ and ARF) (Serrano, Lee et al. 1996). The tumours that these mice developed were predominantly lymphomas and sarcomas and since at the time *ARF* had only just been discovered this tumour prone phenotype was ascribed to the deletion of $p16^{INK4A}$ (Serrano, Lee et al. 1996). The relative roles played by ARF and $p16^{INK4A}$ in the phenotype of the *CDKN2A* knockout mouse have subsequently been unravelled. The *ARF* knockout mouse was generated by deletion of exon1β and thus leaving $p16^{INK4A}$ function intact (Kamijo, Zindy et al. 1997). The *ARF* knockout mice developed tumours at an early age, almost exactly phenocopying the *CDKN2A* knockout mice. The tumours that developed retained detectable levels of $p16^{INK4A}$ (Kamijo, Zindy et al. 1997), indicating that much of the phenotype seen in the *CDKN2A* knock mice was in fact due to the loss of ARF rather than $p16^{INK4A}$. Selective $p16^{INK4A}$ knockout mice have also been developed (Krimpenfort, Quon et al. 2001; Sharpless, Bardeesy et al. 2001). Although these mice are tumour prone and in particular show an increased sensitivity to the effect of chemical carcinogens they do not show the same predisposition to tumours as either the *CDKN2A* or *ARF* knockout mice (Krimpenfort, Quon et al. 2001; Sharpless, Bardeesy et al. 2001). These studies underline both the important role of ARF and that ARF and $p16^{INK4A}$ play subtly different roles in tumour suppression.

ARF function is also lost in a significant percentage of human tumours (Esteller, Tortola et al. 2000; Krassenstein, Sauter et al. 2004). As with the mouse there has been some difficulty in establishing the exact role played by loss of *ARF* as it is often co-deleted with $p16^{INK4A}$, however studies in melanoma have shown a high proportion of *ARF* deletions or mutations in tumours with wild-type $p16^{INK4A}$ again suggesting an important role for ARF in this disease (Freedberg, Rigas et al. 2008). As you would predict from the pathway as a general rule *ARF* and *p53*

mutations are mutually exclusive (Carr-Wilkinson, O'Toole et al. 2010), again indicating that the main effect of ARF in terms of tumour prevention is mediated via its activity on p53.

1.7.4 ARF as a tumour promoter

Although as has been demonstrated by the above discussion ARF is generally seen to act as a tumour suppressor, there is emerging evidence that in certain circumstances the reverse may be true and ARF may promote tumour development. Humbey *et al* showed that in the absence of p53 activity ARF can indeed promote tumour formation in a mouse model of B-cell lymphoma which is driven by constitutively high levels of Myc (Humbey, Pimkina et al. 2008). The authors suggested that this may be due to an ARF induced increase in autophagy, allowing increased tumour cell survival. Another study has demonstrated that in response to oncogenic stress ARF can promote formation of a Myc/Miz complex. This complex represses transcription of genes involved in adhesion. Functionally this resulted in cell detachment and subsequent apoptosis in p53 proficient cells. However, when apoptosis was inhibited the reduction in cell adhesion persisted (Herkert, Dwertmann et al. 2010). This would suggest that in the absence of an apoptosis signal mediated by p53 increased levels of ARF could reduce cell-cell adhesion and promote an invasive phenotype.

1.8 Introduction to mouse models of colorectal carcinoma

Mouse models of human disease have become an invaluable tool in furthering our understanding of the biological processes underlying a number of common human pathologies. In the case of CRC some of the understanding of the step-wise progression of the disease has come from work on animal models. In a reciprocal fashion greater understanding of the biology of CRC has informed the generation of animal models and therefore allowed for models that more closely recapitulate the human disease.

In these studies we have utilised a number of existing mouse models of CRC to study the role of RKIP *in vivo*, these models will be described below. Another aim of this study was to develop a novel model of late stage CRC. Therefore in the final section of this introduction I will discuss models of invasive CRC that already exist.

1.8.1 *Apc*^{MIN} mouse

The Multiple Intestinal Neoplasia (*Apc*^{Min}) mouse was first published in 1990 (Moser, Pitot et al. 1990) and is the result of a truncating mutation of Apc at codon 850, resulting in a non-functional protein (Su, Kinzler et al. 1992). These mice develop generally between 30-100 polyps in both the small and large intestine and recapitulate, to some extent, the phenotype of human FAP patients. However, there are a number of important differences between the mouse and the human, not the least of which is the fact that the tumours in the *Apc*^{Min} mouse only rarely progress beyond the adenoma stage (Moser, Pitot et al. 1990). Following on from the *Apc*^{Min} mouse a number of other mouse models have been generated by using truncations of the *Apc* gene of varying lengths (Fodde, Edelmann et al. 1994; Oshima, Oshima et al. 1995; Colnot, Niwa-Kawakita et al. 2004; Pollard, Deheragoda et al. 2009). A number of these have lead to different phenotypes, in terms of the number of polyps generated. However, the histology in all cases is remarkably similar with mice developing benign adenomas with only a small minority progressing to adenocarcinoma.

1.8.2 *Apc*^{580S} Mouse

Although a good model of early tumour development in FAP the *Apc*^{Min} mouse and other similar models do not recapitulate sporadic CRC, in which mutation of *APC* occurs in the adult. In order to address this the *Apc*^{580S} mouse (from now on referred to as the *APC*^{fl} mouse) was developed (Shibata, Toyama et al. 1997). This is a conditional *Apc* knock-out mouse which makes use of Cre-Lox technology. LoxP sites are inserted into the *Apc* gene in introns 13 and 14. The activation of the bactreriophage enzyme Cre recombinase results in

recombination at the LoxP sites and consequent deletion of exon 14 of the *Apc* gene. This results in a frameshift mutation at codon 580 and the generation of a premature STOP codon (Shibata, Toyama et al. 1997). The Apc protein that is produced is therefore truncated and non-functional. This approach also allows for a more tissue specific approach to the study of Apc function. This is achieved by having the Cre under the control of a tissue specific promoter. One of these is the AhCre, which is under the control of the *Cyp1A1* promoter, which yields activity of the Cre primarily in the small and large intestines and in the liver (Ireland, Kemp et al. 2004). The development of this conditional knock-out mouse also allows for the study of the acute effects of Apc loss within the intestine, this is not possible using any of the germ-line knock-outs as these result in embryonic lethality at day E6.5 (Moser, Shoemaker et al. 1995). The conditional heterozygous knockout of *Apc* therefore produces a better model for the study of sporadic intestinal adenomas, however the problem remains that only a very small minority of these adenomas will progress to carcinoma (Sansom, Meniel et al. 2006). There is a need therefore for development of further mouse models, which more closely recapitulate the later stages of the disease, specifically invasion and metastasis. Such models will allow for delineation of the function of genes involved in tumour progression, such as metastasis suppressor genes.

1.8.3 Carcinogen-induced models of CRC

Methods other than transgenics have been employed to develop models of intestinal tumourigenesis in the mouse. These include tumourigenesis induced by chemical carcinogens. One of the best known and studied examples of this method employs the administration of the carcinogen azoxymethane (AOM),

followed by the repeated oral administration of the irritant dextrane sodium sulphate (DSS). In this model AOM acts as the promoter, inducing mutations (particularly in the ß-catenin gene) and repeated exposure to DSS results inflammation and subsequent tumour formation.

This model has been used successfully in studies to demonstrate the important roles played by toll-like rector 4 (TLR4) and tumour necrosis factor-α (TNF-α) in inflammation induced CRC (Taketo, Edelmann. 2009). This model like all of the other models has a number of advantages and disadvantages. One of the main advantages of the model is that the tumours form in quite specifically in the colon rather than in the small intestine. The relatively short time to tumour formation is another important advantage. A potential drawback of this approach is that although the mutations induced by particular mutations are generally characteristic (such as the example given above, where ß-catenin mutations are induced by AOM), it cannot be exactly predicted what other genetic events are being induced by the carcinogen.

1.8.4 Models of invasive and metastatic colorectal carcinoma

A small number of mouse models exist which demonstrate the potential for invasion. Sansom *et al* demonstrated that the conditional deletion of a single copy of *Apc* combined with expression of an activated, oncogenic form of *KRas* results in an increase in the number of invasive carcinomas, when compared to deletion of *Apc* alone (Sansom, Meniel et al. 2006). However the tumours in this study showed only superficial invasion. Subsequently, it was shown that the combination of conditional deletion of both *Apc* and *Pten* resulted in full

thickness invasion (Marsh, Winton et al. 2008). Metastasis did not occur in either of these models.

Two recent models have been published in which the tumours that develop are capable of metastasis. In the first of these the tumours developed as a result of an inactivating *TGFβR* mutation, combined with the expression of an oncogenic form of *KRas* (Trobridge, Knoblaugh et al. 2009). The tumours that developed in this study showed occasional metastasis to the lymph nodes and lungs. The authors showed that the tumours developed in a Wnt independent fashion (Trobridge, Knoblaugh et al. 2009). This is characteristic of MSI tumours (Issa 2008), which develop along the so-called "serrated pathway". In tumours which develop along this pathway mutations in *APC* are less common and *TGFβR* mutation is more common than in CRC as a whole. In the second of these studies the authors utilised conditional deletion of one copy of *Apc* with expression of oncogenic *Ras*. They also confined the expression of the mutations to the distal colon by administering Adenoviral-Cre per rectum. This resulted in a relatively small number of tumours per mouse which showed invasion and occasional metastasis to the liver and lymph nodes (Hung, Maricevich et al. 2010).

1.9 Thesis aims

It has been shown previously that RKIP protein level is an important determinant of prognosis in CRC as a whole. In this thesis I will address the question of whether RKIP status retains its prognostic utility in Dukes B CRC patients. I will go on from there to examine the use of RKIP as a predictive marker. In the second part of the thesis I will examine the effects of *RKIP* knockout on known mouse models of CRC and attempt to develop a mouse model of CRC which more closely recapitulates the later stages of the human disease, particularly invasion and metastasis. These studies have led me to study the differing effects of *p53* knockout and point mutation in two different models. Therefore, the aims of the thesis are:

- To study the role of RKIP as a prognostic marker in Dukes B CRC.
- To study the role of RKIP as a predictive marker in CRC.
- To investigate the effect of *RKIP* knockout in existing models of CRC, in particular to examine any potential increase in metastatic behaviour.
- To develop and characterise a model of invasive and metastatic CRC, based on the mutation of *p53*.
- To use this and a model of aggressive rhabdomyosarcoma to study the differing effects of *p53* knockout and point mutation.

Chapter 2 Materials and Methods

2.1 Immunohistochemistry

All immunohistochemistry (IHC) was performed on standard paraffin sections. In all cases sections were de-waxed in xylene (3 changes of 3 minutes each) and then rehydrated through decreasing concentrations of ethanol to distilled water. With the exception of RKIP and β-catenin antigen retrieval was performed in citrate buffer (pH 6) either by the water bath or microwave methods (detailed below). Following antigen retrieval endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 10 minutes (with the exception of β-catenin). All washing steps consisted of 3 5-minute immersions in TBST. In all cases (with the exception of ZEB1) staining was visualised using DAB (3,3'-diaminobenzidine) chromogen and slides were counterstained with haematoxylin, prior to being dehydrated in increasing concentrations of ethanol and mounted.

2.1.1 Water bath antigen retrieval

5ml of Citrate or EDTA antigen retrieval buffer (Thermo) was diluted 1/10 with distilled water to a final volume of 50ml in a coplin jar. This was placed in a cold water bath and then heated to 99.9^{o}C, prior to immersion of the slides. Slides were then place in the pre-heated solution for 20 minutes before being allowed to cool in the solution for 30 minutes at room temperature.

2.1.2 Microwave antigen retrieval

Microwave antigen retrieval was performed by pre-heating 1500ml of EDTA or Citrate buffer in an open pressure cooker for ~15minutes until boiling. Slides were then placed into the solution and heated with the lid on until the pressure was optimised. Slides were then heated for a further 3-4 minutes, before being removed and allowed to cool at room temperature for 30 minutes.

EDTA Buffer

EDTA (Sigma): 3.7 g

Distilled water: 1000 ml

Tween 20: 5ml

This 10mM stock solution was diluted 1/10 to yield a 1mM working solution with distilled water and adjusted to pH 8.

Citrate Buffer

Solution A:

10.5g of citric acid

500 mls dH20

Solution B:

29.4g SodiumCitrate

1 litre dH20

27ml of solution A was mixed with 123ml of solution B and then made up to 1500ml with distilled water. The solution was adjusted to pH 6 following dilution.

2.1.3 IHC for $p19^{ARF}$

Sections were de-waxed and rehydrated as in 2.1. Antigen retrieval was performed using the water bath method. Following endogenous peroxidase blocking, slides were blocked in 10% normal goat serum (NGS) for 30 minutes. Primary rabbit anti-$p19^{ARF}$ antibody (Abcam, 1/300 in 10% NGS) was applied overnight at 4°C. After washing secondary anti-rabbit antibody (Vector ABC Kit) was applied for 30 minutes. After washing signal amplification was performed, using the ABC Complex (Vector ABC Kit), applied for 30 minutes. After washing positivity was visualised and slides mounted as described in 2.1.

2.1.4 IHC for β-catenin

Samples that were to be used for β-catenin staining were fixed for no more than 24 hours at 4°C. Sections were de-waxed and rehydrated as in 2.1. Peroxidase block was carried out in a solution of 1.5% hydrogen peroxide for 45 minutes. Antigen retrieval was performed in pre-heated Tris EDTA in a boiling water bath for 50 minutes. Slides were then cooled for 1 hour and blocked with 1% BSA for 30 minutes. Slides were incubated with primary mouse anti-β-catenin antibody (Transduction Laboratories, 1/50 in 1% BSA) for 2 hours at room temperature and with HRP-labelled polymer (Mouse Envision+ system, Dako) for 1 hour at room temperature. After washing positivity was visualised and slides mounted as described in 2.1.

2.1.5 IHC for BRDU

IHC was performed as in 2.1.3 with the following exceptions; Blocking was carried out in 1% BSA for 30 minutes. Primary mouse anti-BRDU antibody (BD Biosciences, 1/500 in 1% BSA) was applied overnight at 4°C. The mouse Envision + system (Dako) was used for both secondary antibody and signal amplification and applied for 1 hour at room temperature.

2.1.6 IHC for c-Myc

IHC was performed as in 2.1.3 with the following exceptions; Antigen retrieval was performed in the water bath for 50 minutes. Primary rabbit anti-c-Myc antibody (Santa Cruz, 1/200) was applied for 48 hours at 4°C. The rabbit Envision + system (Dako) was used for both secondary antibody and signal amplification and applied for 2 hours at room temperature.

2.1.7 IHC for Desmin

IHC was performed as in 2.1.3 with the following exceptions; Blocking was carried out in 10% normal horse serum (NHS). Primary mouse anti-desmin antibody (Thermo, 1/50 in Dako antibody diluent) was applied for 1 hour at room temperature.

2.1.8 IHC for Fascin

IHC was performed as in 2.1.3 with the following exceptions; Antigen retrieval was performed in citrate buffer, using the microwave method. Primary rabbit anti-fascin antibody (Atlas antibodies, 1/100 in Dako antibody diluent) was

applied for 2 hours at room temperature. The rabbit Envision + system (Dako) was used for both secondary antibody and signal amplification and applied for 1 hour at room temperature.

2.1.9 IHC for Ki-67

IHC was performed as in 2.1.3 with the following exceptions; Primary rabbit anti-Ki-67 antibody (Thermo, 1/200 in Dako antibody diluent) was applied for 1 hour at room temperature. The rabbit Envision + system (Dako) was used for both secondary antibody and signal amplification and applied for 1 hour at room temperature.

2.1.10 IHC for myogenin

IHC was performed as in 2.1.3 with the following exceptions; Antigen retrieval was performed in citrate buffer, using the microwave method. Blocking was carried out in 10% NHS. Primary mouse anti-myogenin antibody (Dako, 1/100 in Dako antibody diluent) was applied 1 hour at room temperature.

2.1.11 IHC for p21

IHC was performed as in 2.1.3 with the following exceptions; Primary rabbit anti-p21 antibody (Santa Cruz, 1/500 in Dako antibody diluent) was applied for 1 hour at room temperature. The rabbit Envision + system (Dako) was used for both secondary antibody and signal amplification and applied for 1 hour at room temperature.

2.1.12 IHC for p53

IHC was performed as in 2.1.3 with the following exceptions; Blocking was carried out in 10% NHS. Primary mouse anti-p53 antibody (Vector Laboratories, 1/200 in Dako antibody diluent) was applied for 1 hour at room temperature. The mouse Envision + system (Dako) was used for both secondary antibody and signal amplification and applied for 1 hour at room temperature.

2.1.13 IHC for pAkt^{Ser473}

IHC was performed as in 2.1.3 with the following exceptions; Primary rabbit anti-pAkt^{Ser473} antibody (Cell Signalling, 1/25 in 5% NGS) was applied overnight at 4^{o}C.

2.1.14 IHC for pERK$^{Thr202/Tyr204}$

IHC was performed as in 2.1.3 with the following exceptions; Antigen retrieval was performed in citrate buffer, using the microwave method. Primary rabbit anti-pERK$^{Thr202/Tyr204}$ antibody (Cell Signalling, 1/100 in 10% NGS) was applied overnight at 4^{o}C.

2.1.15 IHC for RKIP

IHC was performed as in 2.1.3 with the following exceptions; Antigen retrieval was performed in EDTA buffer, using the microwave method. Primary rabbit anti-RKIP antibody (Ki-69, 1/1500 in 10% NGS) was applied for 1 hour at room temperature.

2.1.16 IHC for ZEB1

IHC was performed as in 2.1.3 with the following exceptions; Blocking was carried out in 1% BSA. Primary goat anti-ZEB1 antibody (Santa Cruz, 1/100 in Dako antibody diluent) was applied overnight at 4°C. Signal was visualised with alkaline phosphatase chromogen.

2.2 ISH for LGR5

ISH was carried out using labeled riboprobes as has been previously described (Poulsom, Longcroft et al. 1998), with SP6 RNA polymerase and EcoRI linearized sequence verified templates prepared in pGEM3Z. Mouse *Lgr5* 562 bp from 5′ UTR to exon5 (UCSC chr10:114,915,553-115,024,577), introns excluded.

2.3 RKIP monoclonal antibody depletion experiments

To demonstrate binding of the RKIP monoclonal antibody we pre-adsorbed the antibody prior to application to the tissue section. This was done as follows: 200µg of purified GST-RKIP protein was added to binding buffer (PBS, 1mM DTT, pH 7.5). Protease activity was inhibited by the addition of a protease inhibitor tablet (Roche). A 200µl suspension of glutathione sepharose beads was washed 3 times with binding buffer and finally centrifuged for 1 minute at 1000rpm and 4°C. GST-RKIP was added to the washed beads and gently agitated for 1 hour at 4°C to allow GST binding to the beads. The suspension was then centrifuged for 1

minute at 1000rpm and 4°C and washed 3 times, prior to re-suspension. RKIP monoclonal antibody was then exposed to the beads using 2 different methods.

Firstly, 50µl of GST-RKIP beads were placed in a column and the RKIP monoclonal antibody applied above the beads and allowed to flow through them under gentle pressure. The run through was collected and the procedure repeated 9 times. The second method used was to incubate 400µl of RKIP monoclonal antibody with 150µl of GST-RKIP beads, under gentle agitation for 2 hours at 4°C. Following incubation the solution was centrifuged for 1 minute at 1000rpm and 4°C and the supernatant removed. The procedure was repeated once and then the supernatant used for slide staining.

2.4 Tissue Microarrays

The tissue microarray (TMA) used in 3.2.1 contained 4 cores of tumour and normal tissue from 220 patients with Dukes B CRC, all of whom had their primary treatment at St. Vincent's University Hospital, Dublin. The TMA used in 3.2.4 consisted of tumour and normal tissue from 1034 patients with CRC from the Western Australia Tissue Research Network, across all stages. Each sample was present in duplicate. All samples were formalin fixed and paraffin embedded. In the case of the Dublin TMA the slides contained samples of both normal colonic epithelium and normal liver which acted and a positive control, in the case of the TMA from the Western Australia Tissue Research Network normal colonic epithelium acted as the positive control. A negative control, using normal goat serum rather than the primary antibody was included in each staining run. Both TMAs were scored independently by 2 people. Both scorers were blinded to the clinical outcome data. The results of each independent scorer were compared

and any discrepancies were resolved by a review of the sample in question and agreement on a final consensus score.

2.5 Statistical analysis

Statistical analysis was carried out using Minitab 15 or SPSS (IBM) software packages. Survival data was calculated using Kaplan-Meier survival analysis. Univariate and multivariate analyses were carried out using the Cox regression model. Comparison of tumour number and size in the mouse experiments was performed using the Mann-Whitney test for differences in medians. Differences in the numbers of aberrant mitoses and rates of metastases across groups were compared using the Chi-squared test. Depth of invasion into organotypic assay was compared using the Sudent's t-test, follwing a test to ensure the data follwed a normal distribution. In all cases a p-value of less than 0.05 was deemed statistically significant.

2.6 Generation of mouse colonies

All experiments were performed in accordance with UK Home Office guidelines and local ethical approval. Mice were maintained under non-barrier conditions and fed a standard diet (Harlan) and water *ad libitum*.

The alleles used in the experiments were as follows: *RKIP* (Theroux, Pereira et al. 2007), APC^{fl} (Shibata, Toyama et al. 1997), *AhCre* (Ireland, Kemp et al. 2004), $KRas^{G12V}$ (Guerra, Mijimolle et al. 2003), $PTEN^{fl}$ (Groszer, Erickson et al. 2001), $p53^{fl}$ (Jonkers, Meuwissen et al. 2001), $p53^{R172H}$ (Olive, Tuveson et al. 2004) ZE/G GFP (Novak, Guo et al. 2000).

2.6.1 Mouse experiments for chapter 4

To examine the effect of *RKIP* deletion on tumourigenesis in the APC^{fl} mouse $AhCre^{+}$ $APC^{fl/+}$ mice were crossed with $RKIP^{-/-}$ mice to yield $AhCre^{+}$ $APC^{fl/+}$ $RKIP^{+/-}$ mice. These were then inter-crossed to yield experimental cohorts of $AhCre^{+}$ $APC^{fl/+}$ $RKIP^{+/+}$, $AhCre^{+}$ $APC^{fl/+}$ $RKIP^{+/-}$ and $AhCre^{+}$ $APC^{fl/+}$ $RKIP^{-/-}$ mice. *AhCre* expression was induced with 3 intra-peritoneal injections of β-naphthoflavone (80mg/kg), every 4 hours at ~6 weeks of age. Mice were examined 3 times per week for signs of intestinal tumours, such as paling feet, hunching and weight loss. Upon developing any 2 of these signs mice were euthanized and underwent a full necropsy. Tumour invasion was assessed by histology.

To examine the effect of *RKIP* deletion on the acute deletion of *APC*, $AhCre^{+}$ $APC^{fl/+}$ $RKIP^{+/-}$ mice were inter-crossed to yield cohorts of $AhCre^{+}$ $APC^{fl/fl}$ $RKIP^{+/+}$ and $AhCre^{+}$ $APC^{fl/fl}$ $RKIP^{-/-}$ mice. Mice were given 3 intra-peritoneal injections of β-naphthoflavone, every 4 hours at ~6 weeks of age on day 0. Mice were euthanized on day 4 and underwent full necropsy.

To determine the effect of *RKIP* deletion on invasive/metastatic behaviour in the $AhCre^{+}$ $APC^{fl/+}$ $KRas^{G12V}$ mouse $AhCre^{-}$ $APC^{fl/+}$ $KRas^{G12V}$ were crossed with $AhCre^{+}$ $APC^{fl/+}$ $RKIP^{-/-}$ to yield $AhCre^{+/-}$ $APC^{fl/+}$ $KRas^{G12V}$ $RKIP^{+/-}$. These were then inter-crossed to yield cohorts of $AhCre^{+}$ $APC^{fl/+}$ $KRas^{G12V}$ $RKIP^{+/+}$ and $AhCre^{+}$ $APC^{fl/+}$ $KRas^{G12V}$ $RKIP^{-/-}$ mice. Mice were induced and monitored for signs of intestinal tumours as described above. Tumour invasion was assessed by histology.

2.6.2 Mouse experiments for chapter 5

To determine the effects of *p53* deletion or point mutation on the phenotype of the APC^{fl} mouse $AhCre^{+}$ $APC^{fl/+}$ mice were crossed to either $p53^{fl/fl}$ or $p53^{R172H/+}$ mice. The progeny were then inter-crossed to yield cohorts of $AhCre^{+}$ $APC^{fl/+}$ $p53^{+/+}$, $AhCre^{+}$ $APC^{fl/+}$ $p53^{fl/+}$, $AhCre^{+}$ $APC^{fl/+}$ $p53^{R172H/+}$, $AhCre^{+}$ $APC^{fl/+}$ $p53^{fl/fl}$, $AhCre^{+}$ $APC^{fl/+}$ $p53^{R172H/fl}$ mice. *AhCre* expression was induced with 3 intra-peritoneal injections of β-naphthoflavone (80mg/kg), every 4 hours at ~6 weeks of age. Mice were monitored for signs of intestinal tumourigenesis as described above. Tumour invasion was assessed by histology.

To assess the response of mutant p53 and p21 to γ-irradiation, mice were irradiated with 14 Gy irradiation using a Cs^{137} source delivered at a dose rate of 0.423 Gy/min. Mice were euthanized 6 hours post-irradiation and underwent a full necropsy.

To assess the effect of ARF over-expression *in vivo* HCT116 $p53^{-/-}$ cells + pcDNA3 vector, or + pcDNA3-$p14^{ARF}$ were injected subcutaneously into the right flank of 8-10 week old female nude mice (Charles Rivers, Harlan, UK). The mice were monitored regularly and upon developing tumours > 1.7cm, or upon tumour ulceration, mice were euthanized and underwent a full necropsy.

2.6.3 Mouse experiments for chapter 6

To study the differing effects of *p53* deletion and point mutation in the mouse model of pleomorphic rhabdomyosarcoma $AhCre^{-}$ $KRas^{G12V}$ mice were crossed to $AhCre^{+}$ $p53^{fl/fl}$ or $p53^{R172H/+}$ mice. The progeny were then inter-crossed to yield cohorts of $AhCre^{+}$ $KRas^{G12V}$ $p53^{fl/+}$, $AhCre^{+}$ $KRas^{G12V}$ $p53^{R127H/+}$, $AhCre^{+}$ $KRas^{G12V}$

p53$^{fl/fl}$, *AhCre*$^{+}$ *KRas*^{G12V} *p53*$^{R172H/fl}$ mice. Mice were aged until they developed tumours of 1.7cm or ataxia at which point they were euthanized and underwent a full necropsy. Irradiation experiments were carried out as in 2.5.2.

To demonstrate spontaneous AhCre recombination in the skeletal muscle *AhCre*$^{+}$ mice were crossed to Lox-STOP-Lox (LSL) ZE/G GFP mice. The Olympus OV100 Whole Mouse Imaging System (Olympus), containing an MT-20 light source (Olympus Biosystems) and DP70 CCD camera (Olympus), was used for imaging GFP positivity in euthanized mice.

2.7 Tissue isolation

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:acetic acid; 4:2:1) and paraffin embedded. The following 5cm was divided into 1cm lengths, parcelled using surgical tape and then fixed in neutral buffered formalin (NBF) at 4^{o}C for no more than 24 hours before processing. The remainder was fixed in methacarn and then paraffin embedded.

For the tumourigenesis studies the entire intestine was removed and flushed with water. Both small intestine and colon were mounted 'en face' and fixed overnight in either methacarn or NBF. Lesions were then scored macroscopically. Intestines were wound into rolls which were subsequently embedded in paraffin, sectioned at 5-10μm and stained with haematoxylin and eosin prior to microscopic analysis.

Rhabdomyosarcomas were dissected from underlying tissues and sectioned at intervals of ~2mm prior to being fixed in NBF and processed as above. A subset of tumour samples was placed in RNALater (Applied Biosystems) and frozen at -70^{o}C.

2.8 Scoring of apoptosis, mitosis and aberrant mitosis

Apoptosis was identified morphologically; apoptosis was recognised by the appearance of smooth membrane bound apoptotic bodies, the presence of a halo around the apoptotic bodies, nuclei displaying clear chromatin condensation and red cytoplasm (Kerr, Wyllie et al. 1972).

Similarly mitotic figures were identified by morphology and counted if the cell was in any of the stages of mitosis. Aberrant mitoses were defined as follows; Unequal division: gross difference in the amount of nuclear material in the daughter cells, Tripolar mitosis: A mitosis in which 3 mitotic spindles could be clearly identified, Anaphase bridge: Strictly defined as a mitotic figure in which 2 well defined parallel anaphase plates had formed and were connected by a "bridge" of genetic material (Montgomery, Wilentz et al. 2003).

2.9 Genotyping of mice

Mice were genotyped by PCR, with DNA extracted from tail biopsies taken at the time of weaning. All PCR reactions were done in 50μl volumes using 2.5μl of the

tail DNA preparation, with the exception of RKIP in which 3μl was used. PCR products were resolved by electrophoresis on a 2% agarose gel in all cases.

2.9.1 DNA extraction from tails

DNA was extracted from tails using the puregene DNA extraction kit (Qiagen). 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 precipitation solution (Puregene) was added to each tube. These were vortexed and centrifuged at top speed for 5 minutes in a microcentrifuge.

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

Individual PCR protocols are detailed below:

2.9.2 Apc^{fl} 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

*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

Reaction Conditions: 95˚C, 3min (95˚C, 30s; 60˚C, 30s; 72˚C 1min)$_{30}$, 72˚C, 5min. 4˚C, hold.

Bands:FLOX = 314bp

WT = 226bp

2.9.3 Cre 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	

*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

Reaction Conditions: 95˚C, 3min (95˚C, 30s; 55˚C, 30s; 72˚C 1min)$_{30}$, 72˚C, 5min. 15˚C, hold.

Bands:CRE = ~1000bp

2.9.4 LacZ PCR Protocol

PCR Mix	µl
Buffer	5
$MgCl_2$ (25mM)	2.5
dNTPs (10mM)	0.4
Primer (100µM)	0.1 (of each)
Platinum Taq*	0.2
H_2O to final volume of 47.5 µl	

*Platinum Taq (Invitrogen)

Primers:

LACZ A = CTG GCG TTA CCC AAC TTA AT

LACZ B = ATA ACT GCC GTC ACT CCA AC

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

Bands:LacZ = ~500bp

2.9.5 KRas^{G12V} PCR Protocol

PCR Mix	µl
Buffer	5
$MgCl_2$ (25mM)	2.5
dNTPs (10mM)	0.4
Primer (100µM)	0.1 (of each)
Go Taq*	0.4

H_2O to final volume of 47.5 µl

*Go Taq (Promega)

Primers:

F = AGG GTA GGT GTT GGG ATA GC

R = CTG CTC TTT ACT GAA GGC TC

Reaction Conditions: 94°C, 5min (94°C, 1min; 60°C, 1min; 72°C, 1min, 25s)$_{30}$, 72°C, 10min. 15°C, hold.

Bands: WT = 403bp

FLOX = 621bp

2.9.6 p53^{fl} PCR Protocol

PCR Mix	µl
Buffer	5
$MgCl_2$ (25mM)	2.5
dNTPs (10mM)	0.4
Primer (100µM)	0.1 (of each)
Platinum Taq*	0.2
H_2O to final volume of 47.5 µl	

*Platinum Taq (Invitrogen)

Primers:

P53 Fl int1 F CAC AAA AAC AGG TTA AAC CCA G

P53 Fl int1 R AGC ACA TAG GAG GCA GAG AC

Reaction Conditions: 94°C, 3min (94°C, 30s; 58°C, 20s; 72°C, 1min)$_{30}$, 72°C, 5min. 15ºC hold

Bands: WT = 431bp

FLOX = 584bp

2.9.7 $p53^{R172H}$ PCR Protocol

PCR Mix	µl
Buffer	5
$MgCl_2$ (25mM)	2.5
dNTPs (10mM)	0.4
Primer (100µM)	0.1 (of each)
Platinum Taq*	0.2
H_2O to final volume of 47.5 µl	

Note: This is a split PCR

WT Reaction use primers P53R172H WT and P53R172H Universal

LSL Reaction use primers P53R172H Mut and P53R172H Universal

Primers:

P53R172H WT = TTA CAC ATC CAG CCT CTG TGG

P53R172H Universal = CTT GGA GAC ATA GCC ACA CTG

P53R172H Mut = AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A

Reaction Conditions: 94°C 3min (94°C, 30s; 60°C, 1min 30; 72°C 1min)$_{35}$, 72°C, 5min. 4°C hold.

Bands:WT = 170bp

LSL = 270bp

2.9.8 RKIP PCR Protocol

PCR Mix	µl

10x FastStart Taq Buffer	5
$MgCl_2$ (25mM)	3
dNTPs (10mM)	0.4
Primer (100µM)	0.2 (of each)
FastStart Taq*	0.2
H_2O to final volume of 47 µl	

*FastStart Taq from Roche

Primers:

RKIP F2 = GAG CCC TGG CCG GTC TCC CTT GTC CCA AAC TTT

RKIP R2 = CCA AAA GGG TCT TTG AGC ACC AGA GGA CAT CCG

RKIP R4 = AGA CTT CCG TGT CCG GAT GAT AGA TAG CCT CTC C

Split Reaction use primers:

WT Reaction 1: RKIP F2 and R4

HOM Reaction: RKIP F2 and R2

Reaction Conditions: 94ºC, 5min (94ºC, 1min; 57ºC, 1min; 72ºC, 1min)$_{36}$; 72ºC, 7min; 4ºC hold

Bands:WT = 978bp

NULL = 630bp

2.10 RKIP rtPCR

Reverse transcription was performed using the SuperscriptII reverse transcriptase kit (Invitrogen) and Random hexamers (Invitrogen) as per the manufacturer's instructions. rtPCR for RKIP1 and RKIP2 was performed using the following primers and reaction conditions:

RKIP1 Forward: CTG ACT GGC TGG CTG GTA CT

RKIP1 Reverse: TCT GGA GGA AGA AAC GAC AG

RKIP2 Forward: TCT TGA GCT GTT GTA GGG AGG TA

RKIP2 Reverse: TAA CGA GTC CAT CAC AGT GCC

Reaction Conditions: 94°C, 6min $(94°C, 30sec; 58°C, 30sec; 72°C\ 50sec)_{35}$

72°C, 7min. 15°C, hold.

Amplified PCR product was resolved by electrophoresis on a 2% agarose gel.

2.11 Pyrosequencing

2.11.1 DNA Extraction

DNA from both mouse and human tissue samples was extracted using the DNAeasy blood and tissue kit (Qiagen), according to the manufacturer's instructions.

2.11.2 Bisulphite modification

Bisulphite modification was carried out using the Epitect Bisulfite kit (Qiagen) according to the manufacturer's instructions. Briefly this involved bisulphite modification of the DNA, binding of the modified, single stranded DNA to the membrane of a spin column and finally desulphonation and elution of the DNA. In order to confirm complete bisulphite conversion PCR for a segment of the calponin gene was performed using the primers below. This section contains multiple non-CpG cytosine (C) residues. As these cannot be methylated they

should all be converted during the bisulphite modification process. The primers used will only amplify DNA if all of Cs have been converted to U.

Calponin Primers:

Forward: GGA AGG TAG TTG AGG TTG TG

Reverse: CCC AAA CTC AAA ACT CTA ACC TAA C

2.11.3 Pyrosequencing

In the case of the pyrosequencing analysis to determine methylation status, the DNA was bisulphite modified as in 2.10.2. Forward (F), Reverse (R) and Sequencing (S) primers for DNA amplification and sequencing were designed using the PSQ96MA Assay Design Software (Qiagen) and are shown below. DNA was amplified by PCR using the reaction conditions below. Specific amplification was ensured by running 5µl of PCR product on an agarose gel, ensuring the presence of a single clear band of the appropriate size. For the RKIP methylation analysis normal human and 100% in vitro methylated DNA were used as negative and positive controls respectively. For the p53 LOH analysis normal muscle from both a $p53^{+/+}$ animal and a $p53^{R172H/fl}$ animal were used as controls.

Primers

RKIP F: TTT TAG GGC GTT TTT TAT TTT TAT

RKIP R: AAA CTA ACA AAA CAA AAC CTC TC (Biotinylated)

RKIP S: TCC ATA CAA CCT ACT CCC

Reaction Conditions: 94°C, 6min $(94°C, 30sec; 55°C, 30sec; 72°C\ 50sec)_{45}$

72°C, 7min. 15°C, hold.

p53 F: GGC CAT CTA CAA GAA GTC ACA GC

p53 R: CGG TGT TGA GGG CTT ACC A (Biotinylated)

p53 S: GAC GGA GGT CGT GAG A

Reaction Conditions: 94°C, 6min (94°C, 30sec; 61°C, 30sec; 72°C 50sec)$_{40}$

72°C, 7min. 15°C, hold.

Following amplification 40µl of the PCR product was added to 3µl of streptavidin sepharose beads (which bind the biotinylated PCR product) and 37µl of binding buffer (Qiagen). The mixture was then agitated to ensure re-suspension of the beads. The beads were then incubated for 5 seconds each in 70% ethanol, 0.2M NaOH, wash buffer (Qiagen) and high purity water, using the vacuum prep work station (Qiagen). This purifies the DNA and renders it single stranded to allow the pyrosequncing reaction to occur. The beads were then added to 1.5µl of the sequencing primer (10µM solution) and 43.5µl of annealing buffer (Qiagen) and incubated at 80^{o}C for 2 minutes. The sample was the analysed on PSQ96MA pyrosequencing machine (Qiagen), according to the manufacturer's instructions. The data was analysed using the PSQ96MA software (Qiagen)

2.12 Cell Culture

HCT116 cells (wild type and p53$^{-/-}$) were grown in McCoy's 5A medium supplemented with 10% fetal calf serum (FCS) and 2mM L-glutamine, and maintained in a humidified incubator at 37°C with 5% CO_2. Cells were transfected with either pcDNA3 vector or pcDNA3-p14^{ARF} (A kind gift from Prof. Karen Vousden) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, and selected in growth medium containing 500µg/ml G418 sulfate.

2.13 Immunoblotting

Cells were washed with PBS and then lysed in cell extraction buffer (50mM Tris (pH 7.6), 150mM sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10µg/ml aprotinin, 125mM phenylmethylsulfonyl fluoride, 100µM sodium orthovanadate and 0.5mM sodium fluoride). Protein concentration was determined by comparison of absorbance against known BSA concentrations. The resulting lysates were cleared by centrifugation and then resolved by 10% Bis-Tris gel electrophoresis (Invitrogen). Proteins were transferred to PVDF membrane, blocked and probed with either 1:200 anti-$p14^{ARF}$ (AbCam) or 1:10000 anti-ERK (Sigma-Aldrich) antibodies. Bound antibody was detected by incubation with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized by Enhanced Chemiluminescence (Amersham).

2.14 Organotypic Invasion Assay

Organotypic cultures were set up as previously described (Edward, Gillan et al. 2005). Briefly, ~$7.5x10^4$/ml primary human fibroblasts were embedded in a 3 dimensional matrix of rat tail collagen I. Rat tail tendon collagen solution was prepared by the extraction of tendons with 0.5 M acetic acid to a concentration of ~2mg/ml. Detached, polymerised matrix was allowed to contract for approximately 6 days in complete media (DMEM, supplemented with 10% FCS, Invitrogen) until the fibroblasts had contracted the matrix to ~1.5cm diameter. Subsequently, $4x10^4$ HCT116 cells were plated on top of the matrix in complete media and allowed to grow to confluence for 5 days. The matrix was then mounted on a metal grid and fed from below with complete media that was

changed every 2 days. After 12 days, the cultures were fixed using 4% paraformaldehyde and processed by standard methods for haematoxylin and eosin (H&E) staining. Depth of invasion was measured from the surface of the matrix to the deepest point of a group of invading cells.

Chapter 3 RKIP is a prognostic indicator in colorectal carcinoma.

3.1 Introduction

It has been shown that RKIP protein levels are reduced in human cancers compared to normal tissue. These include common tumours such as breast, prostate and CRC and also some rarer tumour such as pituitary adenoma and GIST (Fu, Smith et al. 2003; Hagan, Al-Mulla et al. 2005; Al-Mulla, Hagan et al. 2006; Fougner, Bollerslev et al. 2008; Martinho, Gouveia et al. 2009). Interestingly in both CRC and in prostate cancer it has been shown that the level of RKIP protein in the tumour correlates inversely with the risk of metastatic relapse and therefore with patient prognosis (Al-Mulla, Hagan et al. 2006; Fu, Kitagawa et al. 2006). We set out to study the utility of RKIP as a prognostic marker in a particular subset of CRC patients, namely Dukes B CRC patients. These are patients with locally advanced tumours (the tumour having invaded through the bowel wall) but who at the time of primary surgery have no detectable metastases. The reason for choosing Dukes B was the current controversy regarding the optimal post-operative management of this group, specifically whether post-operative (adjuvant) chemotherapy offers any benefit to these patients.

Patients with Dukes B CRC have a reasonably favourable prognosis with a 5-year survival of ~74-80% (Eisenberg, Decosse et al. 1982; Petersen, Baxter et al. 2002; O'Connell, Maggard et al. 2004). However, this means that although in theory these patients should have been completely cured by surgery alone ~25% will develop recurrent disease and die as a result. This has lead to a debate about the use of adjuvant chemotherapy in this patient group. Recent large-scale clinical trials have been performed in an attempt to answer this question (Andre, Boni et al. 2004; Kuebler, Wieand et al. 2007; Quasar Collaborative, Gray et al.

2007). However, these studies have not provided a definitive conclusion. Clinical guidelines recommend that patients with "high risk" Dukes B CRC should be offered adjuvant chemotherapy (Benson, Schrag et al. 2004; Figueredo, Coombes et al. 2008). These high risk features are broadly defined and there is not yet universal consensus on exactly how the guidelines should be applied. This suggests that additional markers which could refine risk assessment would be beneficial in selecting those patients who are most likely to benefit from adjuvant chemotherapy. We set out to see whether RKIP could act as such a marker and therefore, add value to current methods of risk stratification.

Although there is no doubt that novel prognostic markers in CRC are extremely useful there is also a need for predictive markers. These are markers that not only predict patient survival but also response to therapy. In CRC a number of these already exist, such as testing for KRas mutations to determine the response to anti-EGFR therapy, however there is still a need for further markers (Siena, Sartore-Bianchi et al. 2009). There are reasons to believe that RKIP may function as such a predictive marker in CRC. Studies have shown that RKIP levels are elevated in response to chemotherapy and that this rise in RKIP levels promotes tumour cell apoptosis (Chatterjee, Bai et al. 2004; Jazirehi, Vega et al. 2004). Moreover, it has been shown that cells that cannot upregulate RKIP do not undergo chemotherapy induced apoptosis, suggesting that RKIP is important for this process (Chatterjee, Bai et al. 2004). Indeed, in a recent study of patients with pituitary adenoma RKIP levels were found to correlate with a response to octreotide treatment (Fougner, Bollerslev et al. 2008). To study this we utilised a TMA comprising of samples from patients on whom information was available regarding both treatment and survival.

Although it known that RKIP is downregulated in a large number of human tumours, the mechanism of downregulation remains unclear. To date no mutations have been found in the RKIP gene and this has lead investigators to examine epigenetic mechanisms of RKIP silencing. There has been conflicting evidence in the literature regarding the role of promoter methylation in the silencing of RKIP. Promoter methylation is a process by which CG repeats in the promoter regions of genes (so called CpG islands) are methylated, thus preventing transcription of the gene (Cedar and Bergman 2009). This is an important physiological regulator of gene expression but has also been shown to occur in cancer, resulting in the silencing of tumour suppressor genes (Toyota, Ahuja et al. 1999; Zhu, Qin et al. 2009). Al Mulla *et al* have used MSP to show increased methylation in the RKIP promoter in CRC (Al-Mulla, Hagan et al. 2008), but others have not replicated these results (Minoo, Zlobec et al. 2007). As none of these studies have used gold-standard quantitative methods to assess methylation there is a recognised need to examine the question using such an approach. In the final section of this chapter we have used pyrosequencing to determine the methylation status of a cohort of CRC patients.

3.2 Results

3.2.1 RKIP levels correlate inversely with disease-specific survival in Dukes B CRC.

Studies previously performed in our laboratory had developed a reliable method of staining for RKIP by IHC in human formalin fixed paraffin embedded (FFPE) tissue. Similarly, a method for scoring the sections based on area stained and staining intensity had previously been developed and verified (Al-Mulla, Hagan et al. 2006). Briefly this involved giving a score of 0-3 for both the area stained and staining intensity. These were then summed to give a minimum score of 0 and a maximum score of 6. Scores of 0-2 were classed as negative, 3-4 weak positive and 5-6 strong positive. We stained a TMA consisting of 4 cores of both tumour and adjacent normal tissue from 220 patients with Dukes B CRC. All patients had their primary treatment at a single centre, between 1990 and 2002, giving a median follow-up of 11 years. Median and 5-year survival for the group as a whole was 69%. None of the patients in the study had received chemotherapy. Demographic and clinico-pathological characteristics of the patients in the study are shown in Table 3.1.

		Number of cases (percentage of total)
Gender	Male	118 (56%)
	Female	91 (44%)
Age	<50	7 (3%)
	50-59	24 (11%)
	60-69	58 (28%)
	70-79	85 (41%)
	>80	35 (17%)
Peritoneal Invasion	Absent	168 (80%)
	Present	41 (20%)
Lympho-vascular Invasion	Absent	152 (73%)
	Present	57 (27%)
Tumour Location	Rectum	63 (30%)
	Left	62 (30%)
	Right	84 (40%)

Table 3.1: Demographic and clinico-pathological data relating to the patients in the study.

Two of the most powerful prognosticators currently available in Dukes B CRC are the presence of peritoneal invasion (present in 20% of the patients in this population, Table 3.1) and LVI (present in 27% of patients in this population, Table 3.1) (Shepherd, Baxter et al. 1997; Petersen, Baxter et al. 2002; Lennon,

Mulcahy et al. 2003). To confirm that there was nothing unusual about the outcomes of this particular patient group we performed a Kaplan-Meier survival analysis stratifying patients by the presence or absence of peritoneal invasion and LVI. As expected there was a significant difference in median and 5-year survival between the 2 groups in each case (Figure 3.1). In the case of peritoneal invasion 5-year survival was 50% and 73% for those with and without peritoneal invasion respectively (Log-Rank p=0.001). In the case of LVI 5-year survival was 52% and 75% for those with and without LVI respectively (Log-Rank p=0.017). This indicates that this is a typical group of Dukes B CRC patients and therefore we proceeded with staining for RKIP.

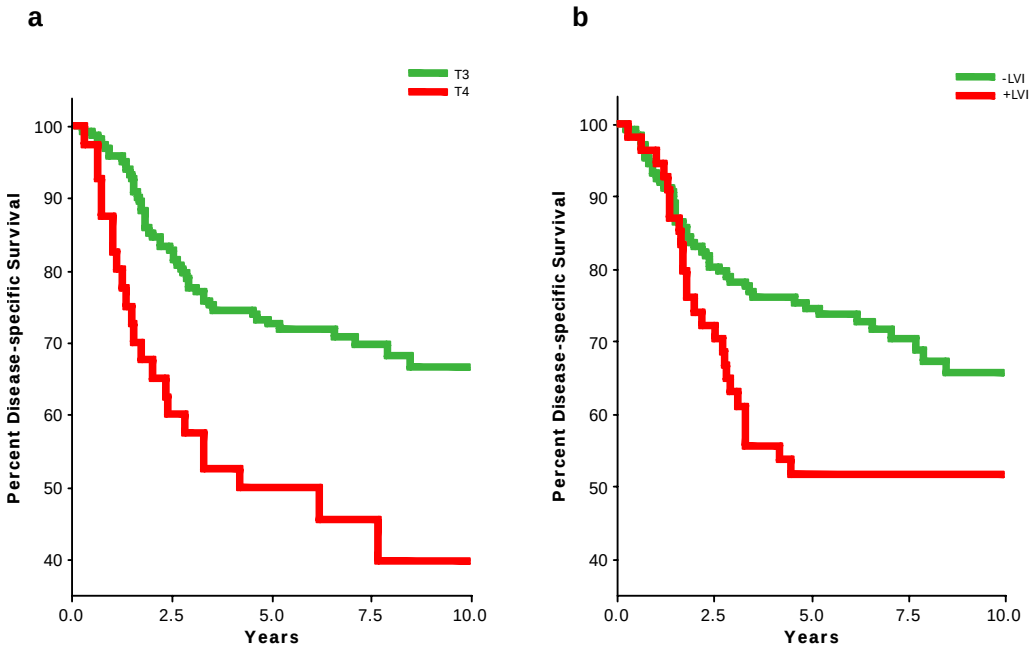


Figure 3.1: Kaplan-Meier analysis of disease-specific survival, stratified by peritoneal invasion and LVI

Tumours in which peritoneal invasion was identified (a, red line) were associated with a statistically significant decrease in disease-specific survival compared to tumours without peritoneal invasion (a, green line) (Log-Rank p=0.001). Similarly the presence of LVI (b, red line was associated with a statistically significant decrease in disease-free survival (Log-Rank p=0.017)

Staining was similar to that which had been seen previously, with a spectrum of RKIP staining seen, from negative through to strongly positive (Figure 3.2). Of the 220 samples 11 could not be scored owing to tissue loss during staining, leaving 209 for analysis. 14 cases were negative, 99 were weakly positive and 96 were strongly positive.

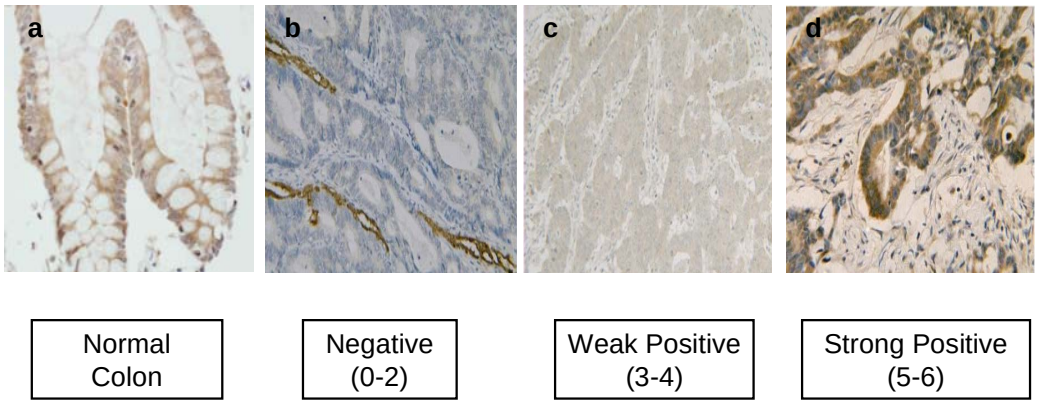


Figure 3.2: RKIP staining in normal colon and CRC

Strong cytoplasmic RKIP staining was seen in normal colonic crypts (a) and score for RKIP staining was higher in normal colon than in tumour tissue (Mann-Whitney $p<0.001$). In the tumour samples RKIP was scored based both on area stained and staining intensity. This scoring system divided tumours into 3 groups, negative (b) with a score of 0-2), weak positive (c) with a score of 3-4 and strong positive (d) with a score of 5-6.

As had been found previously there was a significant difference in the staining seen between normal and tumour tissue. The median score for normal colon was 6 compared to 4 for the tumour tissue, this difference was statistically significant (Mann-Whitney $p<0.001$).

We then went on to examine the relationship between disease-specific survival and RKIP score. The analysis revealed a strong inverse correlation between RKIP expression levels and disease-specific survival (Figure 3.3). As there was no significant difference between the weak positive and the negative groups these 2 groups were combined. The newly formed groups will be referred to as RKIP positive (previously Strong positive) and RKIP negative (previously Weak positive and Negative). 5-year survival was 78% and 60% in the RKIP positive and negative groups respectively. This difference was statistically significant (Log-Rank $p=0.011$) (Figure 3.3).

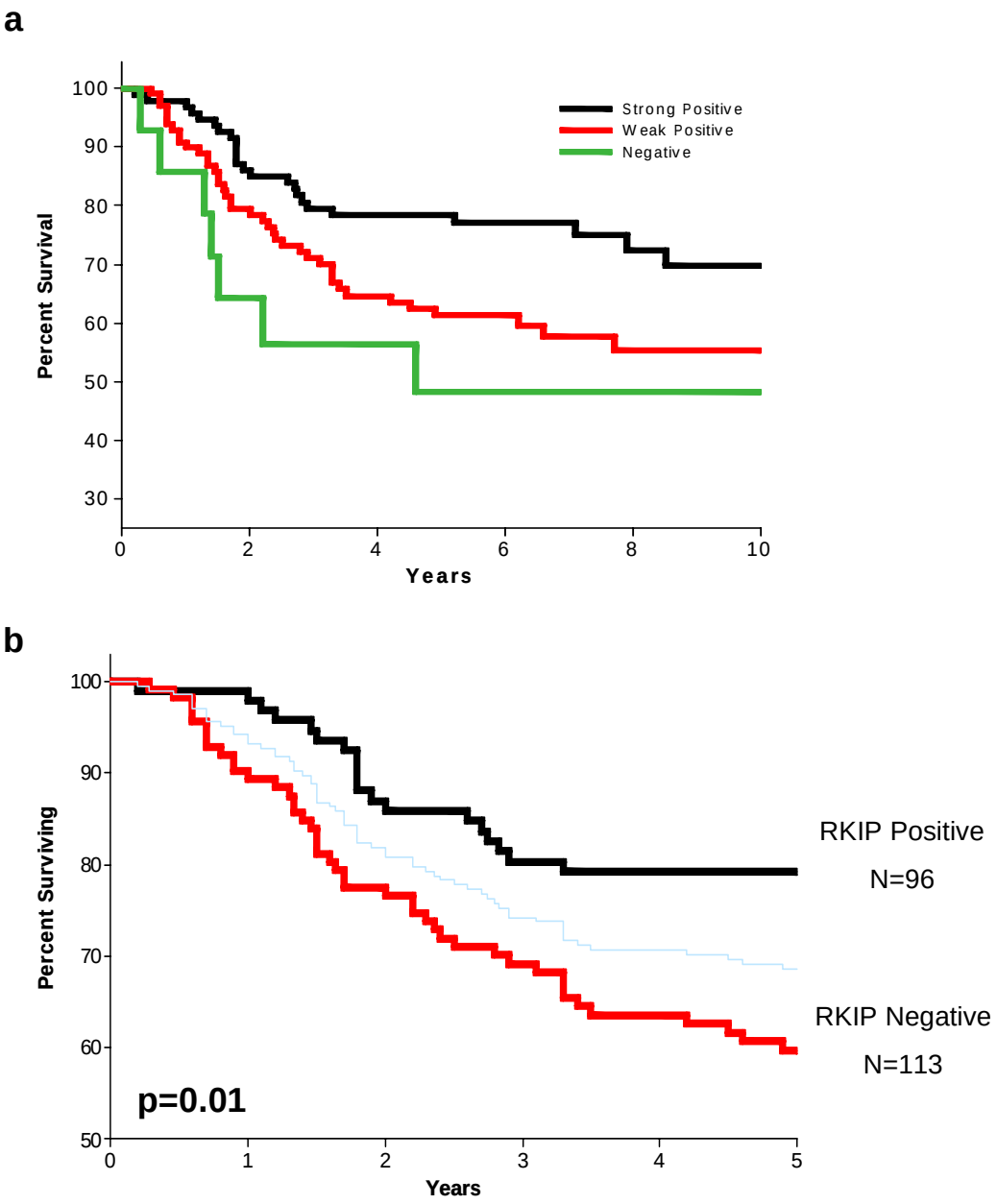


Figure 3.3: Kaplan Meier analysis of disease-specific survival, stratified by RKIP

Disease specific survival was inversely proportional to RKIP score (a). There was no significant difference between the negative and weak positive groups (a, green and red lines respectively) so these were combined. There was a statistically significant difference in disease-specific survival between the 2 newly formed groups (b, Log-Rank p=0.011). The 5-year survival for the cohort as a whole is shown in the narrow blue line.

3.2.2 RKIP is an independent prognostic indicator in multivariate analysis

As mentioned above a number of good prognostic indicators already exist which can accurately predict prognosis in CRC. Therefore, we wanted to examine whether knowledge of RKIP status adds value to the information already available from traditional markers. These markers include demographic features such as age and also clinico-pathological features such as the presence of LVI. Therefore, we performed univariate and multivariate analyses to assess which indicators were independently prognostic. In univariate analysis sex, peritoneal invasion, LVI, tumour grade and RKIP were all significantly associated with disease-specific survival, age was borderline significant (p=0.052) (Table 3.2).

		N	Hazard Ratio (95% CI)	p-Value
Age	Overall	209		0.052
	<50	7	1.00	
	50-59	24	0.57 (0.10 – 3.09)	0.512
	60-69	58	1.08 (0.25 - 4.75)	0.902
	70-79	85	1.61 (0.38 – 6.71)	0.517
	≥80	35	2.41 (0.55 – 10.52)	0.242
Sex	Female	91	1.00	
	Male	118	1.64 (1.00 – 2.69)	0.048
Peritoneal Involvement	Absent	168	1.00	
	Present	41	2.31 (1.40 – 3.82)	0.001
LVI	Absent	152	1.00	
	Present	57	1.78 (1.10 – 2.89)	0.019
Tumour Grade	Overall			0.047
	1	19	1.00	
	2	162	2.36 (0.74 – 7.56)	0.147
	3	28	4.20 (1.19 – 14.76)	0.025
RKIP	Positive	96	1.00	
	Negative	113	1.89 (1.15 - 3.09)	0.011

Table 3.2: Univariate Cox regression analysis of prognostic indicators in this cohort of Dukes B colorectal cancers

(LVI = Lymphovascular Invasion). p-values in italics represent the overall p-value for a given variable.

In the subsequent multivariate analysis only peritoneal invasion, LVI and RKIP were independently predictive of disease-specific survival (Table 3.3).

	Hazard ratio (95% CI)	β-Coefficient	p-Value
Peritoneal Invasion	2.4 (1.5-4.0)	0.89	0.001
LVI Present	1.8 (1.1-3.0)	0.60	0.015
RKIP Negative	2.0 (1.2-3.3)	0.69	0.006

Table 3.3: Results of step-wise multivariate Cox regression analysis, demonstrating the independent prognostic indicators.

3.2.3 Development of a novel prognostic index

The fact that 3 of the variables were shown by the multivariate analysis to be independently prognostic suggests that they give more information together than any one individually. Therefore, we designed a simple prognostic index (PI) based on these variables. To weight the importance of each variable we used the β-coefficient from the multivariate analysis (Table 3.3). This is a measure of the relative contribution of each variable to the overall effect. This approach has previously been used in the development of prognostic indices (Petersen, Baxter et al. 2002). Initially we tried the index using the absolute values of the β-coefficients, however we discovered that since the scores for each variable were similar there was no difference in the results whether we used the exact score or 1. Therefore, for the sake of simplicity we developed the index, assigning a score of 1 to each variable present. These were then summed to give a PI, between 0-3. 5-year disease-specific survival was strongly inversely correlated to the PI (Table 3.4, Figure 3.4).

Score	No of Patients	No Cancer Deaths (%)	5-year survival (95% CI)	Hazard Ratio
0	56	11 (20%)	89% (80-97%)	1.00
1	101	30 (30%)	70% (61-79%)	1.68
2	46	25 (54%)	48% (33-63%)	3.72
3	6	5 (83%)	17% (0-46%)	8.42

Table 3.4: 5-year disease-specific survival, stratified by prognostic index score.

We then spilt the cohort into a good prognosis group (Score of 0 or 1), (which contained approximately 75% of the patients) and a poor prognosis group (Score

of 2 or 3) (Which contained the remaining 25%). There was a dramatic difference in disease specific survival between the two groups, with 5-year survival of 77% (95% CI 70-84%) and 44% (95% CI 30-58%) in the good and poor prognostic groups respectively (Figure 3.4). This difference was highly statistically significant (Log-Rank $p<0.001$).

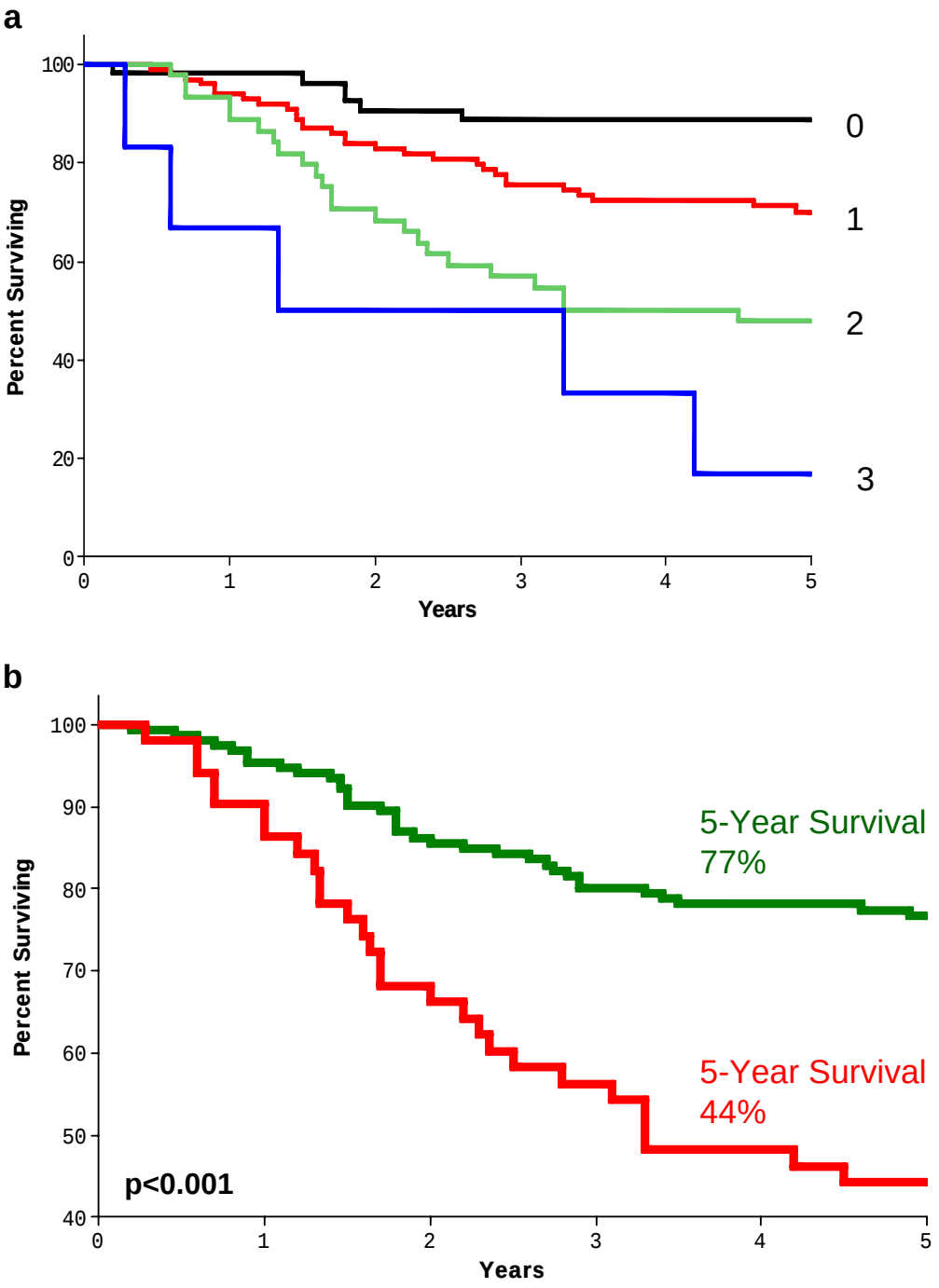


Figure 3.4: Kaplan-Meier analysis of disease specific survival, stratified by prognostic index

There was an inverse correlation between disease-specific survival and prognostic index (a). When we grouped together those patients with scores of 0/1 and those patients with scores of 2/3 this created 2 groups with significantly different disease-specific survival (Log-Rank p<0.001). In the good-prognosis group (b, green line) 5-year survival was 77%, while in the poor prognosis group (b, red line) 5-year survival was 44%, which is similar to patients presenting with Dukes C disease.

3.2.4 RKIP as a predictive marker

Following on from this work demonstrating the utility of RKIP as a prognostic marker in Dukes B CRC, we next wanted to assess whether RKIP is also a predictive marker in this disease. To do this we utilised a TMA from the Western Australia Tissue Research Network. This TMA consisted of 1034 CRC patient samples, with cores present in duplicate and included data on treatment with chemotherapy in 642 of these patients. Demographic information for this patient cohort can be seen in Table 3.5

Total	**1034**
Total for Scoring	915 (88%)
Male/Female	510/524 (49/51%)
Median age at diagnosis (Range)	70.8 (18.8-98.3)
Dukes A	13 (1%)
Dukes B	629 (61%)
Dukes C	386 (37%)
Dukes D	6 (1%)

Table 3.5: Demographic and clinicopathological data relating to the Western Australia cohort.

Firstly, we wished to see whether RKIP retained its prognostic utility in this cohort. Kaplan-Meier analysis revealed a statistically significant difference in median overall survival between the RKIP positive and RKIP negative groups (Median overall survival 102.2 vs 62.2 months in RKIP positive and negative patients respectively, Log Rank $p<0.001$). This translated into 5-year survival of 62% and 51% in RKIP positive and negative patients respectively and a 10-year

survival of 45% and 34% in RKIP positive and negative patients respectively (Figure 3.5). In subgroup analysis RKIP was again prognostic in Dukes B patients; however, in this cohort the effect was less dramatic than in the Dublin cohort (Log-Rank p=0.057). We again performed a multivariate analysis to ascertain which factors provided independent prognostic information. Again we found that RKIP was an independent prognostic indicator, along with age and Dukes stage (Table 3.6).

	Hazard ratio (95% CI)	β-Coefficient	p-Value
Dukes Stage C/D	2.2 (1.7-3.0)	0.80	<0.001
Age (Overall)			<0.001
<50	1.0	0	
50-59	1.5 (1.1-3.0)	0.39	0.293
60-69	2.1 (1.1-4.1)	0.75	0.027
70-79	2.5 (1.3-4.8)	0.91	0.006
≥80	5.4 (2.8-10.5)	1.7	<0.001
RKIP Negative	1.4 (1.1-1.8)	0.3	0.004

Table 3.6: Results of the step-wise multivariate Cox regression analysis, demonstrating the independent prognostic indicators.

Dukes stages A/B and C/D were combined, owing to the low number of Dukes A and Dukes D tumours in the cohort.

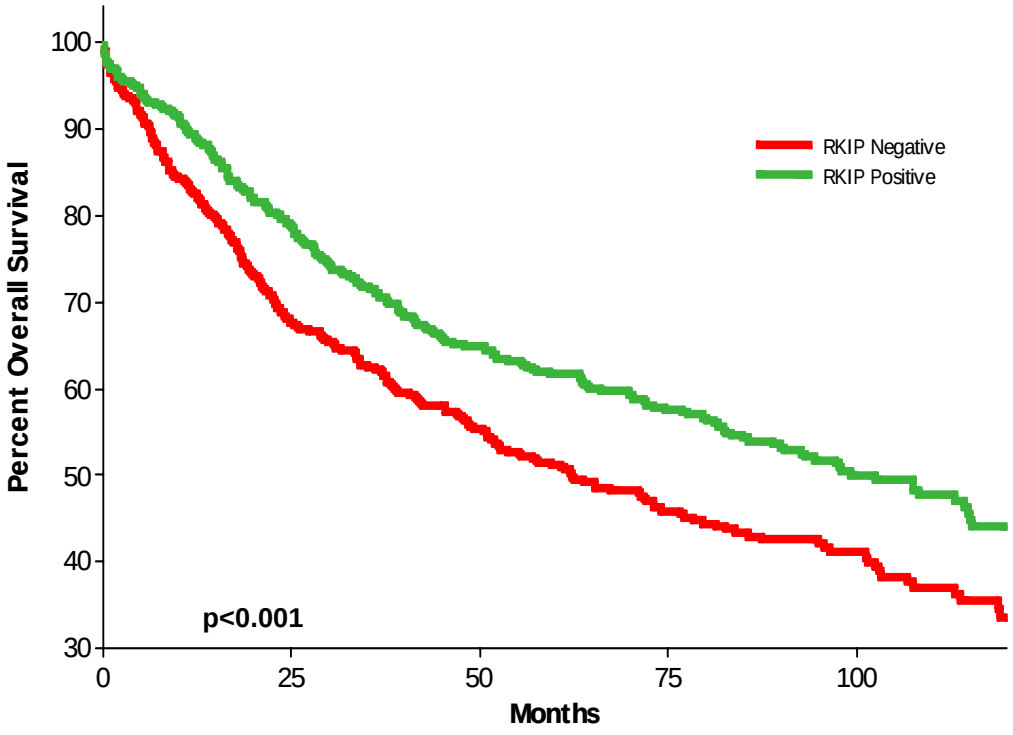


Figure 3.5: Kaplan-Meier analysis of overall survival stratified by RKIP

Again in this cohort there was an inverse correlation between RKIP levels and survival, which resulted in a statistically significant survival difference ($p<0.001$).

Having seen that RKIP was still a useful prognosticator in this cohort we went on to examine the subgroup on which information regarding chemotherapy was available. This group comprised 642 patients. Firstly we compared response to chemotherapy in terms of overall survival separately in the RKIP positive and RKIP negative groups. Kaplan-Meier analysis showed that patients who were negative for RKIP derived a significant benefit from chemotherapy (Log-Rank, p=0.002), whereas patients who were positive for RKIP did not (Log-Rank p=0.120) (Figure 3.6). We then went on to examine this by individual stage. In Dukes B tumours, again there appeared to be a difference in the response between RKIP positive and negative patients. In RKIP positive patients no survival benefit was seen from chemotherapy (5-year-survival 60% vs 64%, Log-Rank, p=0.427). However, in the RKIP negative group a large difference was seen in survival (Figure 3.7), which was statistically significant (5-year survival 86% vs 55%, Log-Rank p=0.050). In Dukes C all patients derived a survival benefit from chemotherapy; however the magnitude of the effect was greater in the RKIP negative group (5-year survival 48% vs 25%, Log-Rank p<0.001) than in the RKIP positive group (5-year survival 58% vs 44% Log-Rank p=0.020) (Figure 3.7).

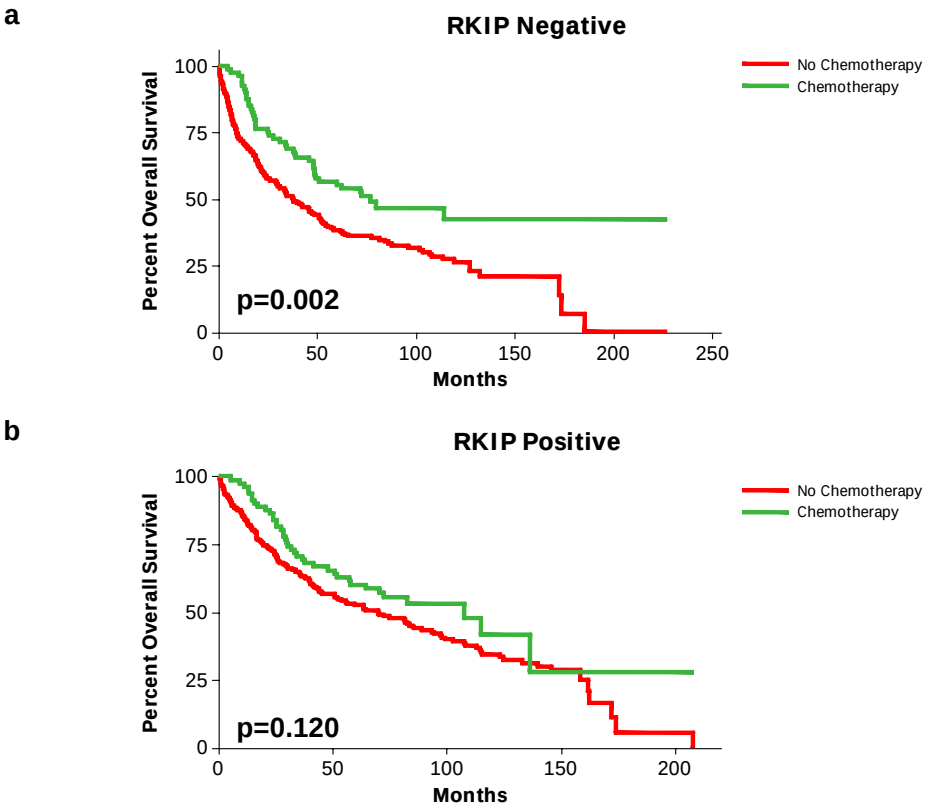


Figure 3.6: Kaplan-Meier analysis of overall survival stratified by treatment with adjuvant chemotherapy and different RKIP status

Patients with RKIP negative tumours (a) derived a significant survival benefit from chemotherapy (Log-Rank p=0.002), whereas patients with RKIP positive tumours did not appear to gain any survival benefit (Log-Rank p=0.120).

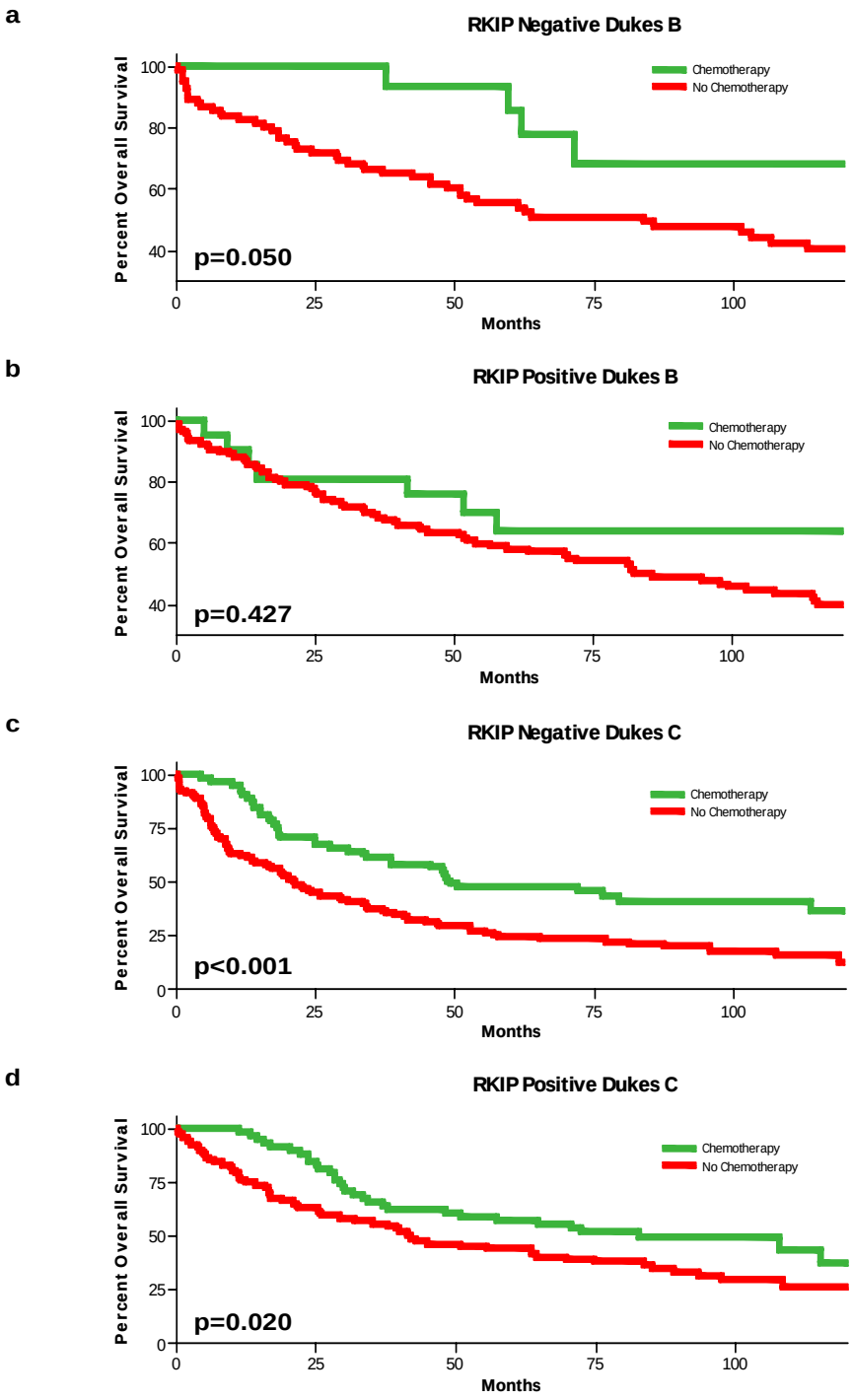


Figure 3.7: Kaplan-Meier analysis of overall survival, stratified by treatment with adjuvant chemotherapy, RKIP status and stage

In Dukes B patients with RKIP negative tumours (a) again there was a statistically significant survival advantage associated with treatment with adjuvant chemotherapy (Log-Rank p=0.050), which was not seen in patients with RKIP positive tumours (b) (Log-Rank p=0.427). Not surprisingly all patients with Dukes C tumours derived a survival benefit from adjuvant chemotherapy, however the effect was greater in patients with RKIP positive tumours (c) than in those with RKIP negative tumours (d).

This indicated that there may indeed be a link between RKIP status and the response to chemotherapy. Namely, those patients with low levels of RKIP were more likely to derive a benefit from systemic chemotherapy than patients with high levels of RKIP, particularly in Dukes B disease. In order to test this more formally we did a statistical interaction analysis to see if RKIP was indeed a factor in the likelihood to derive benefit from chemotherapy. This demonstrated that although RKIP was again an indicator of prognosis in the patient group on whom information on chemotherapy was available, there was no interaction between RKIP status and chemotherapy in terms of survival (Table 3.7).

	Hazard ratio (95% CI)	β-Coefficient	p-Value
RKIP Negative	1.4 (1.1-1.7)	0.32	0.008
Chemotherapy	0.6 (0.4-0.8)	-0.56	0.002
RKIP*Chemotherapy	1.3 (0.8-2.1)	0.24	0.353

Table 3.7: Cox regression analysis analysing the possible interaction between RKIP and chemotherapy

The model shows that although survival depends both on RKIP and chemotherapy there is no evidence that the effect of chemotherapy depends on RKIP status (*indicates the interaction term).

3.2.5 Development and characterisation of an anti-RKIP monoclonal antibody

As we have demonstrated the utility of RKIP as a prognostic indicator in Dukes B CRC, this raises the possibility that it could be useful in clinical practice. However all of these experiments were done using a polyclonal RKIP antibody and before RKIP could be used in clinical practice it would be necessary to develop a monoclonal antibody. A number of monoclonal antibodies were developed by the monoclonal antibody service at the Beatson Institute for characterisation. We tested the antibodies on human sections of normal colon, prostate and liver, all tissues which express RKIP and in which the pattern of staining is well described. Staining was compared to that of the polyclonal antibody. Following selection of the clone which showed the best staining we went on to compare staining with the polyclonal antibody and to demonstrate definite binding of the antibody to RKIP.

The monoclonal antibody compared well to the polyclonal antibody, resulting in specific and crisp cytoplasmic staining of the epithelial cells (Figure 3.8). To demonstrate binding we incubated the RKIP monoclonal antibody with purified GST-RKIP protein attached to glutathione sepharose beads. If the antibody binds RKIP it will bind to the protein attached to the beads. The beads can then be centrifuged and the supernatant applied to tissue sections to see if any residual staining occurs. We carried out the antibody depletion in two different ways, the first passing the antibody solution through a column of the RKIP-GST beads. For the second method the antibody was incubated with the RKIP-GST beads under constant gentle agitation. In both cases the depletion process resulted in near

total removal of the RKIP antibody, as demonstrated by the absence of staining on the sections (Figure 3.8).

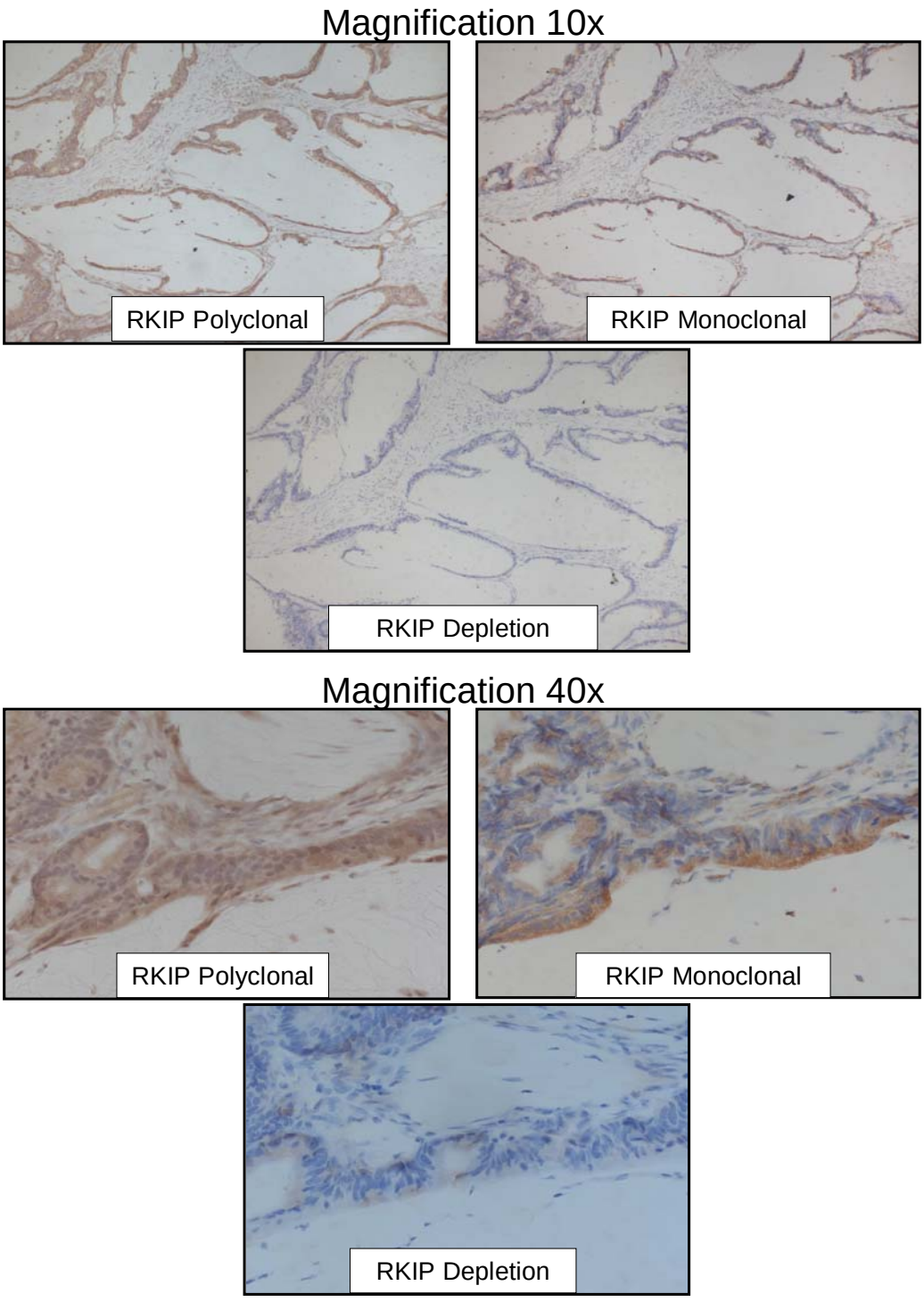


Figure 3.8: Monoclonal anti-RKIP antibody

Sections of CRC were stained with the polyclonal Anti-RKIP antibody (top left panels), the monoclonal anti-RKIP antibody (top right panels) and the monoclonal anti-RKIP antibody, following pre-adsorbtion with purified RKIP protein (bottom panels). The monoclonal anti-RKIP antibody results in sharp cytoplasmic staining, with less background staining than with the polyclonal antibody. The staining is almost completely prevented following the antibody deletion, demonstrating that the antibody is indeed binding RKIP.

3.2.6 Methylation of the RKIP promoter is not an important mechanism of downregulation

In order to determine the importance of promoter methylation as a mechanism for RKIP downregulation in CRC we analysed the *RKIP* promoter of thirty tumours and matched normal tissues for the presence of hypermethylation. A full section from each tumour was stained for RKIP to determine the level of expression. 18/30 tumours were positive and 12/30 were negative. DNA was extracted from freshly frozen tumour and matching normal tissue. DNA was then subjected to chemical bisulphite conversion. This process allows a sequence difference to be created between methylated and unmethylated DNA facilitating subsequent examination of methylation status. The reaction results in the conversion of unmethylated cytosine (C) to uracil (U). However, methylated Cs are resistant to the conversion and remain unchanged. The subsequent PCR reaction matches thymine (T) to the converted U. Therefore, in the PCR product the methylation status of a given CpG dinucleotide may be determined by assessing the ratio of C (methylated CpG) to T (unmethylated CpG).

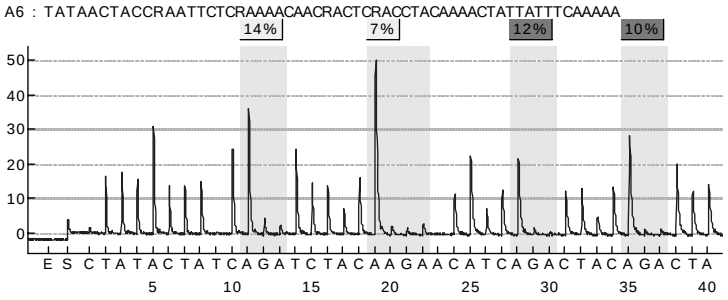
The *RKIP* promoter contains a number of CpG islands which are the potential sites for methylation. We used the sequence of the *RKIP* promoter to design PCR primers which amplified a region of the promoter containing 4 CpG islands. Human unmethylated DNA and 100% *in vitro* methylated DNA were used as negative and positive controls respectively (Figure 3.9) and the presence of a PCR product of the correct length was confirmed by running a sample of the amplified product on an agarose gel. As expected all of the normal tissues showed low levels of RKIP promoter methylation, similar to that seen in the control DNA. Of the tumour samples one tumour could not be sequenced, leaving

29 for analysis. Only one of these tumours showed significant methylation of the RKIP promoter, this tumour was negative for RKIP by IHC (Table 3.8, Figure 3.9).

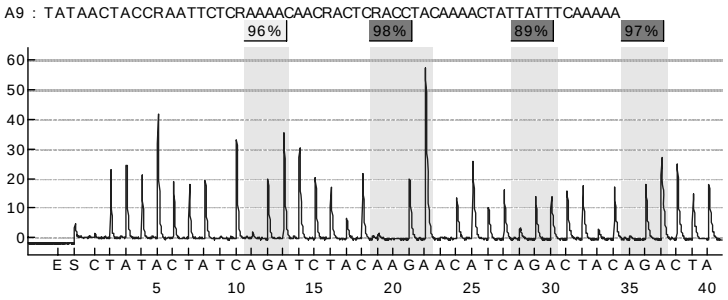
		Tumour	Normal
RKIP Promoter Methylation	High	1	0
	Low	28	30
RKIP Immunohistochemistry	Positive	18	30
	Negative	11	0

Table 3.8: RKIP promoter methylation status of tumour and normal samples.

a

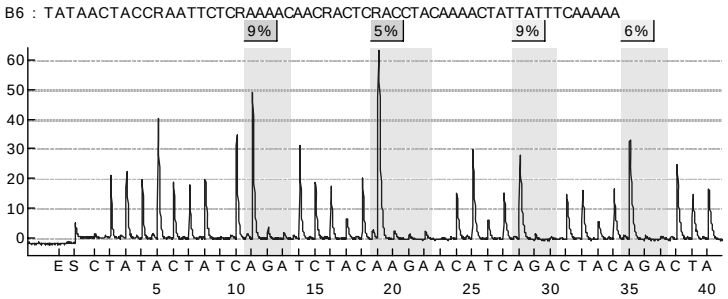


Normal (Unmethylated)

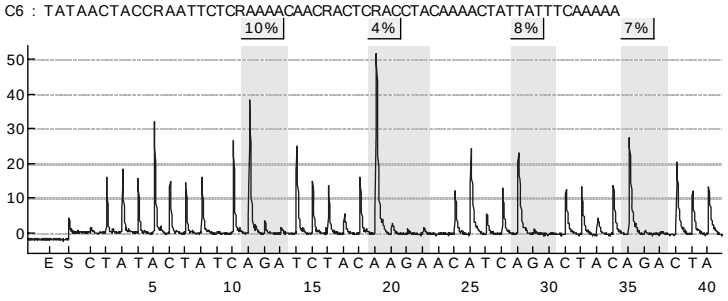


100% In-vitro methylated DNA

b



Normal colon



Tumour

c

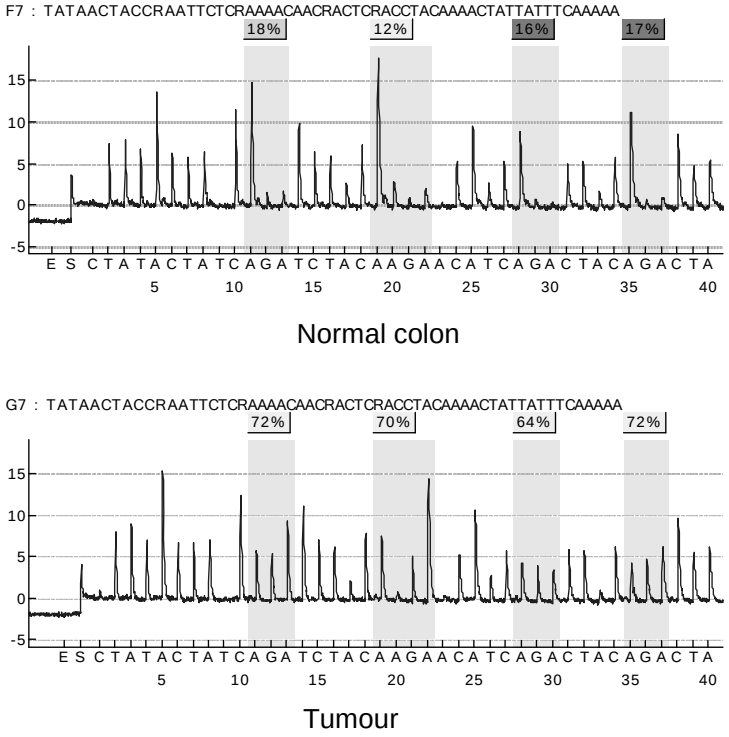


Figure 3.9: Methylation pyrosequencing analysis of the RKIP promoter

Pyrograms demonstrating control (a) normal and 100% in-vitro methylated human DNA. All of the samples of normal colon showed low levels of RKIP promoter methylation (b&c, upper panels). Of the 29 tumour samples that could be analysed 28 also showed low levels of RKIP promoter methylation (b, lower panel). Only 1 tumour (c, lower panel) demonstrated significant methylation of the RKIP promoter.

3.3 Discussion

This study demonstrates the potential of RKIP as a prognostic marker in Dukes B CRC patients. This group comprises approximately 40% of all CRC patients (Shepherd, Baxter et al. 1997; Petersen, Baxter et al. 2002; O'Connell, Maggard et al. 2004; Lindholm, Brevinge et al. 2008) and represents an unsolved management challenge. Although patients with Dukes B CRC have a relatively good outcome, with a 5-year survival of ~75% (Eisenberg, Decosse et al. 1982; Petersen, Baxter et al. 2002; O'Connell, Maggard et al. 2004), this still leaves 1 in 4 patients who should in theory have been cured by surgery developing recurrent disease (Nicastri, Doucette et al. 2007). This has led to controversies regarding the benefit of adjuvant chemotherapy in this group (Sobrero and Köhne 2006). Recent large studies have offered conflicting results with only small, often non-statistically significant benefits seen (Andre, Boni et al. 2004; Kuebler, Wieand et al. 2007; Quasar Collaborative, Gray et al. 2007). These results are summarised in a recent meta-analysis, which showed a small improvement in disease-free survival with the addition of adjuvant chemotherapy, although no improvement in overall survival could be shown (Figueredo, Coombes et al. 2008). The authors suggested that adjuvant treatment should be considered in patients with high-risk features (Figueredo, Coombes et al. 2008), as it is only these patients who are likely to benefit. Andre *et al* demonstrated a trend towards a benefit with the addition of oxaliplatin to standard chemotherapy in a high-risk subgroup of Dukes B CRC, suggesting that further trials should be confined to this subgroup (Andre, Boni et al. 2009). Their high-risk group was broadly defined by featuring at least one of the following: peritoneal invasion, tumour perforation, bowel obstruction,

poorly differentiated tumour, LVI, or <10 lymph nodes examined. These data suggest that a refinement of risk assessment could identify a sub-group of Dukes B patients who will benefit from adjuvant therapy, and therefore there is a need to better define the high-risk group who most likely to derive benefit from adjuvant therapy.

We have shown that low levels of RKIP expression in a tumour is such a high-risk feature, being an independent prognostic marker along with peritoneal invasion and LVI. Peritoneal invasion and LVI are established prognostic markers in Dukes B CRC (Shepherd, Baxter et al. 1997; Petersen, Baxter et al. 2002; Lennon, Mulcahy et al. 2003) and are mentioned in all of the above studies as being "high-risk" features (Figueredo, Coombes et al. 2008; Andre, Boni et al. 2009). As each gives independent prognostic information we have combined them to create a simple PI, which can be used to identify ~25% of patients with a poor prognosis. This poor prognosis group had a 5-year survival of 44%, which is similar to that of patients with lymph node metastases (Dukes C), in whom adjuvant chemotherapy has shown proven benefit (NIH 1990). This PI therefore provides a useful framework for the selection of high-risk patients for close monitoring and for future trials in order to examine the effectiveness of adjuvant therapy in Dukes B CRC.

In the second part of this study we examined RKIP as a potential predictive marker, by examining the effects of chemotherapy on survival separately in RKIP positive and negative patients. We showed an apparent difference, with RKIP negative patients appearing to derive greater benefit from chemotherapy than RKIP positive group. Although this did not prove to be significant in the more stringent test for a statistical interaction, it is interesting to note that the

difference was particularly striking in the Dukes B group. As has been discussed this is the group in which the decision to offer adjuvant chemotherapy is most difficult and this result would again suggest the potential utility of RKIP as an aid in making this decision. The explanation for this result is likely to be that patients with low levels of RKIP expression are more likely to develop metastatic disease (Minoo, Zlobec et al. 2007; Zlobec, Baker et al. 2008). It is presumed that patients with Dukes B CRC who develop metastatic relapse have undetectable, micrometastatic disease at the time of diagnosis. These would therefore be the patients most likely to benefit from systemic chemotherapy.

In this particular group of Dukes B patients, very few (38/251) received chemotherapy and this is likely to have contributed to the failure of the RKIP effect to reach significance in the test for statistical interaction. Another factor which may have affected the result is that there was no standard chemotherapy regime used in the study. Therefore future studies would ideally be conducted as part of a clinical trial examining the effects of a standardised chemotherapy protocol in Dukes B CRC with a large enough sample to allow for the detection of any effect.

In the final section of this study we examined promoter methylation as a possible mechanism for RKIP downregulation in CRC. The mechanism of RKIP downregulation in CRC is not currently known. This has lead investigators to study potential epigenetic mechanisms of downregulation. Al Mulla *et al* used MSP to show methylation to be an important mechanism in RKIP downregulation (Al-Mulla, Hagan et al. 2008); however other groups have not been able to repeat this (Minoo, Zlobec et al. 2007; Beach, Tang et al. 2008). These, conflicting results combined with the potential for false positive results with MSP

led us to examine the question using bisulphite conversion and quantitative analysis by pyrosequencing, which is acknowledged as gold standard in the study of promoter methylation (Clark, Statham et al. 2006). In our study only 1/12 tumours that were negative for RKIP by IHC showed significant methylation of the promoter. The discrepancies between our results and those of others may reflect differences in techniques or cohorts, but in summary suggest a multi-factorial regulation of RKIP expression. For instance, in prostate cancer RKIP expression is partly silenced by transcriptional repression through the Snail transcriptional repressor (Beach, Tang et al. 2008). Thus, different mechanisms alone or in combination may repress RKIP expression in different cancers.

In summary, we have shown that RKIP can function as a prognostic marker in Dukes B CRC. Moreover, it can form part of a prognostic index, including 2 other independent prognostic indicators. We have also shown that patients with low levels of RKIP may derive a greater benefit from chemotherapy than patients with high levels of RKIP, although this would need to be verified in another study. Finally we have shown that promoter methylation is not an important mechanism of RKIP downregulation, at least in the patient group studied.

Chapter 4 The effect of RKIP knockout in established mouse models of CRC

4.1 Introduction

The *RKIP* knockout mouse was first reported in 2007 by Theroux *et al* (Theroux, Pereira et al. 2007). The mouse is a germline knockout of the *RKIP1* gene, which was created using the gene-trap method. This method in essence involves inserting a construct, consisting of a β-galactosidase gene, with an upstream splice acceptor site and a neomycin resistance gene under the control of an autonomous promoter into an intron of the target gene (Stanford, Cohn et al. 2001). This construct results in the transcription of a fusion RNA consisting of the exons upstream from the construct and the β-galactosidase. A separate RNA is transcribed from the neomycin resistance gene owing to its independent promoter and this allows for selection of cells that have incorporated the vector (Stanford, Cohn et al. 2001).

In the mouse there are five *RKIP* family members (*RKIP1-5*) (Theroux, Pereira et al. 2007). *RKIP*1 is located on chromosome 5 and contains 3 introns and therefore 4 exons. The genetrap inserts into intron 1 of the RKIP gene resulting in transcription of a fusion RNA, containing only exon 1 of the native *RKIP1* gene. Very low levels of RKIP1 RNA have been detected in fibroblasts and tissues from these mice. This was thought be due either to read through or to cryptic downstream promoters. However, in either case this would not lead to the translation of a functional protein (Theroux, Pereira et al. 2007). *RKIP3-5* are located on chromosomes 9, 10 and 12 respectively. These loci contain open reading frames but are intronless. This and the fact that no transcript from any of these loci has been detected in any mouse tissue have lead to the conclusion that these are silent pseudogenes (Theroux, Pereira et al. 2007). *RKIP2* is also

intronless but RNA has been detected specifically in the testis of both *RKIP*$^{+/+}$ and *RKIP*$^{-/-}$ mice (Moffit, Boekelheide et al. 2007; Theroux, Pereira et al. 2007).

RKIP$^{-/-}$ mice are viable and reproduce with the expected Mendelian ratios (Theroux, Pereira et al. 2007). However, a slight decrease in reproduction rates has been observed in *RKIP*$^{-/-}$ males, which is thought to be due to premature sperm capacitation (Moffit, Boekelheide et al. 2007). The other reported phenotype of the *RKIP*$^{-/-}$ mouse is a defect in olfactory function. This is in keeping with the high levels of RKIP found in the olfactory neurons of wild-type mice (Theroux, Pereira et al. 2007).

In order to study the role of *RKIP* knockout in CRC we crossed the *RKIP*$^{-/-}$ mouse to known models of CRC. The first of these was the *Apc*^{fl} mouse (Shibata, Toyama et al. 1997). In this mouse the *Apc* gene contains loxP sites in introns 13 and 14. Thus, following recombination by Cre Recombinase enzyme exon 14 is deleted, resulting in a frameshift mutation and the generation of a premature STOP codon (Shibata, Toyama et al. 1997). When crossed to mice expressing the AhCre, which is under the control of the *Cyp1A1* promoter this results in inducible recombination in the small intestine, colon and liver (Ireland, Kemp et al. 2004). Heterozygous *APC*^{fl} mice (*APC*$^{fl/+}$) develop multiple intestinal adenomas (Shibata, Toyama et al. 1997). This mouse model also allows for the study of the acute effects of homozygous deletion of *Apc* in the intestine. Homozygous deletion results in the so-called crypt progenitor phenotype, with a massive increase in size of the crypts, accompanied by an increase in proliferation and in apoptosis (Sansom, Reed et al. 2004). The homozygous loss of *Apc* also leads to a proliferative phenotype in the liver, with resulting hepatomegaly.

Although the *APC*^{fl} is an excellent model of the early stages of tumourigenesis, the tumours that develop only rarely progress to become invasive carcinomas (Sansom, Meniel et al. 2006). As RKIP is thought to be a metastasis suppressor and therefore its loss is likely to be more important in the later stages of tumour development we wanted to examine the effect of RKIP loss in a model of more advanced intestinal tumourigenesis. For this we utilised the *APC*$^{fl/+}$ *KRas*^{G12V} mouse.

The *APC*$^{fl/+}$ *KRas*^{G12V} mouse combines the *APC*^{fl} mouse with a mouse conditionally expressing the oncogenic *KRas*^{G12V} mutation (Guerra, Mijimolle et al. 2003). *KRas* is commonly mutated in human CRC and the G12V mutation is among the more common seen in human tumours (Bos 1989). The mutant KRas allele is under the control of the endogenous Ras promoter and is preceded by a STOP cassette, flanked by LoxP sites. The STOP cassette prevents transcription of the mutant allele until it is deleted following Cre recombination. As the same *AhCre* is used the oncogenic Ras is expressed in the small intestine, liver and colon. This mouse has been shown to develop intestinal tumours at an increased rate compared to the *APC*$^{fl/+}$ mouse and also develops superficially invasive tumours (Sansom, Meniel et al. 2006).

4.2 Results

4.2.1 RKIP is not detectable in the RKIP$^{-/-}$ mouse

In order to confirm the absence of RKIP in the tissue that we were studying we stained sections of intestine and liver (tissues which are known to express high levels of the protein) for RKIP by IHC. The staining showed that while sections from the wild-type type mice revealed strong cytoplasmic staining. In contrast sections from the *RKIP*$^{-/-}$ mice showed no staining in the intestine and only weak background staining in the liver (Figure 4.1).

In order to confirm that the weak staining seen in the liver, was indeed non-specific we performed reverse transcriptase PCR to determine whether RKIP RNA could be detected in these tissues. RNA was extracted from freshly frozen tissue and converted to cDNA. This was amplified by PCR, using primers that specifically amplify RKIP cDNA, using tissue from *RKIP*$^{+/+}$ mice as a control. RKIP RNA could be clearly detected in control tissues but no RKIP RNA could be detected in either the liver or intestine of the *RKIP*$^{-/-}$ mice (Figure 4.1).

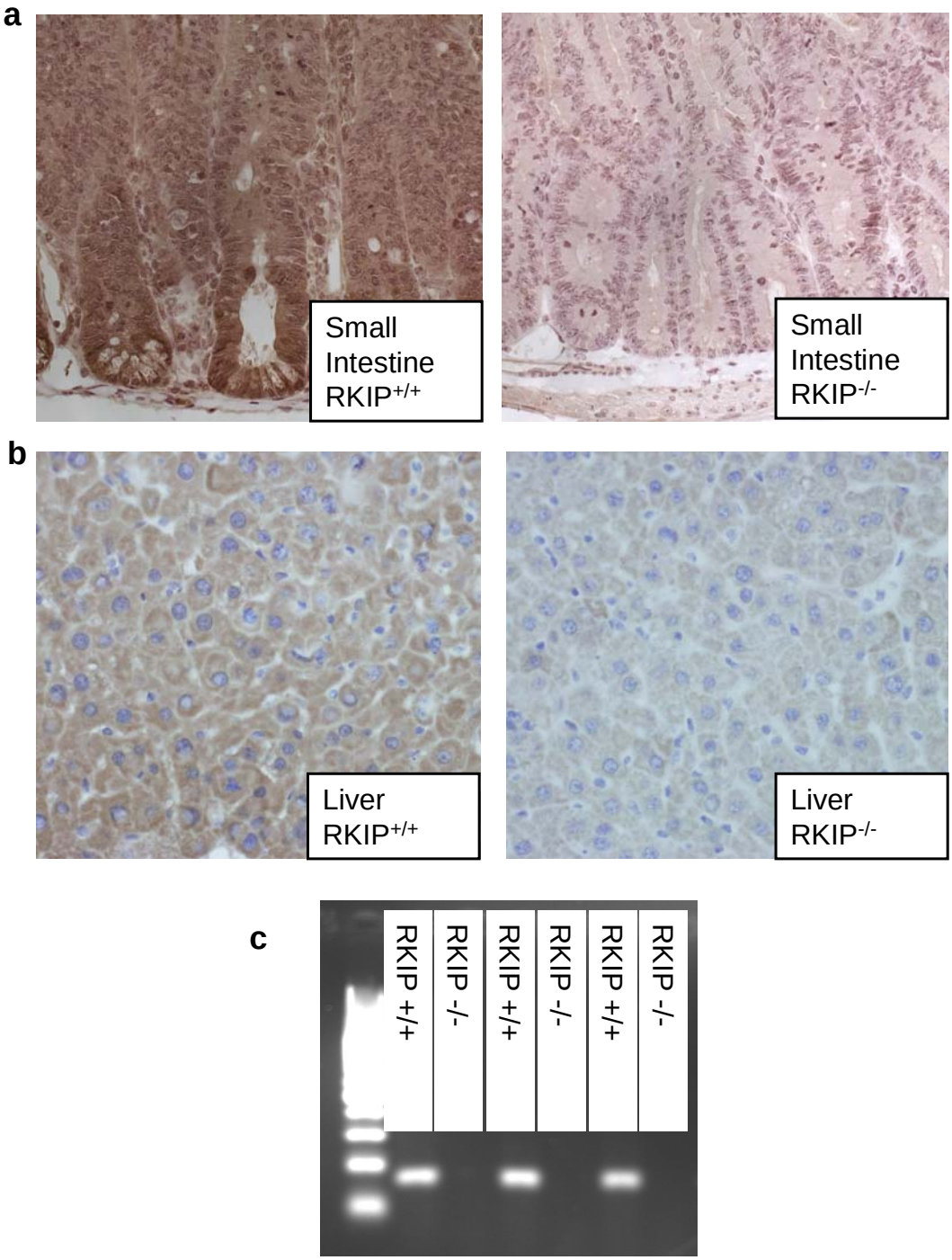


Figure 4.1: RKIP protein and mRNA are not detectable in the RKIP$^{-/-}$ mouse

Immunohistochemistry in the small intestine (a) reveals readily detectable RKIP protein in the wild-type mouse (left panel), but not in the RKIP$^{-/-}$ mouse (right panel). The same is true in the liver (b) although there is some background staining seen in the RKIP$^{-/-}$ mouse (right panel). To ensure that this did not represent low-level RKIP expression rtPCR was performed (c) and demonstrated that although RKIP mRNA could be detected in tissues from wild-type mice none could be detected in the RKIP$^{-/-}$ mouse.

4.2.2 RKIP levels are low in tumours in the mouse

In addition to confirming the absence of detectable RKIP in the *RKIP*$^{-/-}$ mouse we wanted to confirm that levels of RKIP in the mouse mimic the patterns seen in human tumours. Namely, that the level of RKIP is lower in tumours than in normal tissue and moreover that RKIP levels are lower still in metastases. To test this we stained normal, tumour and metastatic tissue from different tumour models in *RKIP*$^{+/+}$ mice.

We showed that in a renal tumour model, in which there is homozygous deletion of *Apc* combined with oncogenic mutation of *KRas* (*APC*$^{fl/fl}$ *KRas*^{G12V}) RKIP was expressed at lower levels in the tumour tissue than in normal tissue (Figure 4.2). Next we examined intestinal adenomas and carcinomas from mice and again demonstrated lower RKIP levels in the tumours when compared to adjacent normal tissue (Figure 4.2). In many cases there was a progressive decrease in RKIP staining from normal to adenoma to carcinoma. However this was not universal with some adenomas showing similar staining to the invasive carcinomas. Finally, we examined a metastatic pancreatic cancer model in which oncogenic *KRas* is expressed along with mutant *p53* (*KRas*^{G12D} *p53*^{R172H}) conditionally in the pancreas. Again in this model we saw a similar pattern of expression to that seen in human tumours. RKIP was expressed at low levels in the primary tumour, but expression was almost completely absent in liver metastases (Figure 4.2).

As we had demonstrated that RKIP is indeed decreased in tumours in a number of different mouse models we then went on to examine the effect of *RKIP* deletion in mouse models of CRC.

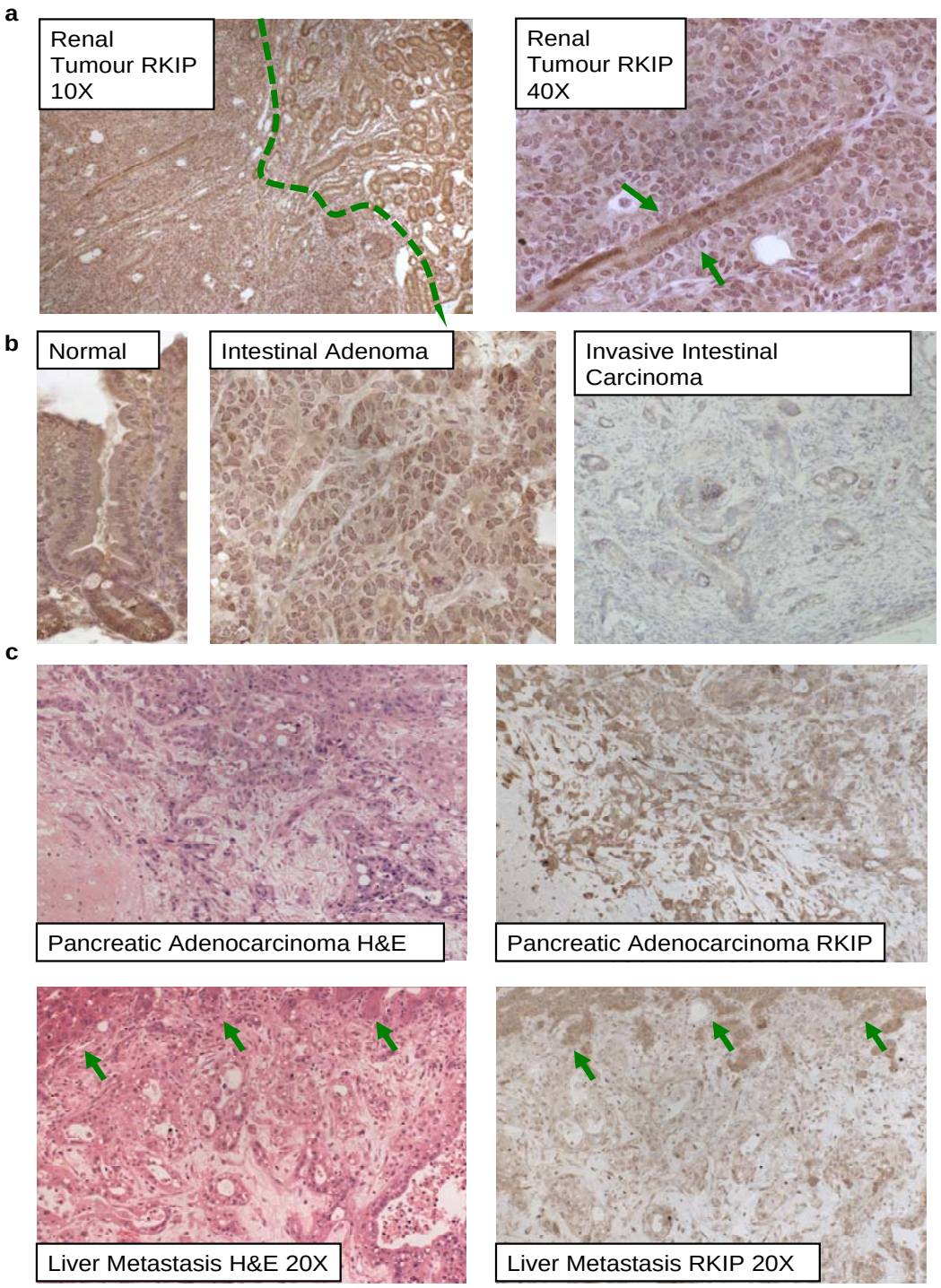


Figure 4.2: RKIP in mouse tumours

To determine if RKIP expression levels followed a similar pattern in the mouse as in human a number of different mouse tumours were stained for RKIP. In the kidney (a) normal tissue (right of green line in left panel and arrow in right panel) showed higher levels of RKIP expression than tumour tissue. Similarly in the intestine (b) RKIP levels were higher in normal intestine (left panel) than in tumours (middle and right panels). Finally in a model of pancreatic cancer (c) RKIP levels were lower in liver metastases (upper panels) than in the primary tumour (upper panels). Arrows indicate residual normal liver.

4.2.3 *RKIP* deletion does not alter tumourigenesis in the *APC^{fl}* mouse

Given that RKIP levels were low in the mouse tumour models we then went on to examine the effect of *RKIP* deletion on intestinal tumourigenesis. To test this we crossed *RKIP*$^{-/-}$ mice to *APC^{fl}* mice to yield 3 experimental cohorts; *APC*$^{fl/+}$ *RKIP*$^{+/+}$ (N=22), *APC*$^{fl/+}$ *RKIP*$^{+/-}$ (N=21) and *APC*$^{fl/+}$ *RKIP*$^{-/-}$ (N=24). These cohorts were induced with β-naphthoflavone to activate expression of the *AhCre* recombinase and were then aged until they developed signs of intestinal tumours, such as paling of the feet, hunching and development of a "starry coat". Mice were then euthanized and the intestinal tumours examined both grossly and histologically.

The first parameter that we examined was survival. There was no difference seen in survival between the 3 cohorts (Figure 4.3), strongly indicating that RKIP deletion has no effect on tumourigenesis following heterozygous deletion of *Apc*. We also assessed tumour number and size both in the small intestine and colon and again found no difference in tumour number (Figure 4.3) or average size across the different cohorts. This indicates that the mice were euthanized at approximately the same stage of tumour development.

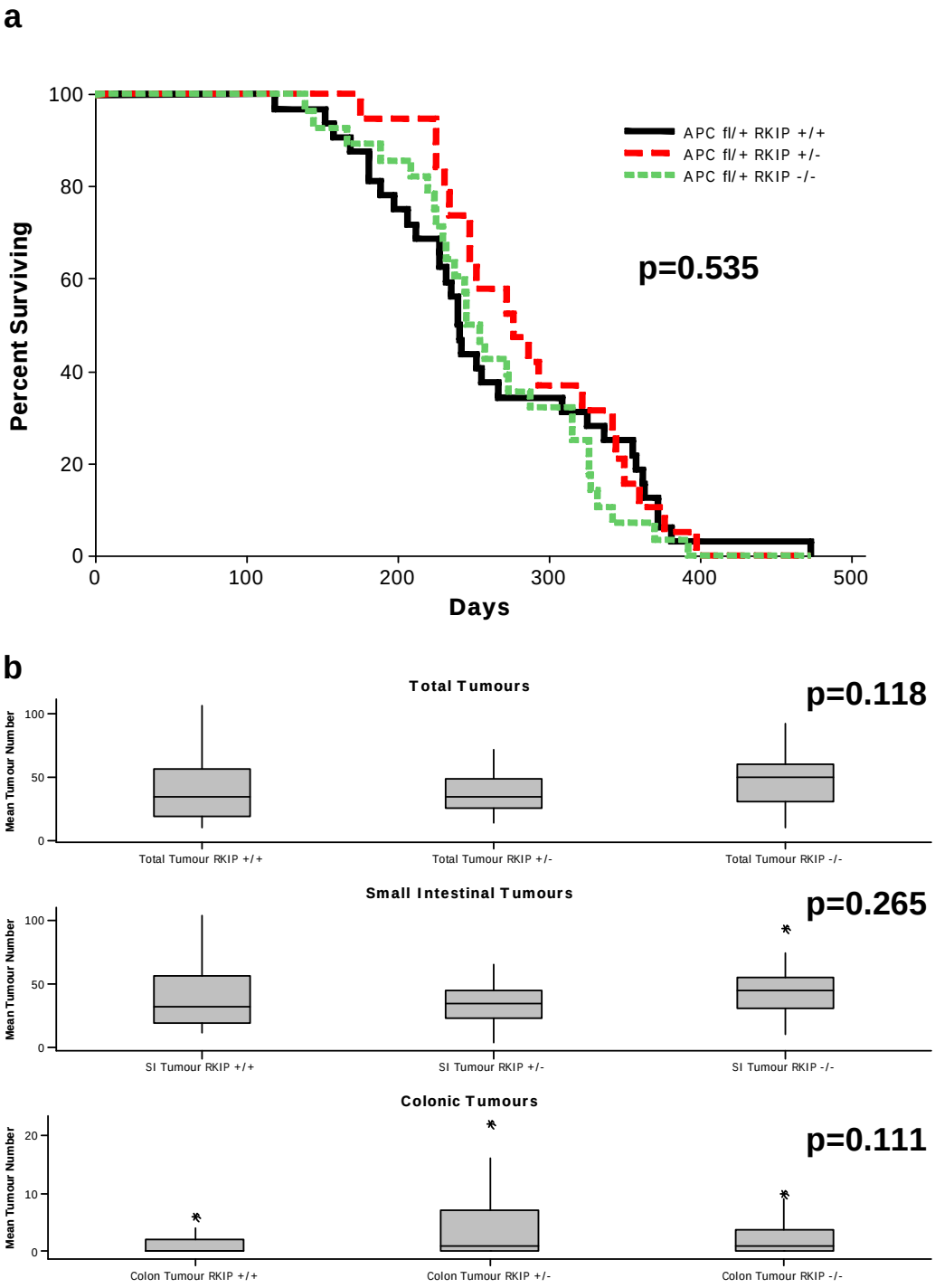


Figure 4.3: RKIP deletion does not alter tumourigenesis in the $APC^{fl/+}$ $RKIP^{-/-}$ mouse

RKIP deletion did not result in any difference in survival in the $APC^{fl/+}$ $RKIP^{-/-}$ mouse, when compared to either the $APC^{fl/+}$ $RKIP^{+/-}$ or the $APC^{fl/+}$ $RKIP^{+/+}$ mouse (a) (Log-Rank p=0.535). No difference was seen in tumour number across the different genotypes (b) (* indicates outlying values).

Although there was no difference between the groups in terms of survival and therefore rate of tumourigenesis we wanted to determine if *RKIP* deletion affected the invasive characteristics of the tumours. Therefore we examined haematoxylin and eosin (H&E) stained sections of tumours, to determine the number of mice developing either high grade dysplasia (HGD) or invasive carcinoma. Although mice from the *APC$^{fl/+}$ RKIP $^{-/-}$* cohort did develop occasional invasive carcinomas, the proportion was no greater than that seen in the control mice (Chi-square p=0.597) (Table 4.1). No metastases were seen in any of the cohorts.

	APC$^{fl/+}$ RKIP$^{+/+}$	**APC$^{fl/+}$ RKIP$^{+/-}$**	**APC$^{fl/+}$ RKIP$^{-/-}$**
Invasive Tumour	4	3	7
No invasive Tumour	16	15	17

Table 4.1: APC$^{fl/+}$ RKIP$^{-/-}$ mice do not have an increased incidence of invasive carcinoma.

4.2.4 Effect of RKIP deletion on homozygous APC knockout

Although *RKIP* deletion did not lead to a detectable phenotype following heterozygous *Apc* deletion we wanted to determine whether there was any effect of *RKIP* deletion in the setting of acute homozygous deletion of *Apc*. Given that RKIP is seen to be lower in tumour than in normal tissue it is possible that tumours may arise in cells with low levels of RKIP. Since intestinal tumours will have lost all APC function homozygous deletion of the Apc gene in the mouse may reveal the phenotype of RKIP deletion. To examine this we crossed $RKIP^{-/-}$ mice to APC^{fl} mice to yield cohorts of $APC^{fl/fl}$ $RKIP^{+/+}$ mice and $APC^{fl/fl}$ $RKIP^{-/-}$ mice. These were induced as before with β-naphthoflavone on day 0 and then euthanized on day 4. Previous studies in the lab have shown that the $APC^{fl/fl}$ mice develop a dramatic crypt progenitor phenotype and also increased liver proliferation (Sansom, Reed et al. 2004). As a result these mice cannot be aged any longer as they become sick due to the massive phenotype induced by deletion of *Apc*.

Comparing the small intestine of $APC^{fl/fl}$ $RKIP^{+/+}$ mice with those of $APC^{fl/fl}$ $RKIP^{-/-}$ mice we noted that although there was no difference in crypt size or in the number of mitotic figures seen we did see a small but statistically significant decrease in apoptoses per crypt (Figure 4.4). This is in keeping with previous reports suggesting a pro-apoptotic role for RKIP (Chatterjee, Bai et al. 2004; Jazirehi, Vega et al. 2004) and also in keeping with the observation that human CRC with low levels of RKIP have decreased apoptoses compared to those with high levels of RKIP (Al-Mulla, Hagan et al. 2006).

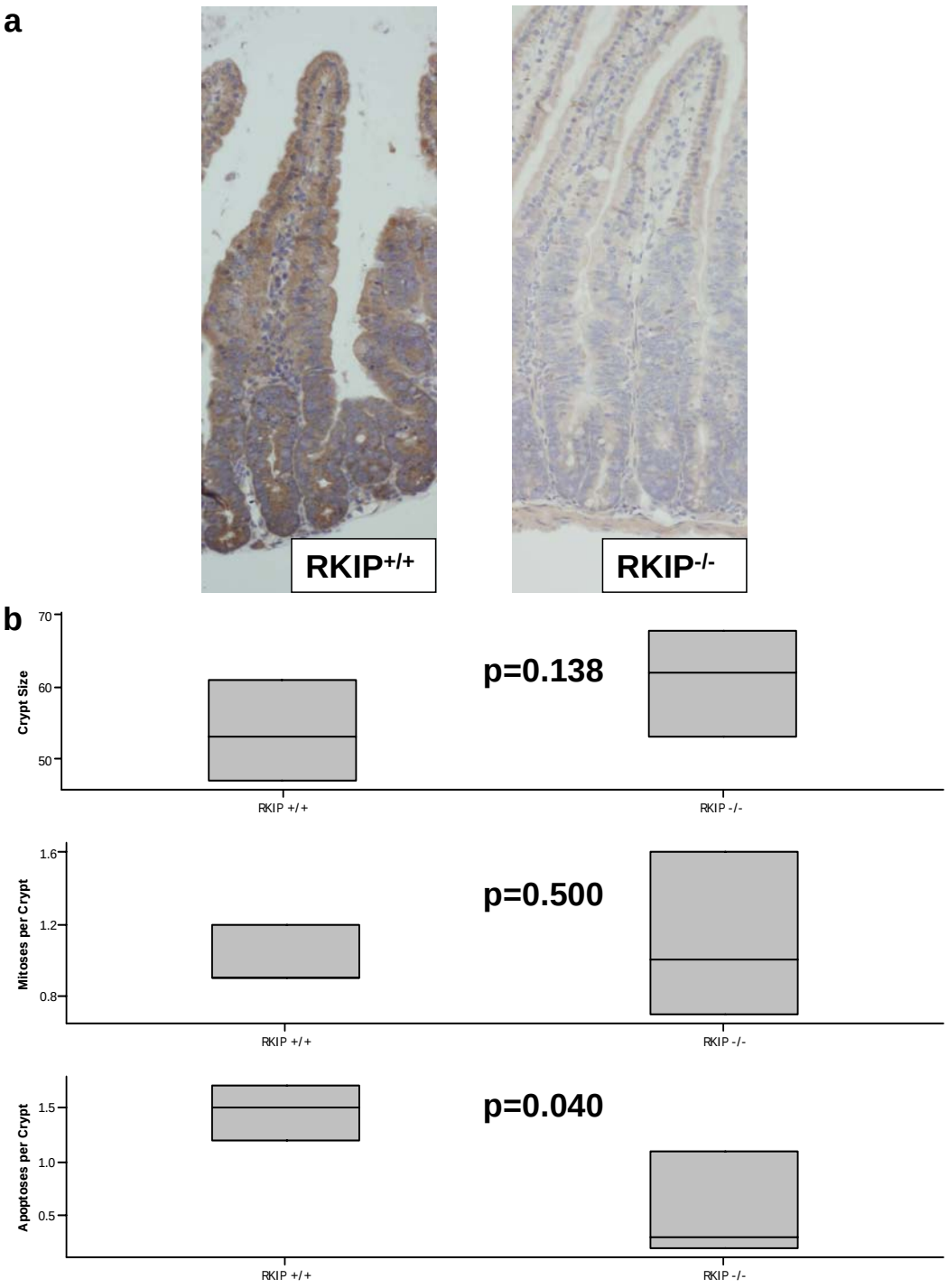


Figure 4.4: RKIP deletion results in a decrease in apoptoses in the small intestine of $APC^{fl/fl}$ mice.

RKIP staining again revealed no RKIP protein in the intestine of $APC^{fl/fl}$ $RKIP^{-/-}$ mice (a). Although there was no difference in crypt size or number of mitoses between $APC^{fl/fl}$ $RKIP^{-/-}$ and $APC^{fl/fl}$ $RKIP^{+/+}$ mice, the $APC^{fl/fl}$ $RKIP^{-/-}$ mice did show an increased number of mitoses per crypt than did the $APC^{fl/fl}$ $RKIP^{+/+}$ mice (b).

As it has also been shown that the *APC*$^{fl/fl}$ mice develop a liver phenotype with increased hepatocellular proliferation we also examined the liver to see if there was an effect of *RKIP* deletion following homozygous *Apc* deletion in this organ. Although no increase in liver mass was seen in the *APC*$^{fl/fl}$ *RKIP*$^{-/-}$ mice compared to controls there was a massive and statistically significant difference in the number of mitotic figures seen in the livers of the *APC*$^{fl/fl}$ *RKIP*$^{-/-}$ mice (Mann-Whitney p=0.04) (Figure 4.5).

On closer examination it was clear that there was a large number of aberrant mitotic figures present in the *APC*$^{fl/fl}$ *RKIP*$^{-/-}$ livers. To more precisely quantify this we divided aberrant mitoses into 3 classes; unequal divisions, tripolar mitoses and anaphase bridges (Figure 4.6). As there was an overall increase in mitotic activity in the livers of the *APC*$^{fl/fl}$ *RKIP*$^{-/-}$ mice we expressed the amount of aberrant mitoses as a percentage of the total mitoses seen. There was a clear difference in the proportion of aberrant mitoses, unequal mitoses and anaphase bridges between the livers of the *APC*$^{fl/fl}$ *RKIP*$^{-/-}$ mice and those of controls (Figure 4.6). Given that it has been shown that aberrant mitoses and anaphase bridges in particular correspond with the presence of CIN (Montgomery, Wilentz et al. 2003) this would suggest that there is an increase in CIN in the livers of the *APC*$^{fl/fl}$ *RKIP*$^{-/-}$ mice.

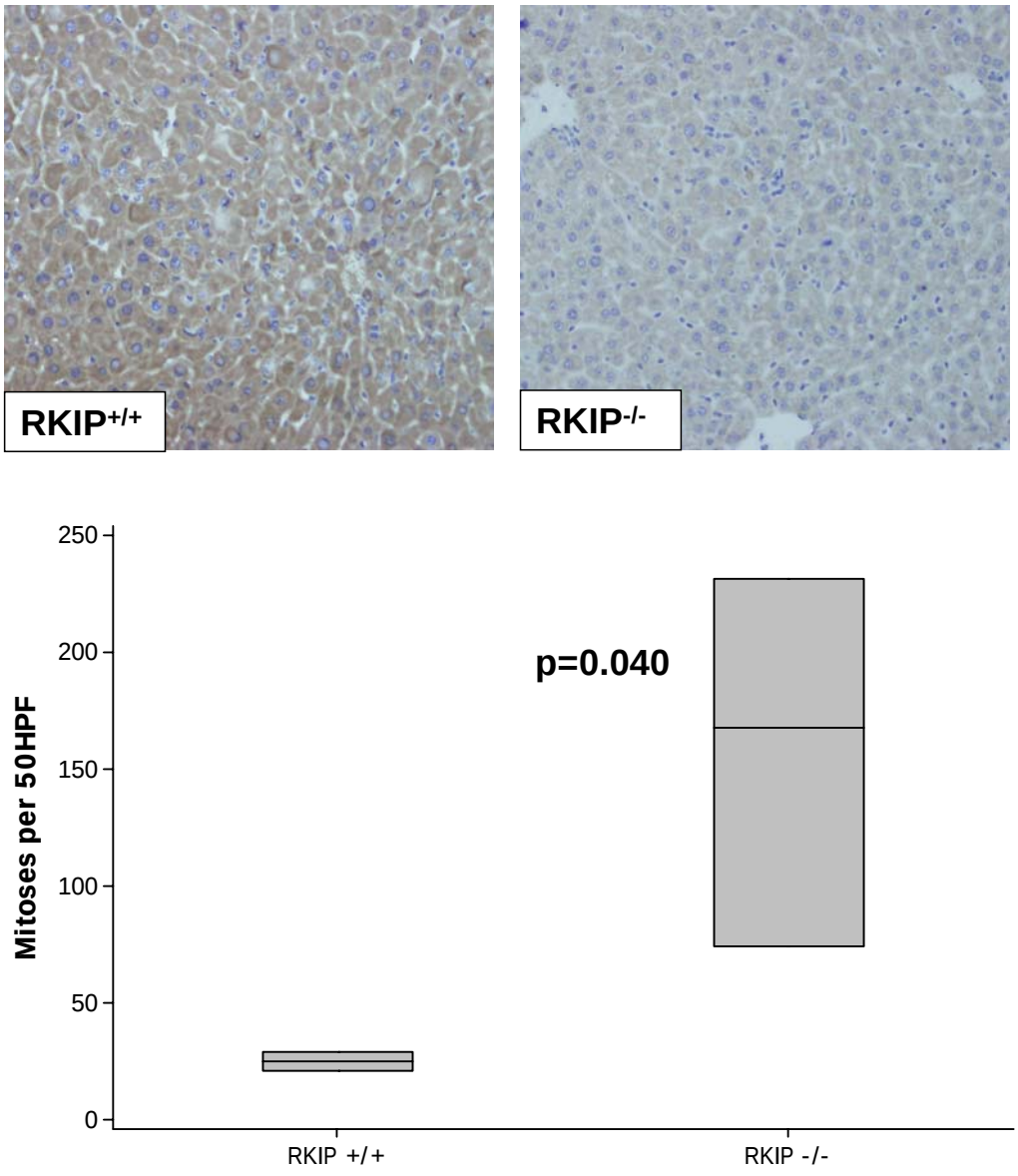


Figure 4.5: RKIP deletion increases the number of mitotic figures in the livers of $APC^{fl/fl}$ mice.

The number of mitoses per high power field (HPF) was assessed for $APC^{fl/fl}$ $RKIP^{+/+}$ and $APC^{fl/fl}$ $RKIP^{-/-}$ mice. There was a marked increase in mitotic figures seen in the livers of the RKIP knockout mice with a median number of mitotic figures per 50HPF of 168.1 compared to 28.6 in the wild-type mice. This difference was statistically significant (Mann-Whitney p=0.040).

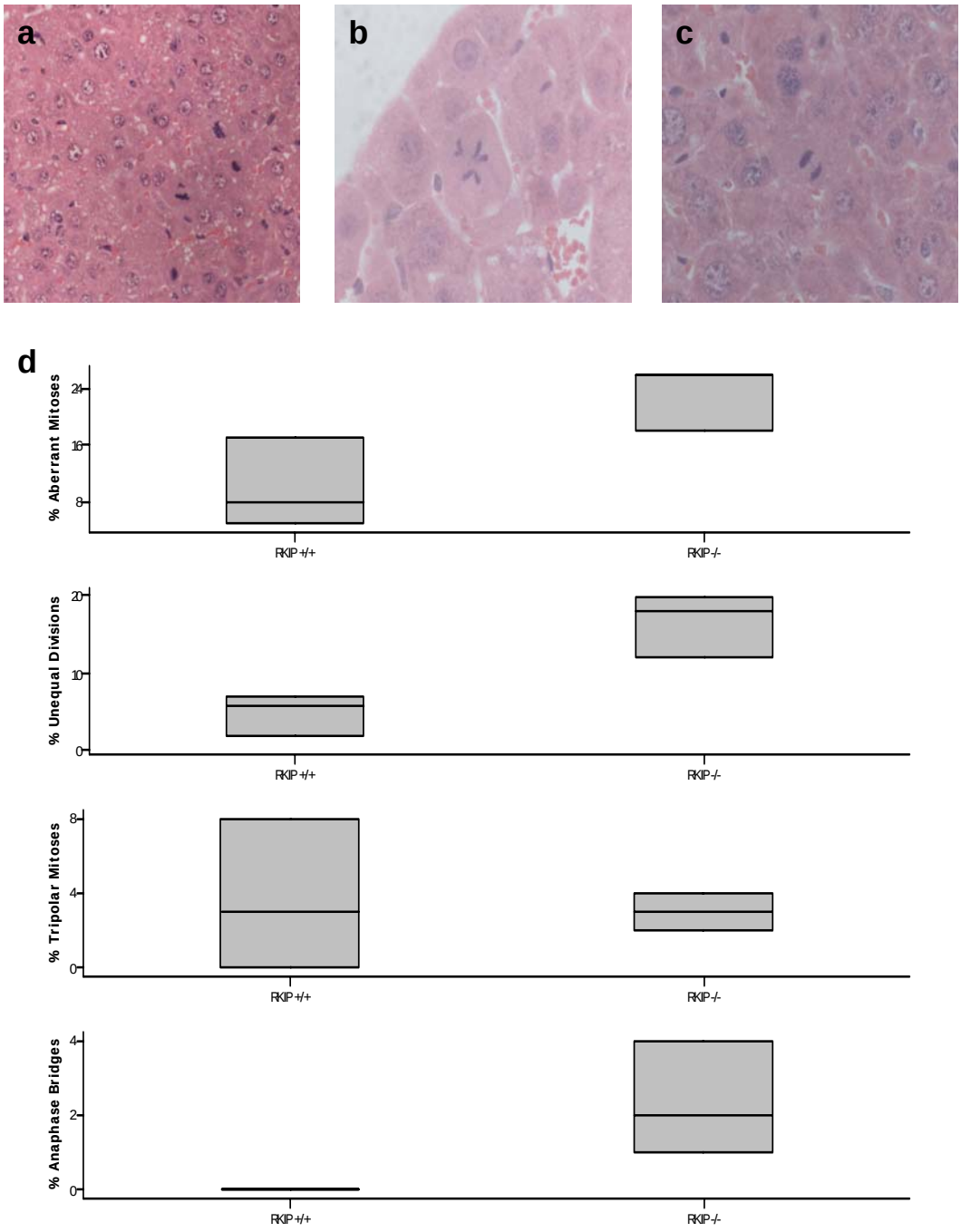


Figure 4.6: RKIP deletion resulted in an increase in aberrant mitoses in the livers of $APC^{fl/fl}$ mice.

Aberrant mitoses (unequal divisions (a), tripolar mitoses (b) and anaphase bridges (c)) were counted and expressed as a percentage of the total number of mitoses. There was a statistically significant increase in the percentage of aberrant mitoses in the livers of the $APC^{fl/fl}$ $RKIP^{-/-}$ mice when compared to controls (d) (Mann-Whitney p=0.040). On examining the individual types of aberrant mitoses, there was an increase seen in both unequal divisions and anaphase bridges (d).

Given the absence of an increase in liver mass this apparent increase in mitotic index was puzzling.

We hypothesised that the increase in mitotic figures may be due to cells being arrested in mitosis. To test this we injected mice with bromodeoxyuridine (BRDU) either 2 or 24 hours prior to euthanasia. BRDU is incorporated into the DNA in S-phase and is retained in the DNA, being passed on to daughter cells after mitosis. BRDU can thus be used as a marker of dividing cells and by comparing the amount of BRDU incorporation at 2 and 24 hours we can determine if the cells that enter S-phase 24 hours prior to euthanasia do indeed go on to complete mitosis.

We saw a marked increase in the number of BRDU positive cells in the livers of the $APC^{fl/fl}$ $RKIP^{-/-}$ mice compared to controls at the 2 hour timepoint, demonstrating an increase in cells entering S-phase in these mice. However, while the number of BRDU positive cells had increased by a factor of ~7 at the 24 hour timepoint in the $APC^{fl/fl}$ $RKIP^{+/+}$ mice; there was no significant difference in the $APC^{fl/fl}$ $RKIP^{-/-}$ livers between the 2 and 24 hour timepoints, indicating that these cells are arrested in mitosis (Figure 4.7).

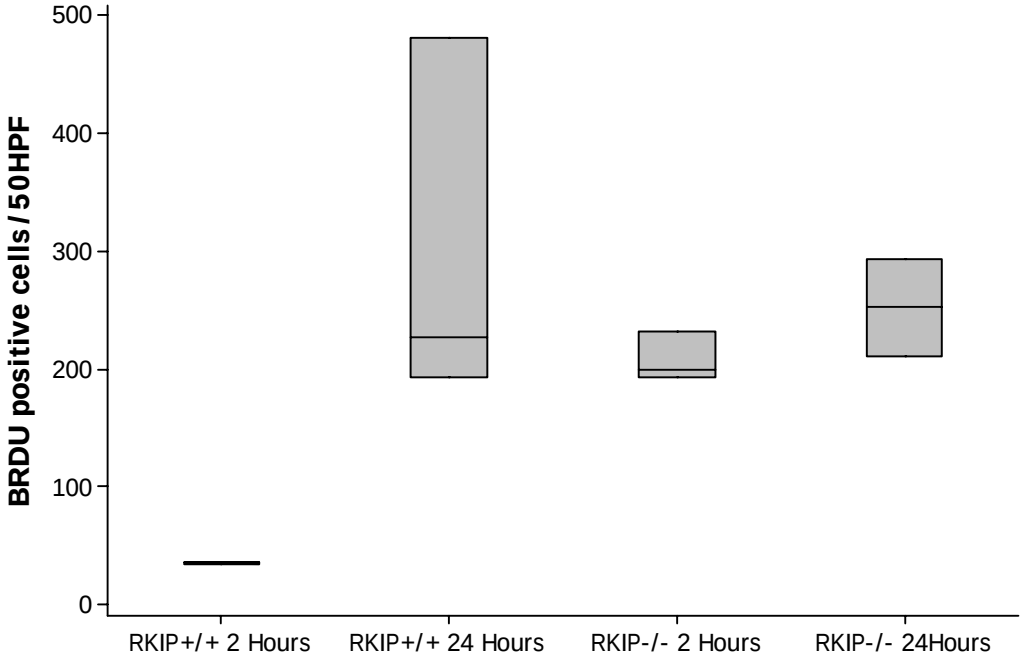


Figure 4.7: Cells in the livers of $APC^{fl/fl}$ $RKIP^{-/-}$ mice appear to be arrested in mitosis.

To determine whether the increase in mitotic figures seen in the livers of $APC^{fl/fl}$ $RKIP^{-/-}$ mice was due to a true increase in mitotic activity mice were injected with BRDU 2 or 24 hours prior to being euthanized. This marks cells in S-phase and is retained in daughter cells, following mitosis. In the RKIP wild-type mice there was a ~7 fold increase in the number of BRDU positive cells, between 2 and 24 hours, indicating that these cells are progressing through mitosis. However in the RKIP knockout mice there was virtually no difference between the number of BRDU positive cells between 2 and 24 hours, indicating that these cells are arrested in mitosis.

4.2.5 RKIP deletion results in a shift in tumour location from the small intestine to the colon in *APC$^{fl/+}$ KRas^{G12V}* mice

We have seen that deletion of *RKIP* did not result in a measurable phenotype in the *APC^{fl}* tumour model. One possible reason for this is that the tumours in this model very rarely progress beyond benign adenomas to invasive carcinomas. As RKIP is thought to be a metastasis suppressor gene it is possible that any effect of RKIP deletion will only be realised in a more advanced tumour. Indeed it has been shown in an orthotopic model of prostate carcinoma that reduced levels of RKIP led to an increase in lung metastases but had no effect on primary tumour formation (Fu, Smith et al. 2003).

Therefore, we set out to study the effect of *RKIP* knockout in a model of invasive CRC. The model we chose was the *APC$^{fl/+}$ KRas^{G12V}* mouse, which combines conditional heterozygous deletion of *Apc* with the expression of an activated, oncogenic form of *KRas*. The mutant *KRas* is present at the endogenous *KRas* locus, preventing massive overexpression of the mutant (Guerra, Mijimolle et al. 2003). This mouse develops more invasive tumours than the *APC^{fl}* mouse (Sansom, Meniel et al. 2006). Therefore we crossed APC$^{fl/+}$ KRas^{G12V} mice to *RKIP$^{-/-}$* mice to yield experimental cohorts of *APC$^{fl/+}$ KRas^{G12V} RKIP$^{+/+}$* (N=15) and *APC$^{fl/+}$ KRas^{G12V} RKIP$^{-/-}$* (N=12) mice. The mice were again aged until they developed signs of intestinal tumours.

Firstly, we examined the effect on survival, we noted that as has been shown previously *APC$^{fl/+}$ KRas^{G12V}* mice develop tumours much more rapidly than *APC^{fl}* mice (Sansom, Meniel et al. 2006). However, the deletion of *RKIP* did not lead to

an increase in the rate of tumourigenesis (Figure 4.8) (Log-Rank p=0.379). Following histological examination of the tumours, no difference in the proportion of invasive tumours was seen (3/13 vs 3/12, Chi-square p=0.906).

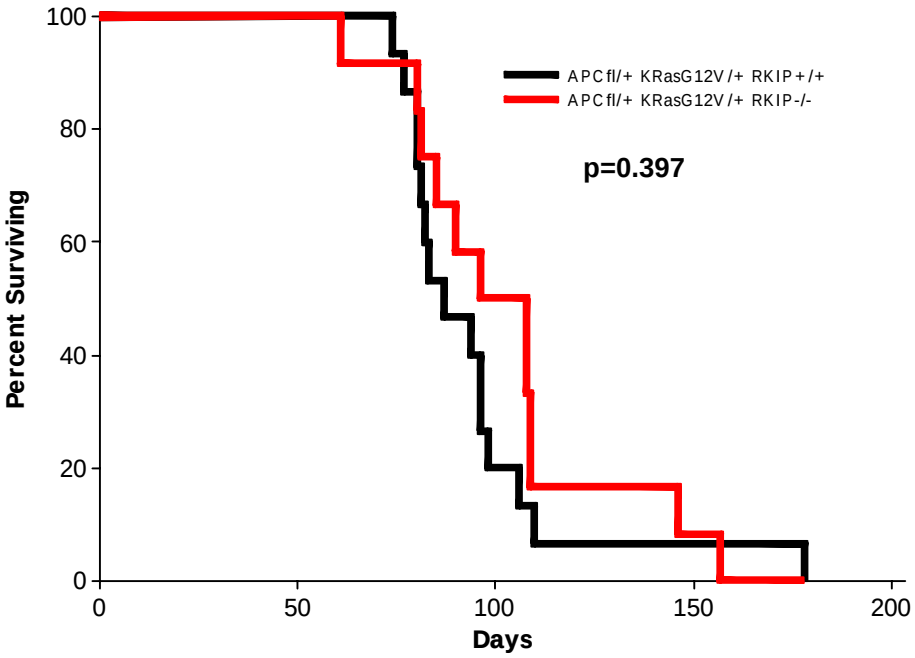


Figure 4.8: RKIP deletion does not affect survival in the $APC^{fl/+}$ $KRas^{G12V}$ mouse

Kaplan-Meier survival analysis demonstrates that there is no difference in survival between $APC^{fl/+}$ $KRas^{G12V}$ $RKIP^{+/+}$ and $APC^{fl/+}$ $KRas^{G12V}$ $RKIP^{-/-}$ mice (Log-Rank p=0.397).

To determine if the there was any difference in tumour number or size between the cohorts, these were measured at the time of necropsy. Although there was no difference in overall tumour number or size between the 2 cohorts, there was a marked increase in the number of colonic tumours seen in the *APC*$^{fl/+}$ *KRas*^{G12V} *RKIP*$^{-/-}$ mice (Mann-Whitney p=0.006) (Figure 4.9). This increase was offset by a statistically non-significant decrease in the number of small intestinal tumours (Mann-Whitney p=0.075) (Figure 4.9) Histological examination revealed that not only was this increase apparent in the number of tumours visible grossly, but also in small microscopic tumours (Figure 4.9).

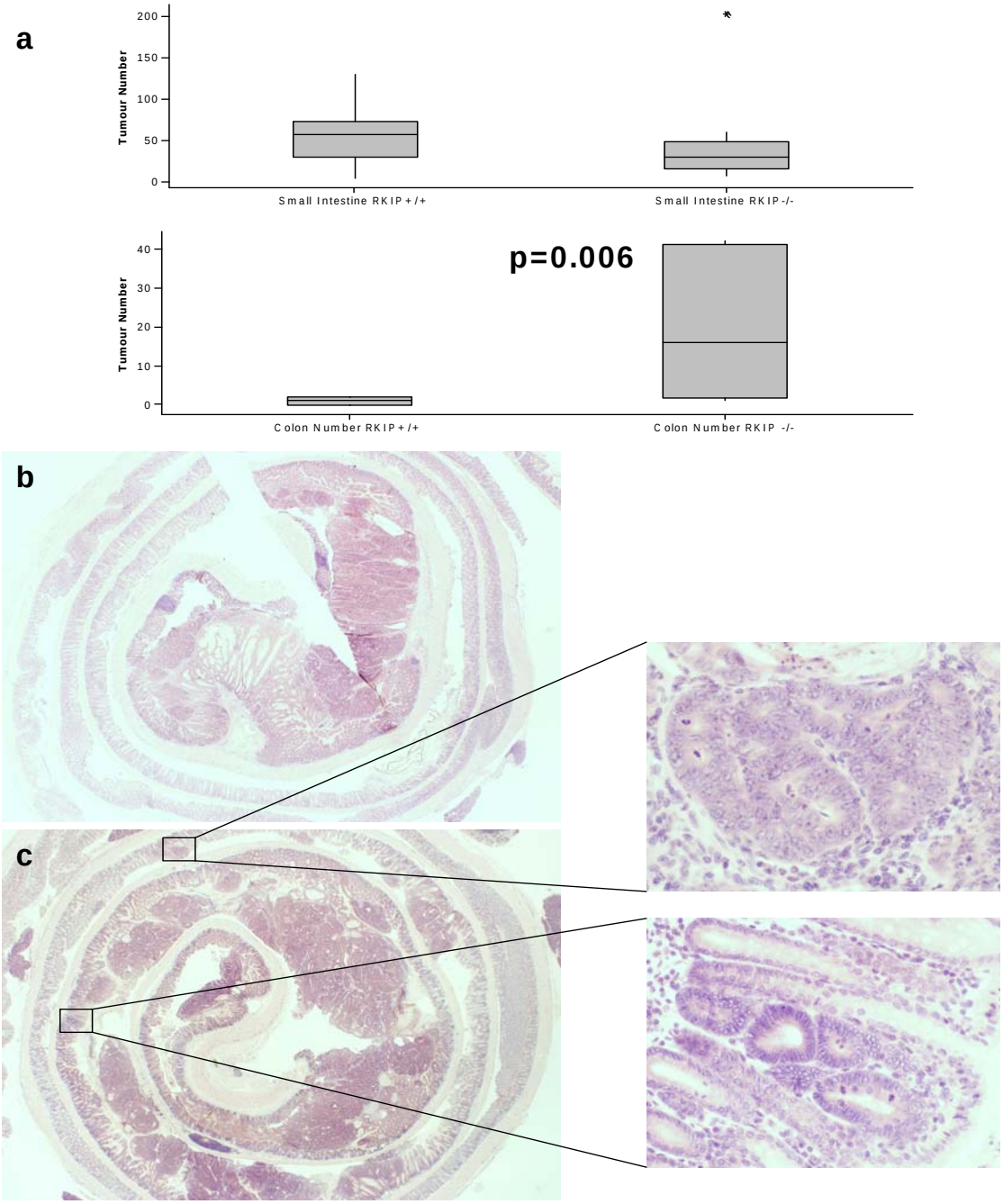


Figure 4.9: RKIP deletion results in an increase in colonic tumours in the APC$^{fl/+}$ KRas^{G12V} mouse

Although there was no difference in the overall tumour number between APC$^{fl/+}$ KRas^{G12V} RKIP$^{+/+}$ and APC$^{fl/+}$ KRas^{G12V} RKIP$^{-/-}$ mice there was a marked difference in the number of colonic tumours between the 2 cohorts (a, lower panel) (Mann-Whitney p=0.006, * indicates outlying values). This could also be demonstrated at the microscopic level, where there was a clear difference in the number of very early lesions between the 2 cohorts (b). In addition to the increase in macroscopically visible tumours there was a marked difference in microscopic lesions. In APCfl/+ KRas^{G12V} RKIP+/+ mice any tumours seen were single and large (b) whereas in the APCfl/+ KRas^{G12V} RKIP-/- mice many microadenomas (c, upper enlargement) and single crypt lesions (c, lower enlargement) were seen.

4.2.6 RKIP2 is not increased in the tumours from any of the models

In the above studies we did not see the phenotype that we expected in terms of increased invasion and metastases in the different tumour models. One possible explanation for this would be compensation for the absence of RKIP by upregulation one of the RKIP family members. As *RKIP3-5* have been shown to be silent pseudogenes (Theroux, Pereira et al. 2007) we looked for the expression of RKIP2. RKIP2 has been shown to expressed specifically in the testis and has not been detected in other organs (Moffit, Boekelheide et al. 2007; Theroux, Pereira et al. 2007). However it is possible that in response to stress induced by the tumour model that RKIP2 may be elevated to compensate for the low levels of RKIP1. As there is no antibody available that will differentiate between RKIP1&2 we used reverse transcriptase PCR to test for the presence of RKIP2 RNA in tumours from the different models, using testis as a positive control. Although we could detect strong expression of RKIP2 in the testis, no RKIP2 RNA could be detected in any of the tumour samples (Figure 4.10), indicating that compensation by RKIP2 is not occurring in these tumours.

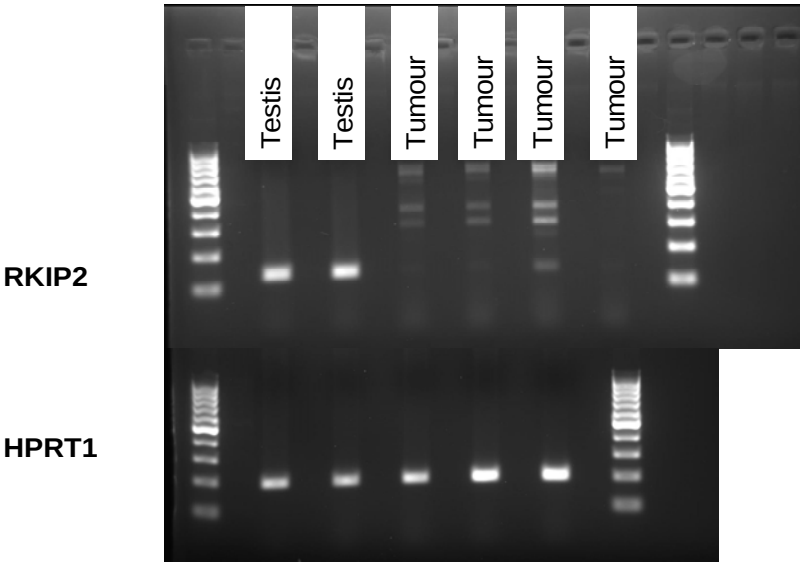


Figure 4.10: RKIP2 is not upregulated in tumours from the RKIP$^{-/-}$ mice

rtPCR was performed to determine if there was a compensatory upregulation of RKIP2 in tumours from the RKIP-/- mice. RKIP2 was easily detected in the testis as a positive control, but despite 35 cycles of PCR no RKIP2 could be detected in the tumours. rtPCR for HPRT1 (hypoxanthine phosphoribosyltransferase 1) was performed as a positive control (Note no control present for tumour 4).

4.3 Discussion

Although our work and that of others have shown that the loss of RKIP expression is a significant event in CRC, very little is known with regards to the mechanisms of how this comes about. We were attempting to address this question by studying the effect of *RKIP* knockout in a number of established mouse models of CRC. The results of these experiments have been somewhat unexpected as we did not observe increased invasion and/or metastasis in the tumour models and the phenotypes that we did observe were quite subtle and did not appear to relate directly to the known role of RKIP as a metastasis suppressor. The phenotypes that we observed are interesting in their own right and appear to relate to the role that RKIP is known to play in the maintaining the integrity of the spindle checkpoint and preventing the development of CIN (Eves, Shapiro et al. 2006; Al-Mulla, Hagan et al. 2008).

In the liver we saw that deletion of *RKIP* led to a markedly increased mitotic index and an increase in aberrant mitoses and in particular anaphase bridges. It has previously been shown in a number of human tumours that the presence of anaphase bridges correlates with the presence of CIN (Montgomery, Wilentz et al. 2003). This suggests that *RKIP* deletion is cooperating with homozygous *Apc* deletion to promote CIN in the livers of these mice. RKIP has been shown both *in vitro* (Eves, Shapiro et al. 2006) and in human CRC (Al-Mulla, Hagan et al. 2008) to be an important component in regulating CIN. APC can also influence the spindle checkpoint and affect the development of CIN. It is known that APC is localised to the kinetochore in metaphase chromosomes, where it forms a complex with checkpoint proteins BUB (Budding Uninhibited By Benzimidazoles) 1 and 3, and is thought that this is important for the correct attachment of

microtubules to the kinetochore (Fodde, Kuipers et al. 2001; Kaplan, Burds et al. 2001). Indeed cells that lack APC develop multiple spindle and centrosomal defects which can result in CIN both through disjunction defects, resulting in aneuploidy (Fodde, Kuipers et al. 2001; Kaplan, Burds et al. 2001) and chromosomal translocations, through the formation of multiple centrosomes (Kaplan, Burds et al. 2001). It has also been shown that APC loss leads to CIN both in the intestine both in mice and humans (Cardoso, Molenaar et al. 2006; Alberici, de Pater et al. 2007). There is no evidence to suggest that in the mouse loss of *Apc* alone in the liver results in CIN; however our data suggest that the addition of a second hit, namely RKIP loss is enough to induce this. In these studies we also observed a mitotic arrest as measured by a lack of progression from S-phase. These data support the previous studies showing that decreased RKIP levels are associated with CIN (Eves, Shapiro et al. 2006; Al-Mulla, Hagan et al. 2008). However, the finding that cells in the $APC^{fl/fl}$ $RKIP^{-/-}$ livers are arrested in mitosis is contrary to the previous report, in which RKIP depletion was shown to speed the progression of cells through mitosis (Eves, Shapiro et al. 2006). This discrepancy may reflect the cooperation between *Apc* and *RKIP* deletion and also the differences in experimental approaches used. It is possible that alternative checkpoint mechanisms may be activated *in vivo* that would not be present in tumour cells in culture.

The phenotype of the $APC^{fl/+}$ $KRas^{G12V}$ $RKIP^{-/-}$ mice was a shift in tumour localisation from the small intestine to the colon. Interestingly this is a phenotype that has been seen before in a mouse model of CRC, in which the Apc^{Min} mouse was crossed to *BubR1* knockout mouse. BubR1 is a component of the spindle assembly checkpoint, which prevents activation of the anaphase promoting complex until chromosomes are appropriately lined up and

microtubules from 2 centromeric poles are attached to kinetochores (Kops, Weaver et al. 2005). Moreover, reduction of BubR1 has been shown *in vitro* to result in an increase in CIN (Baker, Jeganathan et al. 2004). A similar result has also been reported by Baker *et al*. who showed an increase in colonic tumours in the Apc^{Min} mouse following deletion of the checkpoint regulator *Bub1* (Baker, Jin et al. 2009). This would suggest that on a background of *APC* heterozygous deletion the addition of an element which perturbs the correct functioning of the spindle checkpoints, thus promoting CIN will result in a shift of tumour location to the colon; this also suggests that colonic cells are more sensitive to this kind of insult. We did not see the same result in the $APC^{fl/+}$ $RKIP^{-/-}$ mice, indicating that the effect may be specific to the hyperactivation of the MAPK pathway induced by both KRas activation and *RKIP* deletion.

A number of possible reasons could account for the fact that the *RKIP* knockout mice did not demonstrate increased invasiveness in any of the models studied. One of these is a compensatory increase expression of other members of the RKIP family. *RKIP3-5* have been shown to be silent pseudogenes, therefore we examined the potential upregulation of RKIP2. RKIP2 has previously been shown to be expressed specifically in the testis (Moffit, Boekelheide et al. 2007; Theroux, Pereira et al. 2007) and even in the *RKIP* knockout mouse no RKIP2 could be detected in any other organs, indicating that it does not increase to compensate for the loss of RKIP1 (Theroux, Pereira et al. 2007). Despite this there is the possibility that increased cellular stresses in a tumour may induce a compensatory increase in RKIP2. We examined tumours from all of the different models by rtPCR and showed that although we could detect the transcript in the testis, none was detectable in any of the tumours, demonstrating that there is no compensatory upregulation of RKIP2 in these tumours.

A second possibility is that there is a difference in the effect of germline *RKIP* deletion and the loss that occurs during the progression of a tumour. Although this is very difficult to prove, in the next chapter we will demonstrate the very different effects of *p53* germline mutation and *p53* mutation in the adult in a model of CRC, which could be due, at least in part to such a phenomenon.

In summary we have shown that deletion of *RKIP* does not increase the invasive or metastatic potential of the tumour models studied. It does however appear to potentiate the CIN, induced by loss of *Apc* and expression of oncogenic *KRas*. The lack of invasive phenotype in the *RKIP* knockout mice is not due to compensatory upregulation of RKIP2.

Chapter 5 *p53* mutation promotes invasion in a novel model of CRC

5.1 Introduction

In addition to studying the role of *RKIP* knockout in established mouse models of CRC an important aim of this project was to develop new models of CRC which more closely recapitulate the human disease, particularly the progression from adenoma to carcinoma to metastasis. These models will be useful in developing a greater understanding of the biology of CRC and in the preclinical testing of novel therapies. We attempted to take a rational approach to this problem by basing the model on genetic events that are common in human CRC and which occur at different stages in the adenoma to carcinoma sequence. To this end we have crossed the APC^{fl} mouse with a *p53* mutant mouse.

APC is mutated in the majority of human CRC (Miyoshi, Nagase et al. 1992; Powell, Zilz et al. 1992) and is generally considered the gatekeeper mutation in CRC, occurring early in the disease process (Vogelstein, Fearon et al. 1988). Similarly, *p53* is mutated in a large proportion (~50%) of human CRC (Baker, Preisinger et al. 1990), however, in contrast to *APC*, *p53* mutations are rare in early adenomas and much more common in invasive carcinomas, suggesting that they are a later event in tumour progression (Vogelstein, Fearon et al. 1988; Baker, Preisinger et al. 1990). While the mutations that occur in many tumour suppressor genes (including *APC*) typically result in the expression of truncated and non-functional protein (Cottrell, Bicknell et al. 1992; Miyoshi, Nagase et al. 1992; Miyaki, Konishi et al. 1994), mutations of *p53* tend to be point mutations, resulting in the expression of mutant forms of the p53 protein, which often accumulate in tumour cells (Bartek, Bartkova et al. 1991; Brosh and Rotter 2009). The majority of these mutations affect the DNA binding domain, thus preventing normal function of p53 as a transcription factor (Hollstein, Sidransky

et al. 1991; Brosh and Rotter 2009). It has been shown that these mutant forms of p53 can have a dominant negative effect, or can indeed exert an oncogenic effect beyond simple loss of the normal protein function (Brosh and Rotter 2009). For this reason we have used 2 different *p53* mutant mice. The first is the $p53^{fl}$ mouse (Jonkers, Meuwissen et al. 2001) in which *p53* is deleted conditionally in a similar manner to the APC^{fl} mouse. The second is the $p53^{R172H}$ mouse; in this case a R172H point mutant is conditionally expressed from the endogenous locus following deletion of a preceding STOP cassette by Cre recombinase (Olive, Tuveson et al. 2004). This has allowed us to compare the effects of these 2 different types of *p53* mutation in CRC.

Although it is clear that mutation of *p53* occurs regularly in CRC, the reason for this remains unclear, indeed Fazeli and colleagues showed that it does not result in a decrease in apoptosis in either early or late stage adenomas (Fazeli, Steen et al. 1997). Previous studies investigating *p53* loss at the early stages of intestinal tumorigenesis *in vivo*, using either the Apc^{Min} mouse or acute *Apc* loss, have found little or no effect of *p53* loss (Fazeli, Steen et al. 1997; Halberg, Katzung et al. 2000; Reed, Meniel et al. 2008). *p53* loss did not affect crypt size, proliferation or apoptosis following *Apc* deletion, reflecting the finding that p53 expression is only increased in a small number of cells (Reed, Meniel et al. 2008). Likewise adenomas from Apc^{Min} mice show little p53 accumulation and *p53* mutations are never observed in the Apc^{Min} polyps, suggesting that there is no selective advantage to lose p53 in these tumours. By using the APC^{fl} mouse we felt that we could overcome this given the much longer latency of tumour development in this mouse. This longer latency and the fact that APC^{fl} mice develop occasional invasive carcinomas could produce the environment which reveals the transforming properties of *p53* mutation.

5.2 Results

5.2.1 Loss or mutation of *p53* accelerates APC induced tumourigenesis

In order to determine the effect of *p53* loss on *Apc* induced tumourigenesis, *AhCre*$^{+}$ *APC*$^{fl/+}$ mice were crossed to either *p53*$^{fl/+}$ mice or to *p53*$^{R172H/+}$ mice. In the *p53*$^{fl/+}$ mice, a LoxP site is present in introns 2 and 11. Thus, on expression of the Cre-recombinase exons 2-10 are deleted, resulting in a null allele (Jonkers, Meuwissen et al. 2001). Similar to the *KRas*$^{G12V/+}$ mice (see chapter 4.2.5), expression of the mutant *p53*^{R172H} allele is prevented by the presence of an upstream STOP cassette, which is flanked by LoxP sites. Upon activation of Cre, the STOP cassette is deleted and the mutant allele expressed (Olive, Tuveson et al. 2004). The breeding strategy resulted in cohorts of *AhCre* positive *APC*$^{fl/+}$ *p53*$^{+/+}$ (N=15), *APC*$^{fl/+}$ *p53*$^{fl/+}$ (N=20), *APC*$^{fl/+}$ *p53*$^{R172H/+}$ (N=14), *APC*$^{fl/+}$ *p53*$^{fl/fl}$ (N=15) and *APC*$^{fl/+}$ *p53*$^{R172H/fl}$ (N=18). Mice were induced with β-naphthoflavone at approximately 6 weeks of age. All of the mice were aged until they developed signs of intestinal tumours (such as pale feet, hunching or abdominal swelling).

Loss or mutation of a single *p53* allele led to a significant acceleration in tumourigenesis (*APC*$^{fl/+}$ *p53*$^{fl/+}$ and *APC*$^{fl/+}$ *p53*$^{R172H/+}$ cohorts) compared with controls (*APC*$^{fl/+}$ *p53*$^{+/+}$) (Log-Rank p=0.008) (Figure 5.1). Genetic deletion of both copies of *p53* (*APC*$^{fl/+}$ *p53*$^{fl/fl}$) or mutation of one copy, with concomitant deletion of the second copy (*APC*$^{fl/+}$ *p53*$^{R172H/fl}$) led to a marked acceleration in tumorigenesis compared to both control and heterozygous mice (Log-Rank p<0.001) (Figure 5.1). Indeed, median tumour onset was more than halved in

APC$^{fl/+}$ *p53*$^{fl/fl}$ and *APC*$^{fl/+}$ *p53*$^{R172H/fl}$ mice compared with controls. There was no difference in survival between the *APC*$^{fl/+}$ *p53*$^{fl/+}$ and *APC*$^{fl/+}$ *p53*$^{R172H/+}$ cohorts or between the *APC*$^{fl/+}$ *p53*$^{fl/fl}$ and *APC*$^{fl/+}$ *p53*$^{R172H/fl}$ cohorts. There was no difference in tumour number or size across the cohorts, indicating that all of the animals were euthanized at approximately the same stage of tumour development (Figure 5.1).

To assess the effect of loss of *p53* alone on the development of intestinal tumours, *AhCre* positive *APC*$^{+/+}$ *p53*$^{fl/fl}$ mice were induced and aged in an identical manner to the other cohorts. Despite a longer survival time than *APC*$^{fl/+}$ *p53*$^{fl/fl}$ mice none of these mice developed intestinal tumours. The majority of the tumours were lymphomas or sarcomas with rare mammary tumours, squamous cell carcinomas and one endometrial carcinoma (the *AhCre* transgene yields low levels of Cre-mediated recombination in these tissues). This indicates that loss of *p53* alone is insufficient for intestinal tumour development and an initiating event, such as *APC* loss is required.

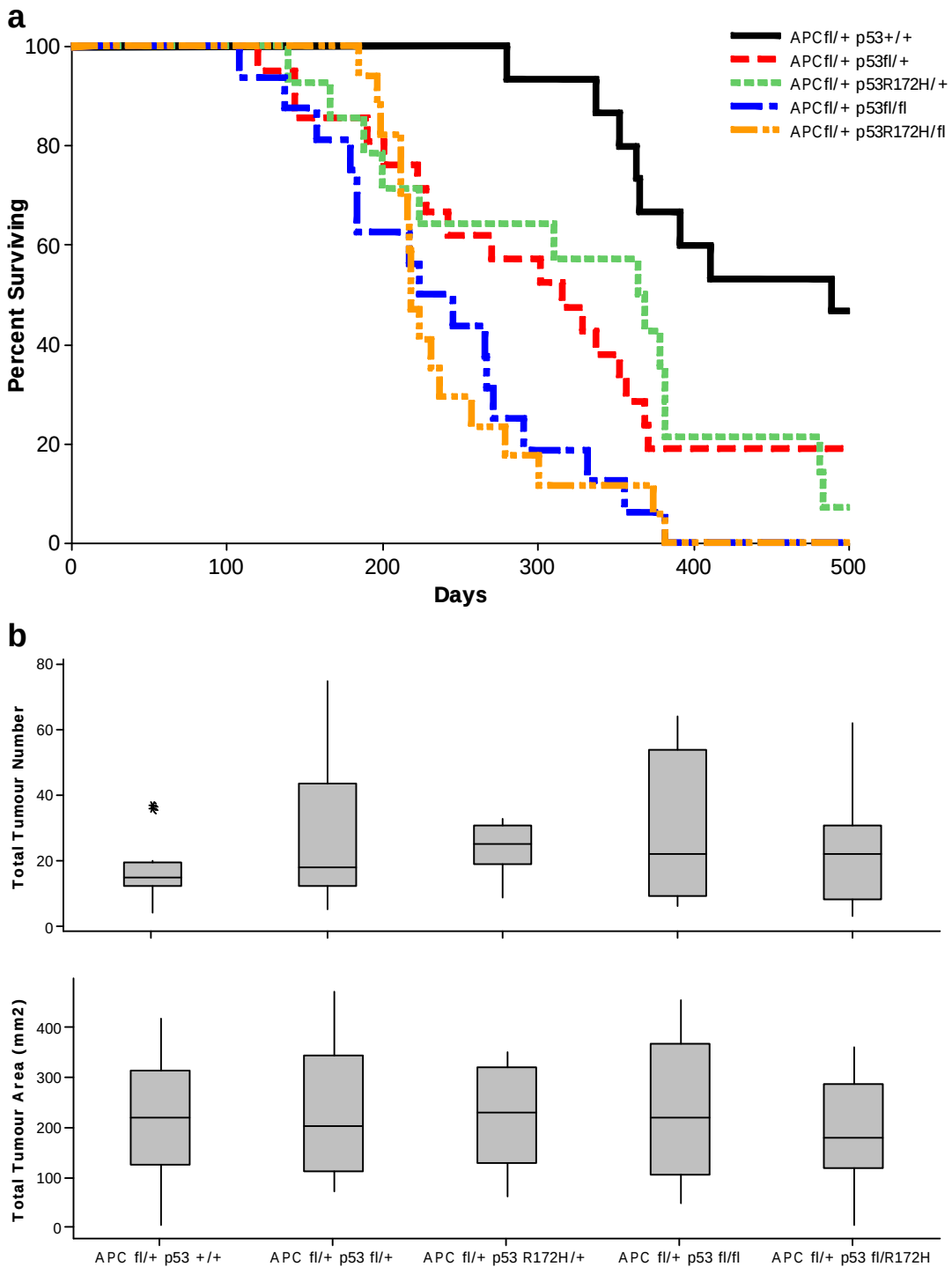


Figure 5.1: APC driven tumourigenesis is accelerated by p53 mutation

Kaplan-Meier survival analysis showing the survival of the 5 cohorts studied. There was a significant difference in survival between the $APC^{fl/+}$ $p53^{+/+}$ cohort and both $APC^{fl/+}$ $p53^{fl/+}$ and $APC^{fl/+}$ $p53^{R172H/+}$ cohorts (Log-Rank p=0.008)). A further acceleration in tumourigenesis between these cohorts and both the $APC^{fl/+}$ $p53^{fl/fl}$ and the $APC^{fl/+}$ $p53^{R172H/+}$ cohorts (Log-Rank p<0.001) (a). There was no difference in either tumour number or size across the 5 cohorts studied (b) (* indicates outlying values).

5.2.2 Point mutation of a single copy of *p53* is sufficient for the progression to invasive carcinoma

To examine progression, tumours from all genotypes were histologically examined. High grade dysplasia (HGD) and invasive carcinoma were identified in all cohorts but at very different rates. In the controls (*APC*$^{fl/+}$ *p53*$^{+/+}$), HGD and invasive carcinoma were seen in 5/15 (33%) mice and 4/15 (27%) mice respectively. Rates were similar in the *APC*$^{fl/+}$ *p53*$^{fl/+}$ cohort, with 8/20 (40%) and 5/20 (25%) mice developing HGD and invasive carcinoma respectively. Strikingly, the rates of HGD and invasive carcinoma were much greater in the other 3 cohorts, at 13/14 (93%) and 12/14 (86%) in the *APC*$^{fl/+}$ *p53*$^{R172H/+}$ cohort, 13/15 (87%) and 12/15 (80%) in the *APC*$^{fl/+}$ *p53*$^{fl/fl}$ cohort and 18/18 (100%) and 16/18 (89%) in the *APC*$^{fl/+}$ *p53*$^{R172H/fl}$ cohort respectively (Figure 5.2).

These data demonstrate that while deletion of both copies of *p53* and the mutation of 1 copy with concomitant deletion of the second copy result in an identical shortening of lifespan and promote an invasive phenotype, there is a marked difference in the heterozygous situation. Despite an almost identical lifespan, total tumour number and average tumour size, invasive carcinoma developed in almost 90% of the *APC*$^{fl/+}$ *p53*$^{R172H/+}$ mice, compared with only 25% of *APC*$^{fl/+}$ *p53*$^{fl/+}$ mice (Figure 5.2). This would suggest that while mutant p53 does not have an effect on tumour initiation it has a profound effect on tumour progression, as evidenced by the increase in invasive carcinomas.

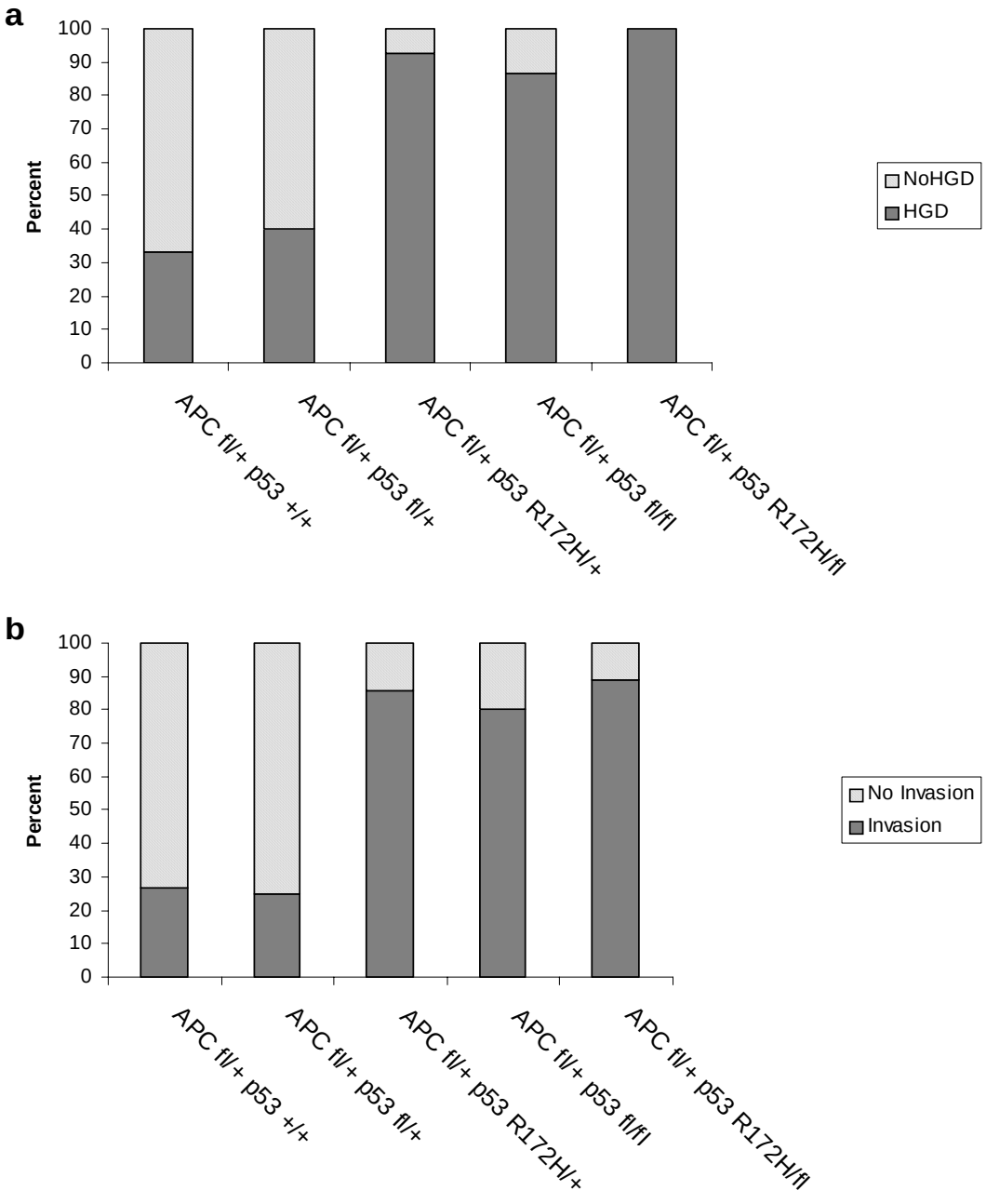


Figure 5.2: p53 point mutation promotes high grade dysplasia and an invasive phenotype

Tumours were analysed histologically for the presence of high grade dysplasia (HGD) (a) and invasive carcinoma (b). The proportion of mice developing invasive carcinoma in the control group was approximately 25%. Mutations affecting both copies of p53 (either loss of function of both $APC^{fl/+}$ $p53^{fl/fl}$, or point mutation of one and loss of function of the other $APC^{fl/+}$ $p53^{R172H/fl}$) resulted in a dramatic increase in invasion with ~90% of mice developing invasive tumours. Interestingly, while there was no difference in survival between the $APC^{fl/+}$ $p53^{fl/+}$ or $APC^{fl/+}$ $p53^{R172H/+}$ cohorts there was a marked increase in invasive tumours in the $APC^{fl/+}$ $p53^{R172H/+}$ cohort, indicating that point mutation of p53 promotes invasion but is unlikely to play a role in tumour initiation.

As we wanted to develop a model that closely recapitulated the human disease, we examined the tumours microscopically to assess similarities at the level of histology. Variation in the depth of invasion, similar to the spectrum seen in human CRC was observed. Some tumours showed superficial invasion, equivalent to T2-T3 human tumours (Figure 5.3). Other tumours invaded through the full thickness of the muscularis and peritoneum, resulting in tumour cells in the peritoneal cavity and occasional direct invasion into local organs (Figure 5.3). This would be equivalent to T4 disease in human CRC. The abdominal organs and lymph nodes were harvested for histology at necropsy. A single $APC^{fl/+}$ $p53^{fl/fl}$ mouse was found to harbour a metastasis in a pancreatic lymph node (Figure 5.3). The primary tumour was a moderate-poorly differentiated invasive carcinoma. This demonstrates that the invasive tumours that developed in this model are capable of metastasising. However, the fact that only one metastasis was detected indicates that somatic events other than those induced are necessary before this occurs. Aside from this single metastasis in the $APC^{fl/+}$ $p53^{fl/fl}$ cohort the invasive tumours that developed in the $APC^{fl/+}$ $p53^{R172H/+}$, $APC^{fl/+}$ $p53^{fl/fl}$ and $APC^{fl/+}$ $p53^{R172H/fl}$ mouse cohorts showed similar differentiation and depth of invasion.

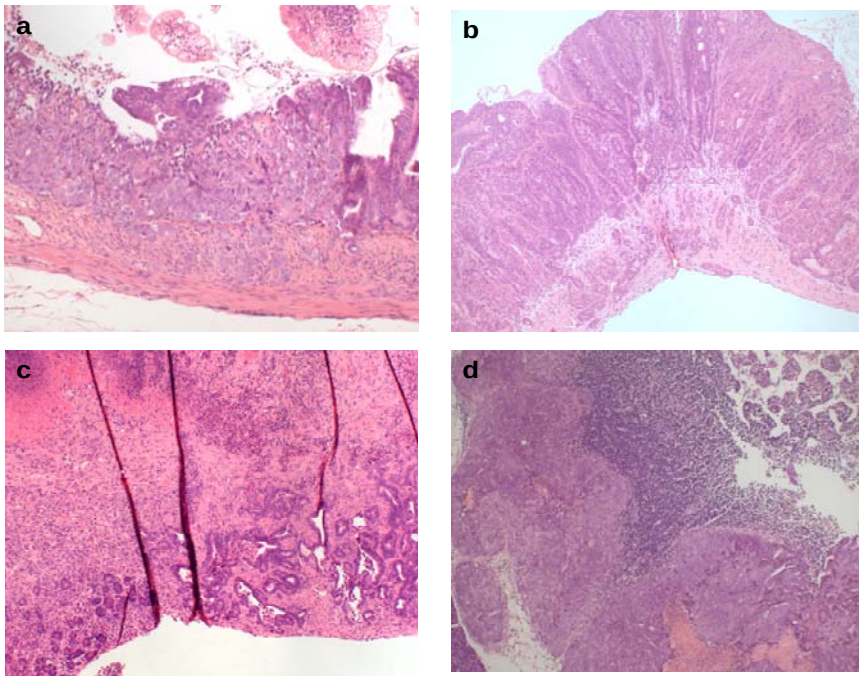


Figure 5.3: Examples of invasive tumours and metastasis

As in human CRC the invasive tumours that developed in this model showed a variety of depth of invasion, ranging from superficial muscle invasion (a), through complete muscle invasion to reach the peritoneal surface (b). A number of tumours perforated the bowel wall, resulting in a massive inflammatory reaction and direct invasion of local structures (c). A single metastasis was identified in a pancreatic lymph node (d).

Having seen that the tumours in the mice bare a close resemblance to human CRC at the level of histology we wanted to determine if those similarities were also present at the molecular level. Firstly we examined the epithelial to mesenchymal transition (EMT). EMT is thought to be a key step in the development of invasive carcinoma and in the progress towards metastasis. Two markers of EMT are loss of E-cadherin and expression of ZEB1 (Schmalhofer, Brabletz et al. 2009), a member of the zinc finger homeobox family, which represses E-cadherin transcription and has been reported to be upregulated at the invasive front of CRC (Spaderna, Schmalhofer et al. 2006). To see if this was the case in the tumours from this study, we performed ZEB1 and E-cadherin IHC on a subset of invasive tumours. We observed selective elevation of ZEB1 expression and a decrease in E-cadherin expression (Figure 5.4) at the invasive front. These data suggest that EMT is occurring at the invasive front of these tumours, and further demonstrates the similarity between the tumours in this model and human CRC. The expression of these markers was similar in the invasive tumours from the $APC^{fl/+}$ $p53^{R172H/+}$, $APC^{fl/+}$ $p53^{fl/fl}$ or $APC^{fl/+}$ $p53^{R172H/fl}$ cohorts.

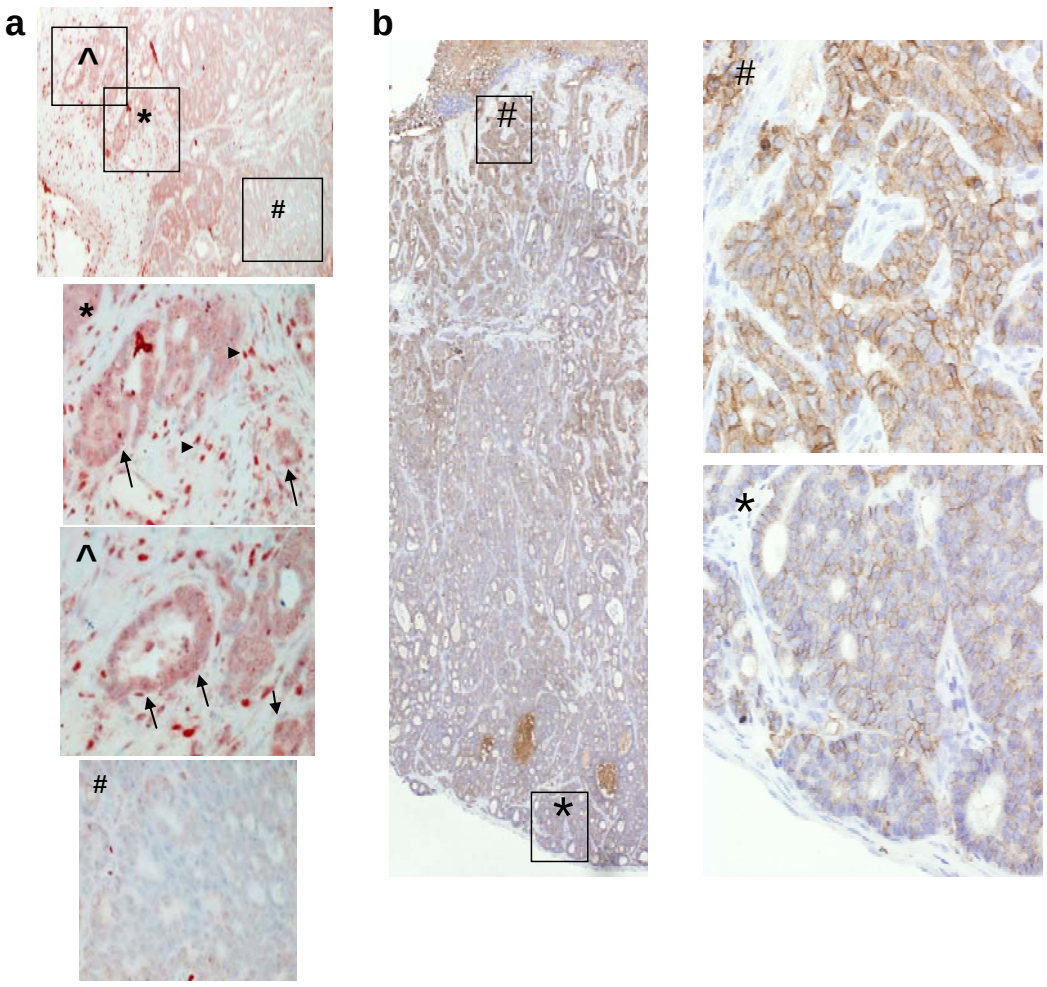


Figure 5.4: EMT markers are present at invasive edge of the tumours

ZEB1 is a repressor of e-cadherin and has been shown to be a marker of EMT and raised at the invasive edge of human CRC. Staining for ZEB1 in the invasive tumours from our model demonstrated a positivity for ZEB1 at the invasive front of the tumour (a, ^ & *) when compared to the centre of the tumour (a, #). Similarly, the loss of nuclear e-cadherin is associated with EMT. Tumours from our model showed high levels of membranous e-cadherin on the surface and in the centre of tumours (b, #) but much lower levels at the invasive front (b, *).

CIN also occurs in the majority of human CRC (Issa 2008). To determine if this was the case in the mouse tumours we examined them for evidence of CIN by quantifying abnormal mitoses, in particular tripolar mitoses and anaphase bridges (Figure 5.5). These have been shown to correlate with the presence of CIN in CRC and other tumours (Montgomery, Wilentz et al. 2003; Petersen, Kotb et al. 2009). A higher percentage of tripolar mitoses and anaphase bridges were seen in tumours taken from the $APC^{fl/+}$ $p53^{R172H/+}$, $APC^{fl/+}$ $p53^{fl/fl}$ and $APC^{fl/+}$ $p53^{R172H/fl}$ cohorts when compared to $APC^{fl/+}$ $p53^{+/+}$ and $APC^{fl/+}$ $p53^{fl/+}$ cohorts (Chi square p=0.002) (Table 5.1).

	$APC^{fl/+}$ $p53^{+/+}$	$APC^{fl/+}$ $p53^{fl/+}$	$APC^{fl/+}$ $p53^{R172H/+}$	$APC^{fl/+}$ $p53^{fl/fl}$	$APC^{fl/+}$ $p53^{fl/R172H}$
Total Mitoses	198	251	223	188	214
Aberrant Mitoses (% of Total)	0 (0%)	2 (0.8%)	9 (4.0%)	5 (2.7%)	6 (2.8%)

Table 5.1: Chromosomal instability is increased in those cohorts with a high proportion of invasive carcinomas.

Aberrant mitoses were defined as either tripolar mitoses or those showing definite anaphase bridges. The difference in aberrant mitoses between those cohorts with a high frequency of invasive carcinoma ($APC^{fl/+}$ $p53^{R172H/+}$, $APC^{fl/+}$ $p53^{fl/fl}$ and $APC^{fl/+}$ $p53^{R172H/fl}$) and those with a low frequency of invasive carcinoma ($APC^{fl/+}$ $p53^{+/+}$ and $APC^{fl/+}$ $p53^{fl/+}$) was statistically significant (Chi square: p=0.002).

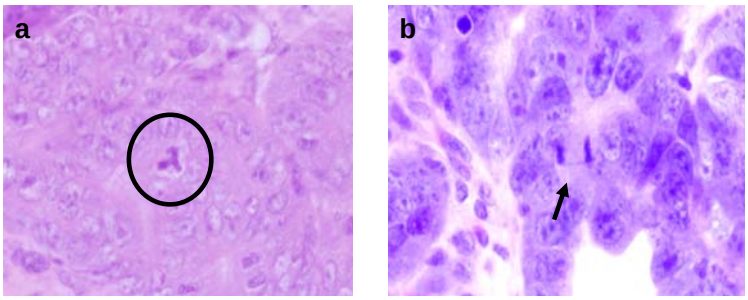


Figure 5.5: Example of a tripolar mitosis and an anaphase bridge

Tumours were studied for the presence of tripolar mitoses (a), in which 3 mitotic spindle poles can clearly be seen and for anaphase bridges (b), in which 2 parallel anaphase plates have formed but remain joined by a bridge of nuclear material (arrow).

5.2.3 p53 and p21 levels in the tumours

In human CRC p53 accumulation has been shown to be associated both with *p53* mutation and with poorer prognosis (Munro, Lain et al. 2005). In order to see if this was the case in the mouse tumours, we stained a selection of both invasive and non-invasive tumours for p53. Tumours from the $APC^{fl/+}$ $p53^{+/+}$ and $APC^{fl/+}$ $p53^{fl/+}$ cohorts showed generally low levels of p53 expression (Figure 5.6). Not surprisingly no p53 expression was seen in the $APC^{fl/+}$ $p53^{fl/fl}$ cohort indicating total loss of p53 protein in these tumours (Figure 5.6). In the $APC^{fl/+}$ $p53^{R127H/fl}$ cohort there was almost universally high levels of p53 protein seen within tumour cells but not in normal cells (Figure 5.6). In the $APC^{fl/+}$ $p53^{R172H/+}$ cohort two patterns of p53 staining were observed. In 2/7 tumours low levels of p53 staining, similar to that seen in control tumours, was observed. The remaining 5 tumours showed at least focally high levels of p53 staining (Figure 5.6), but interestingly there was a wide variation in the percentage of tumours cells staining (10-70%).

As it has been previously reported that loss of the wild-type allele of p53 occurs in a proportion of tumours which develop in the setting of *p53* mutation in the mouse (Olive, Tuveson et al. 2004), we were interested to see if the $APC^{fl/+}$ $p53^{R172H/+}$ tumours still express wild-type p53. This was assessed functionally, by examining levels of p21 by IHC. p21 is a recognised downstream target of p53 but the R172H mutant used in these experiments is not capable of inducing transcription of p21. In the $APC^{fl/+}$ $p53^{+/+}$ and $APC^{fl/+}$ $p53^{fl/+}$ cohorts, although p53 was strongly expressed in only a small number of cells, back to back sections demonstrated high levels of p21 staining in those cells in which p53 expression was high (Figure 5.6). The $APC^{fl/+}$ $p53^{fl/fl}$ tumours showed no p53 expression and

little or no p21 expression (Figure 5.6), consistent with the total loss of p53 protein in these tumours. $APC^{fl/+}$ $p53^{R172H/fl}$ tumours demonstrated high levels of p53 staining but little or no p21 staining (Figure 5.6); this is consistent with there being only mutant p53 present in these cells. In the $APC^{fl/+}$ $p53^{R172H/+}$ cohort, back to back sections revealed that those cells which showed high levels of p53 staining also showed high levels of p21 staining (Figure 5.6). This was also the case, even in those tumours where the overall levels of p53 were low. This would indicate that these tumours retain p53 protein which is transcriptionally active and is able to bring about the upregulation of p21.

Interestingly, although high levels of p53 were seen in tumours from both cohorts expressing the mutant protein, there was a concentration of expression at the invasive front of many of the tumours (Figure 5.7). This may suggest selection for cells expressing mutant p53 in these regions.

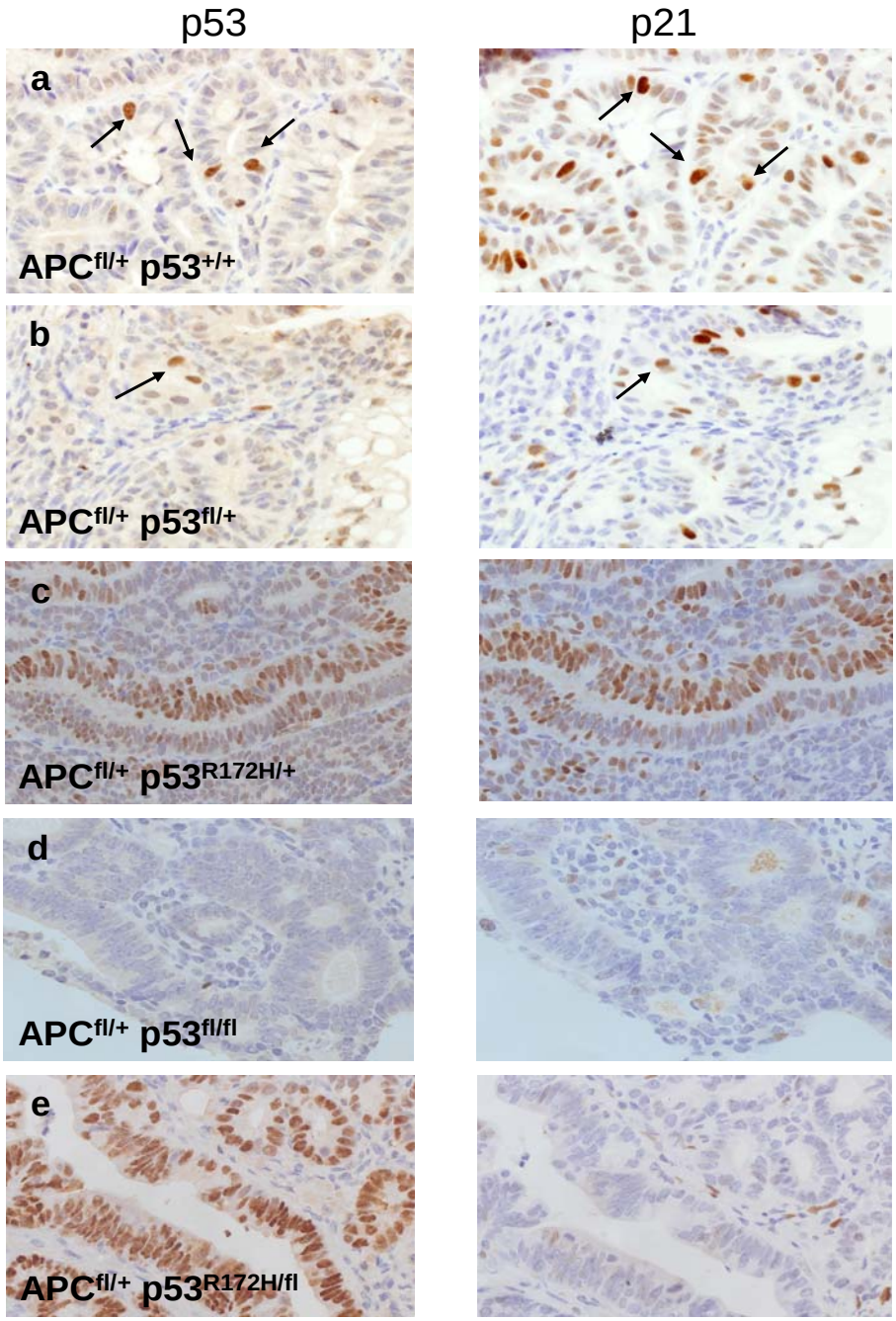


Figure 5.6 p21 levels mirror p53 in tumours from the APC$^{fl/+}$ p53$^{R172H/+}$ mice

To test whether functional p53 was present in the tumours they were stained for both p53 and p21. In APC$^{fl/+}$ p53$^{+/+}$ and APC$^{fl/+}$ p53$^{fl/+}$ tumours only low levels of p53 were seen (a&b, left panels). Staining of back to back sections revealed p21 staining in these cells (a&b, right panels), indicating the presence of functional p53. In the APC$^{fl/+}$ p53$^{fl/fl}$ tumours no p53 or p21 could be detected (d), while in the APC$^{fl/+}$ p53$^{R172H/fl}$ tumours there was accumulation of mutant p53 but no p21 staining (e), confirming that this p53 point mutation cannot activate p21 transcription. Interestingly, in the APC$^{fl/+}$ p53$^{R172H/+}$ tumours high levels of p53 staining was seen, at least focally and in these areas the p53 staining was mirrored by p21 (c), indicating that in these tumours the cells retain a functioning (wild-type) copy of p53.

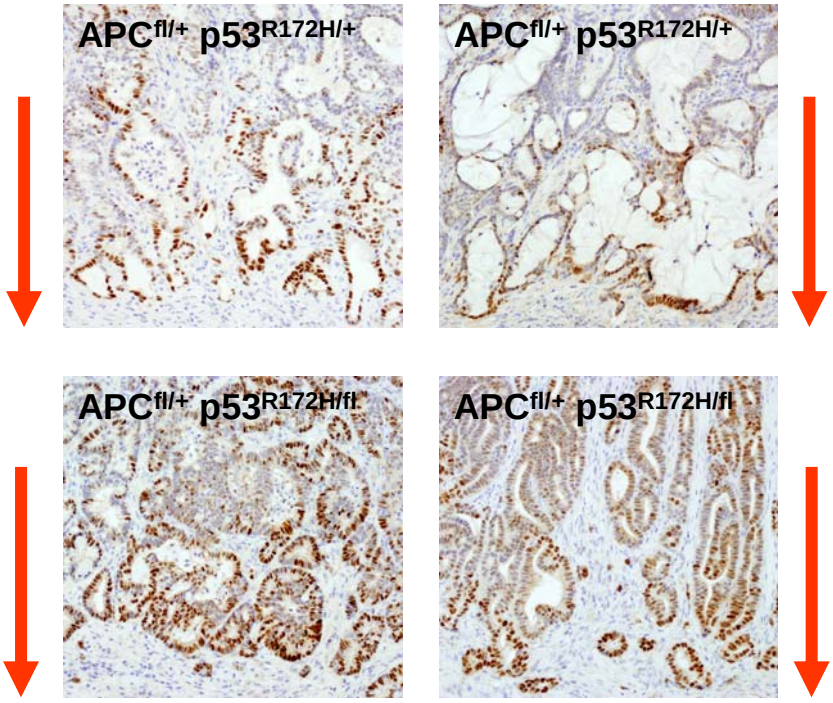


Figure 5.7: Mutant p53 accumulation is greatest at the invasive front

Although mutant p53 was seen to accumulate throughout tumours from the $APC^{fl/+}$ $p53^{R172H/+}$ and $APC^{fl/+}$ $p53^{R172H/fl}$ cohorts, the staining was at its most intense at the invasive front of the tumours. This can be seen in the above examples where the red arrow indicates the direction of invasion.

In order to confirm these findings, a subset of mice were irradiated with 14Gy 6 hours prior to being euthanized. Irradiation leads to stabilisation of p53, expression of p21 and apoptosis in the mouse intestine (Wilson, Pritchard et al. 1998). Tumours from the $APC^{fl/+}$ $p53^{fl/+}$ cohort showed an increase in p53 expression when compared to unirradiated animals, which was mirrored by an increase in p21 levels (Figure 5.8). Tumours from the $APC^{fl/+}$ $p53^{R172H/fl}$ cohort showed high levels of p53 with little or no p21 expression (Figure 5.8). In tumours from the $APC^{fl/+}$ $p53^{R172H/+}$ cohort there was an increase in p53 expression levels, when compared to unirradiated animals and this was closely mirrored by an increase in p21 levels in these cells (Figure 5.8). This would suggest that the wild type copy of p53 is not lost in these tumours and therefore that the loss of wild type p53 is not necessary for the development of an invasive tumour in this setting.

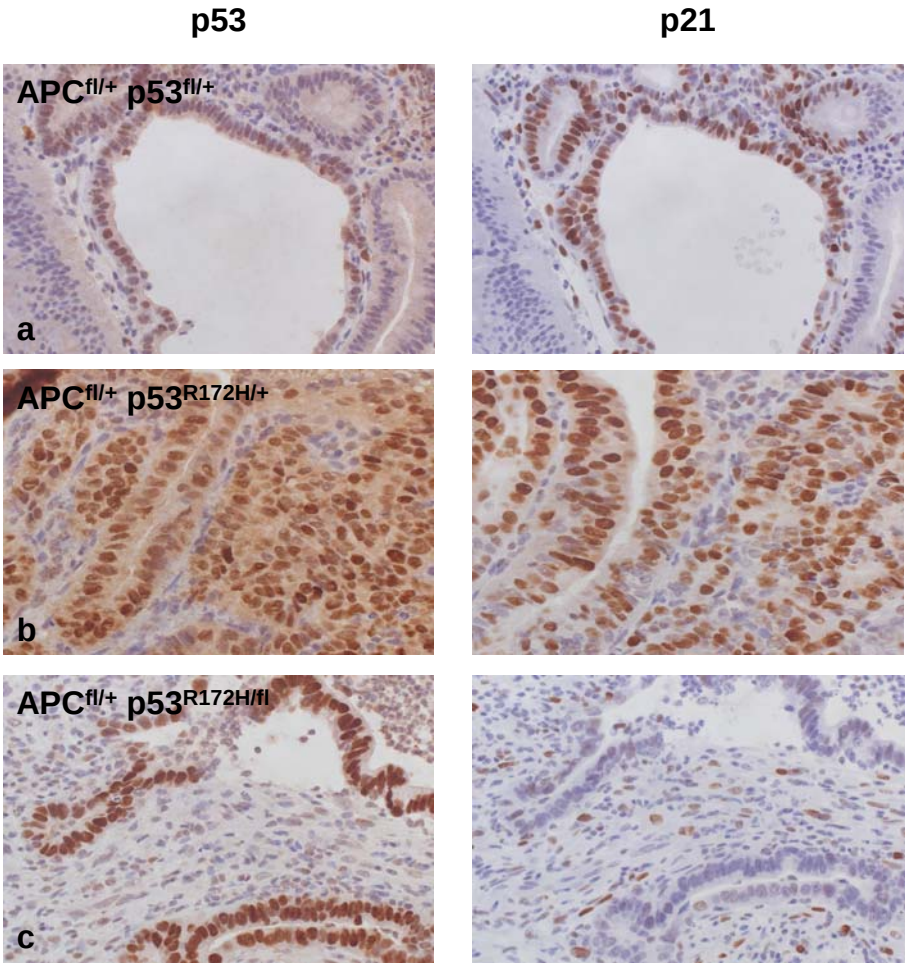


Figure 5.8: p21 is increased in APC$^{fl/+}$ p53$^{R172H/+}$ tumours following irradiation

To confirm the earlier result showing that p21 is upregulated along with p53 in APC$^{fl/+}$ p53$^{R172H/+}$ tumours a subset of mice were treated with 14Gy irradiation. Tumours from the APC$^{fl/+}$ p53$^{fl/+}$ mice showed increased staining with both p53 and p21 (a), while tumours from the APC$^{fl/+}$ p53$^{R172H/fl}$ tumours mice showed high p53 but no p21 (c), again showing that the point mutant cannot activate transcription of p21. As with the earlier result in the APC$^{fl/+}$ p53$^{R172H/+}$ tumours p53 and p21 were both seen (b) again indicating the presence of functional p53 in these tumours.

5.2.4 Levels of β–catenin and Wnt target genes are highest at invasive fronts

As mentioned previously, there is debate over the levels of Wnt signalling in human CRC, as despite *APC* mutation, nuclear β-catenin only appears to be localised at the leading edges of tumours (Brabletz, Jung et al. 2001). This has been described as the β-catenin paradox. In our previous studies we have found that both adenomas and invasive carcinomas that develop within *AhCre APC*$^{fl/+}$ mice have nuclear β-catenin. To investigate whether the levels of β-catenin and downstream Wnt target genes are altered by *p53* mutation we first stained for levels of β-catenin by IHC. There was a marked increase in levels of β-catenin (both in the nucleus and the cytoplasm) at the invasive fronts of tumours, in a manner analogous to human CRC. Interestingly, using our IHC protocol we still detect clear nuclear positivity throughout the tumours (Figure 5.9).

To test if this increase in β-catenin was functionally active, we performed IHC for a subset of Wnt targets. We were able to show markedly higher levels of c-Myc and Sox9 at the invasive fronts by IHC, and of the Wnt target/stem cell marker LGR5 by in-situ hybridisation (ISH). Once again, these Wnt target genes are still expressed in the centre of tumours though expression is higher at the leading edge (Figure 5.9).

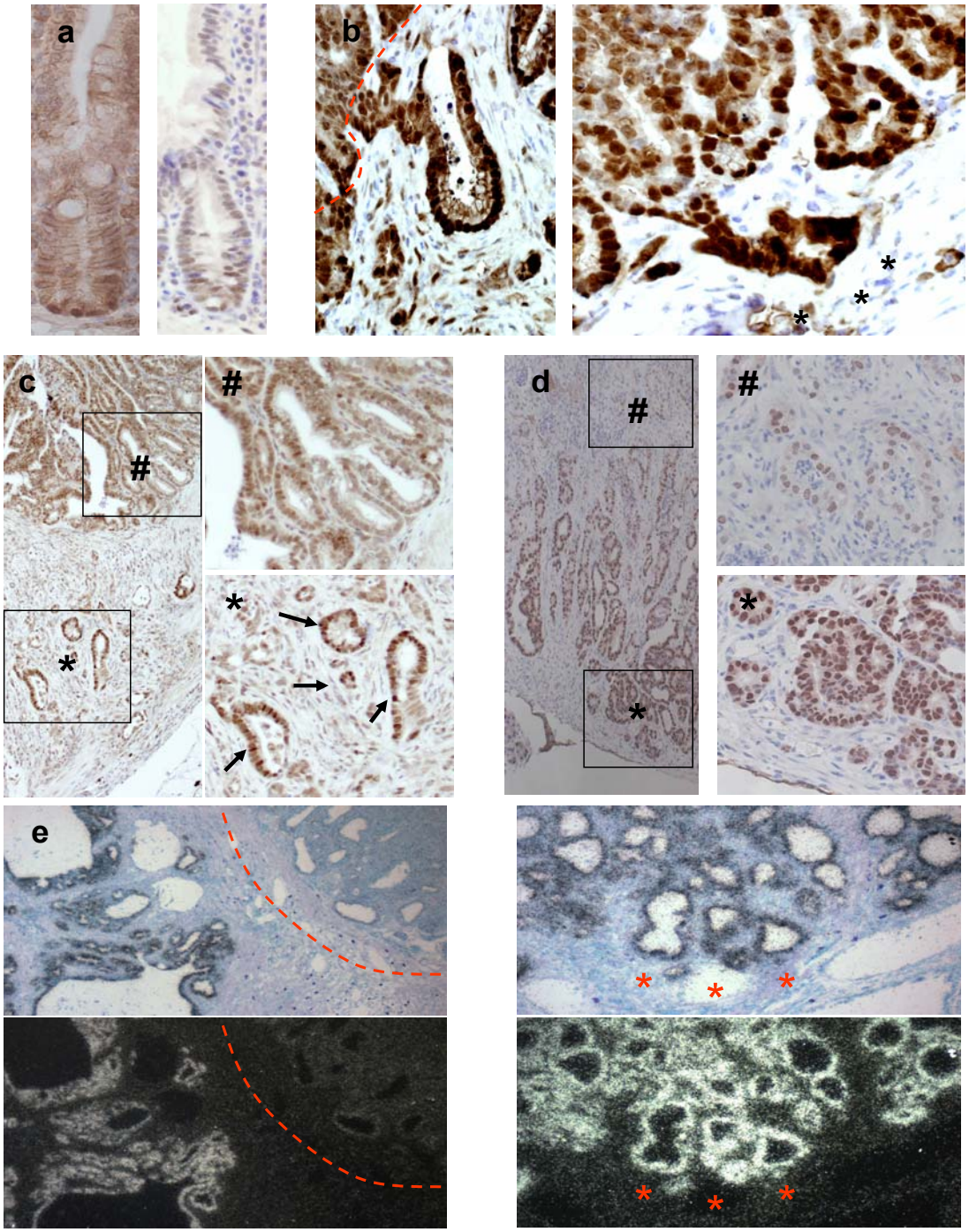


Figure 5.9: β-catenin and Wnt targets are increased at the invasive front

In the normal intestine β-catenin can be seen at cell membranes (a), in the tumours we noted that although nuclear β-catenin could be seen throughout the tumour it was highest at the invasive front (b). To confirm that this increase in nuclear β-catenin was functional we stained for Wnt targets and saw increases in c-Myc (c) and Sox9 (d) were increased at the invasive front (*) compared to the centre of the tumour (#). We also stained for the Wnt target and intestinal stem cell marker LGR5 by ISH (e). Staining could seen in the invasive component of the tumour (e, left panels, below the line) but not in the adenomatous component (e, left panels, above the line). Staining was most intense at the invasive front (e, right panels).

5.2.5 Fascin, pERK and ARF levels are highest at the invasive edge

From previous microarray studies in our laboratory, we found that a number of Wnt targets such as *Cyclin D1* and *Fascin* are not immediately deregulated following APC loss (Sansom, Meniel et al. 2007). This is consistent with human CRC where both have been associated with activation at the invasive front of tumours and a poor prognosis (Jung, Schrauder et al. 2001; Vignjevic, Schoumacher et al. 2007; Ogino, Nosho et al. 2009). To assess if these higher levels of β-catenin signalling were driving expression of a wider repertoire of targets we examined a number of these by IHC. Fascin and pERK, both of which can promote invasion (Pollock, Shirasawa et al. 2005; Vignjevic, Schoumacher et al. 2007), were upregulated at the invasive front of tumours (Figure 5.10).

Studies have shown that increased levels of c-Myc lead to heightened ARF activity, which, in the presence of functional p53 leads to apoptosis (Zindy, Eischen et al. 1998). IHC for $p19^{ARF}$ showed a dramatic upregulation at the invasive edge of tumours from the $APC^{fl/+}$ $p53^{R172H/+}$, $APC^{fl/+}$ $p53^{fl/fl}$ and $APC^{fl/+}$ $p53^{R172H/fl}$ cohorts (Figure 5.11) but not $APC^{fl/+}$ $p53^{+/+}$ tumours (Figure 5.11).

To show that this was specific to invasive carcinomas lacking p53, we stained invasive carcinomas from $APC^{fl/+}$ $PTEN^{fl/fl}$ mice. Tumours from these mice failed to show any upregulation of Myc, ARF or pERK at the leading edge, and instead showed massive upregulation of pAkt (Figure 5.12).

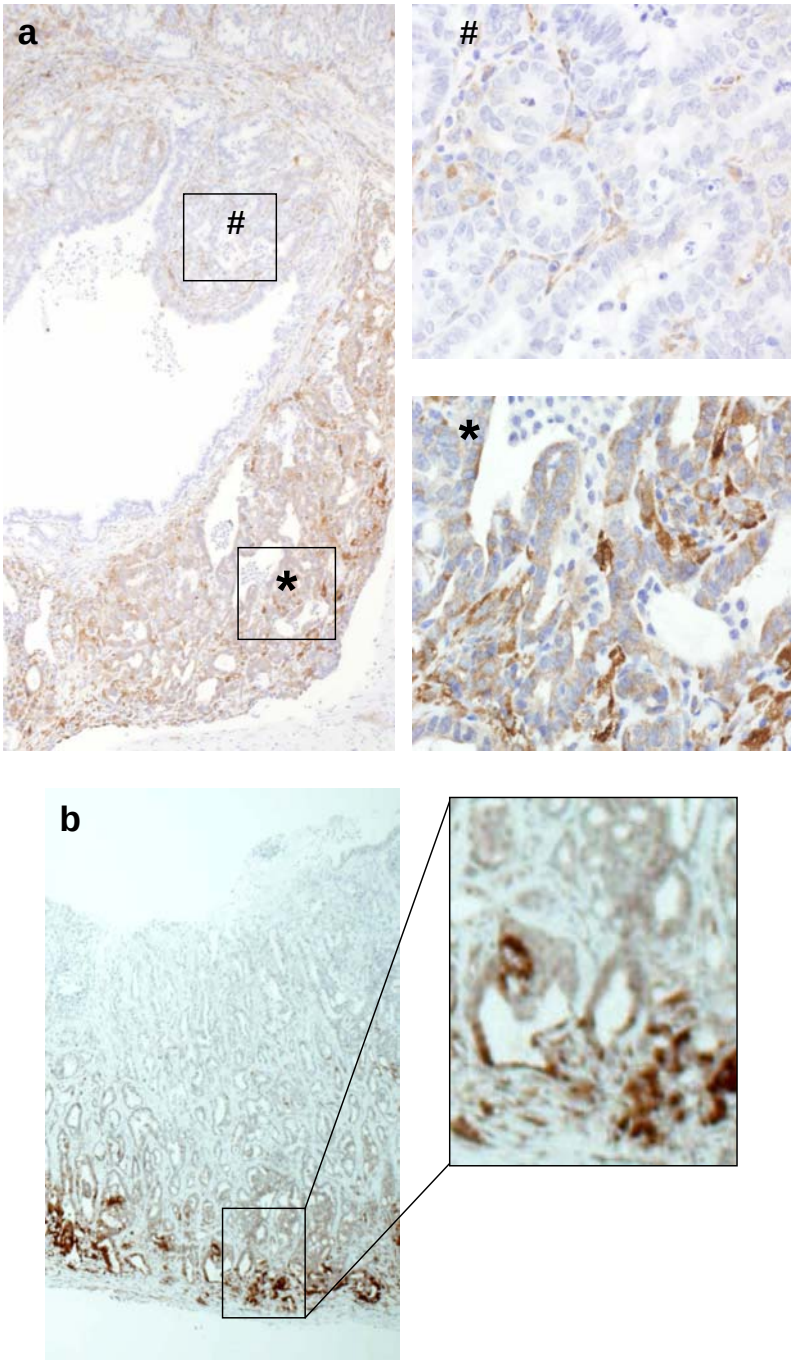


Figure 5.10: Fascin and pERK are increased at the invasive front

Both fascin (a) and pERK (b) showed specific upregulation at the invasive front of tumours. In the case of fascin there is virtually no staining seen in the tumour cells at the centre of the tumour (a, #), while clear cytoplasmic staining can be seen in the cells at the invasive front (a, *). Similarly in the case of pERK (b) the low power view shows clear selective upregulation at the invasive front, the enlargement shows that the staining is both cytoplasmic and nuclear.

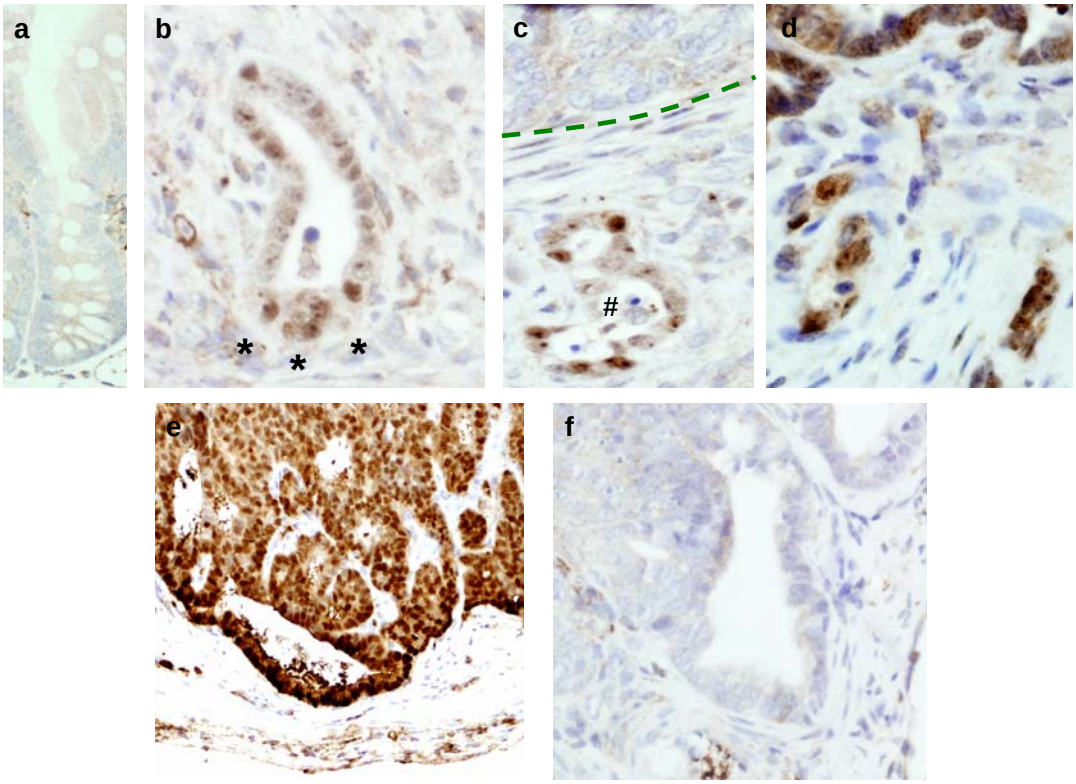


Figure 5.11: ARF is upregulated specifically at the invasive front

In normal cells ARF is undetectable (a). However in invasive tumours ARF was seen staining in a characteristic nucleolar pattern in tumour cells and glands specifically at the invasive front (b). In the centre of tumours (c, above line) although many prominent nucleoli can be seen no ARF staining is present, while in the adjacent tumour gland form the invasive front most of the cells show nucleolar staining (c, #). Tumour buds (small groups of cells separate from the main tumour mass) ARF staining was particularly prominent (d). In contrast in the few invasive tumours that developed in $APC^{fl/+}$ $p53^{+/+}$ mice although there was an upregulation of β-catenin at the invasive front (e) no upregulation of ARF was seen (f).

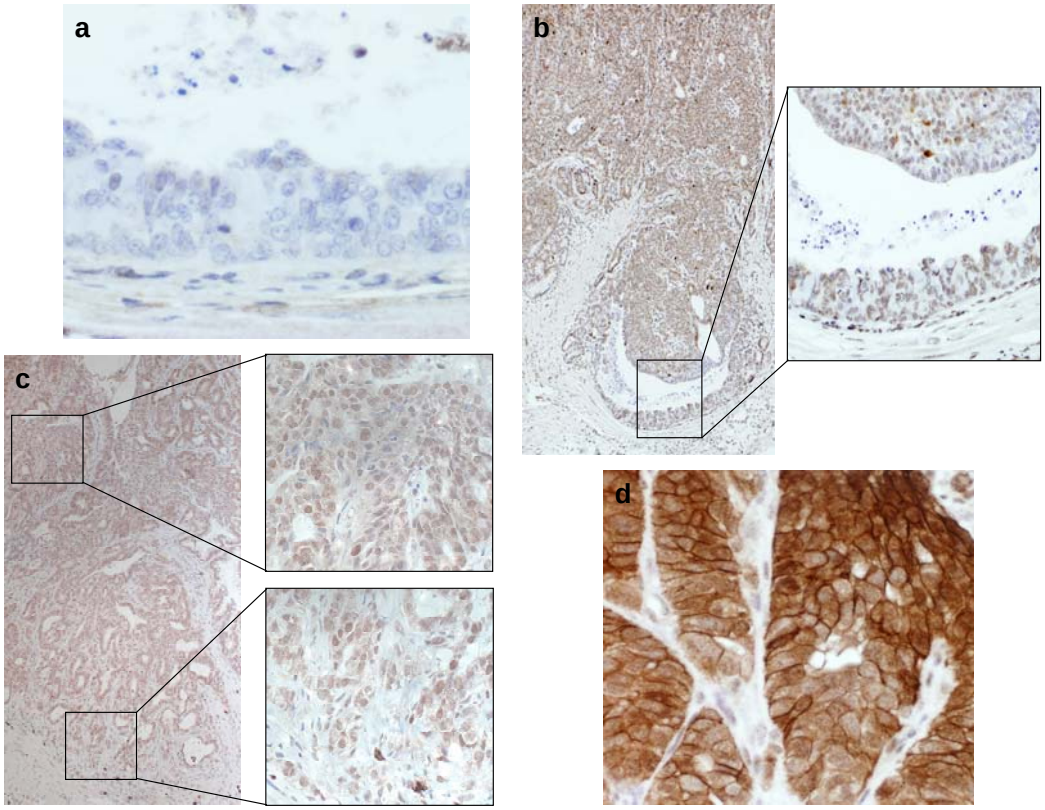


Figure 5.12: ARF is not upregulated at the invasive front of APC$^{fl/+}$ PTEN$^{fl/+}$ tumours

To see if the increase in ARF and Wnt targets at the invasive front was specific to the model studied, sections of invasive tumours from APC$^{fl/+}$ PTEN$^{fl/+}$ mice were stained for ARF, pERK and c-Myc. These tumours did not show any detectable ARF (a) and levels of pERK (b) and c-Myc (c) showed no appreciable difference between the tumour centre and invasive front. Instead these tumours showed high levels of pAKT (d) indicating strong activation of the PI-3Kinase pathway.

5.2.6 p14/19^{ARF} as a potential regulator of invasive tumours in the absence of p53.

Unlike many human cancers, the *CDKN2A* locus encoding the *p16^{INK4A}* and *p14^{ARF}* gene products is neither mutated nor epigenetically inactivated in the majority of sporadic CRC (Burri, Shaw et al. 2001; Konishi, Shen et al. 2009). Indeed previous studies have shown that p16 is expressed at the invasive front of human CRC and may correlate with a poor prognosis (Wassermann, Scheel et al. 2009). These findings suggested that this distinctive upregulation of ARF at invasive edges, rather than simply marking deregulated c-Myc, may play a functional role. A previous study has suggested that ARF may have tumour promoting functions in the absence of p53 (Humbey, Pimkina et al. 2008) despite the fact that it is mainly seen as a tumour suppressor.

Therefore we wished to test whether ARF can confer a selective advantage in the absence of p53. To assess this, we transfected *p53$^{-/-}$* HCT116 cells with either p14^{ARF} or vector control, and grew the cells under identical conditions of serum starvation. The expression of p14^{ARF} was confirmed by western blot analysis (Figure 5.13). Cells in the control group showed a dramatic drop in number between days 3-5, with a slight recovery by day 7. In the p14^{ARF} group the drop in cell numbers was much less and they also made a stronger recovery, so that by day 7 there was a statistically significant increase in cell numbers over controls, indicating that upregulation of p14^{ARF} may confer a survival advantage in *p53$^{-/-}$* cells (Figure 5.13). To test this more rigorously we then assessed if p14^{ARF} *p53$^{-/-}$* HCT116 cells had a growth advantage *in vivo.* To do this we injected stably transfected cell lines subcutaneously in nude mice and aged the mice until tumours reached 1.7cm in size. We found that the p14^{ARF} tumours

grew much more rapidly than vector controls (Figure 5.13, Log-Rank p=0.003). The resulting tumours were examined for invasive potential. 3/6 tumours from the $p14^{ARF}$ group resulted in skin ulceration, requiring early euthanasia, before they could reach 1.7cm. The remaining tumours demonstrated invasion and adherence to the body wall and ribcage that was not seen in any of the controls. This body wall invasion was confirmed microscopically, where it was also seen that the tumours displayed poorly defined edges and both vascular and perineural invasion. In contrast, control tumours were well circumscribed and did not invade the muscle of the body wall (Figure 5.13).

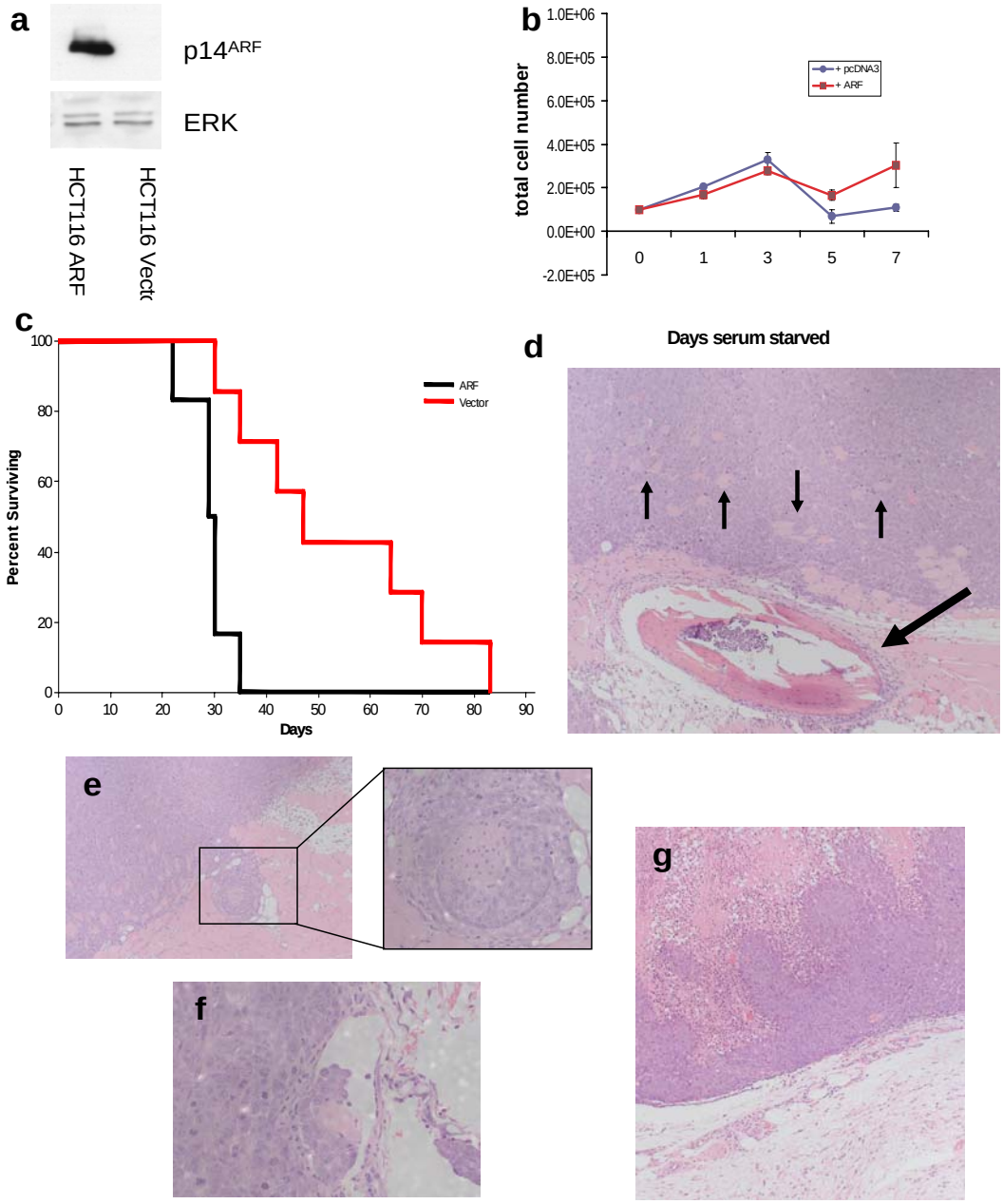


Figure 5.13: ARF promotes cell viability in p53$^{-/-}$ HCT116 cells

To determine if ARF was playing a functional role at the invasive edge p53$^{-/-}$ HCT116 cells were transfected with exogenous ARF (referred to as ARF cells) or an empty vector control (referred to as control cells). The presence of ARF in the cells was confirmed by western blot analysis (a). After 7 days of serum starvation ARF cells showed a statistically significant increase in survival compared to controls (b). To confirm this result both ARF and control cells were injected into the flank of nude mice, which were culled when tumours reached 1.7cm. The ARF cells grew significantly more rapidly, resulting in a shorter lifespan for these mice (c). In addition to growing more rapidly the tumours formed by the ARF cells demonstrated marked invasion of the body muscle wall (d, small arrows) in some cases reaching the ribs (d, large arrow). These tumours also demonstrated perineural (e) and vascular (f) invasion. In contrast the tumours formed by the control cells were well circumscribed and did not invade (g).

As we have suggested that the increase in ARF levels may play a role in the development of invasion, we went on to examine the effect of increased ARF levels in *p53*$^{-/-}$ cells in an *in vitro* invasion assay. *p53*$^{-/-}$ HCT116 cells transfected either with p14^{ARF} or vector control were seeded as a monolayer on the surface a matrix, consisting of rat tail collagen and human fibroblasts. An EGF gradient across the matrix encouraged cells to invade. Both the p14^{ARF} and vector control cells invaded into the matrix. However, the depth of invasion was almost doubled in the p14^{ARF} group compared with controls (Mean depth of invasion 198.80μm vs 102.00μm, Student's T-test $p<0.001$) (Figure 5.14). Furthermore, although both sets of cells invaded as groups, in many cases cells from the p14^{ARF} group were seen to break off into smaller groups at the invading edge (Figure 5.14). To ensure that this difference was not due merely to an increase in proliferation Ki-67 staining was performed and showed no difference in the proliferative index between the two groups (43% vs 37%, Chi-square $p=0.87$).

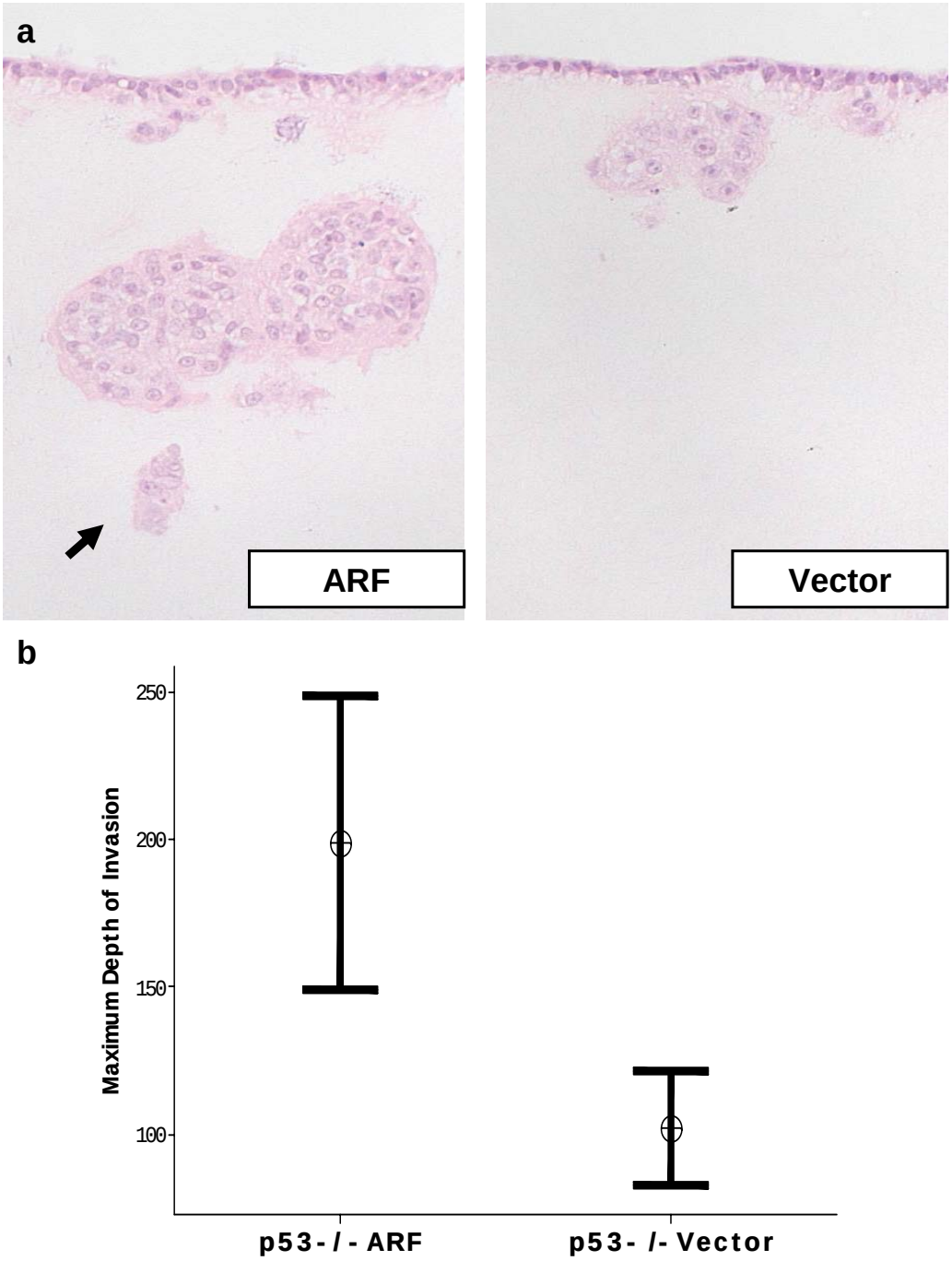


Figure 5.14: ARF promotes invasion in an organotypic invasion assay

HCT116 $p53^{-/-}$ cells stably transfected with ARF (a, left panel) invaded deeper into the matrix than identical cells transfected with vector control (a, right panel). Although both the ARF and control cells invaded as groups, in the case of the ARF over-expressing group, small groups of cells were seen to break off from the main group in many cases (d, left panel, arrow), similar to the tumour budding seen in invasive tumours *in vivo*. This phenomenon was not seen in the control cells. The increase in maximal depth of invasion is quantified in b. The depth of invasion was nearly doubled in the ARF over-expressing cells (Student's T-test $p<0.001$).

5.3 Discussion

Although many excellent animal models of the early stages of CRC exist there is a recognised need for new paradigms, which recapitulate the later stages of the disease, particularly invasion and metastasis (Taketo and Edelmann 2009). In this study we have used mutations in *APC* and *p53*, 2 of the most commonly mutated genes in human CRC to develop a mouse model of CRC which invades in ~90% of cases and is capable of metastasis.

While invasive tumours consistently developed in both the $APC^{fl/+}$ $p53^{fl/fl}$ and $APC^{fl/+}$ $p53^{R172H/fl}$ cohorts, there was a dramatic difference between the $APC^{fl/+}$ $p53^{fl/+}$ and $APC^{fl/+}$ $p53^{R172H/+}$ cohorts, demonstrating the enhanced oncogenic effect of *p53* point mutation over loss in the setting of CRC. The fact that these 2 cohorts had almost identical lifespans and developed very similar numbers of tumours suggests that the difference is not in tumour initiation, but instead in tumour progression and invasion. The accumulation of mutant p53 at invasive fronts also supports this. This observation is in keeping with previous reports on the differences between *p53* deletion and mutation. Both Olive *et al* and Lang *et al* showed that in a model of Li-Fraumeni syndrome *p53* knockout mice and mice expressing mutant *p53* have identical lifespans but develop a different tumour spectrum. They also observed an increase in aggressiveness in the mutant *p53* group (Lang, Iwakuma et al. 2004; Olive, Tuveson et al. 2004). Other studies, in mouse models of both skin and pancreatic cancer have also shown a more aggressive phenotype associated with *p53* mutation (Caulin, Nguyen et al. 2007; Morton, Timpson et al. 2010).

Although it has long been known that *APC* and *p53* mutations co-exist in human CRC, this is the first time that this cooperation has been demonstrated in an animal model. Previous studies have examined the potential *in vivo* cooperation between *APC* and *p53* (Clarke, Cummings et al. 1995; Fazeli, Steen et al. 1997; Halberg, Katzung et al. 2000). In only one of these studies was a slight difference in tumour invasiveness demonstrated (Halberg, Katzung et al. 2000). However, the number of invasive carcinomas in that study was very small (only 4 were identified), and the effects observed were strain specific. Although our model utilises similar genetic events to these previous studies, we observed a dramatic difference in invasion. A possible explanation for this is the tumour burden in the different models. Previous studies used the Apc^{Min} mouse, which develops 100's of tumours and the animals succumb to the overall tumour burden rather than tumour progression. Consistent with this is the finding that adenomas from the Apc^{Min} mouse do not show CIN (Haigis, Caya et al. 2002). In our model the animals develop fewer tumours (median 20). This decreased overall tumour burden allows for the progression of individual tumours. Moreover, this is closer to the situation in humans, where only a small number of tumours initiate, allowing progression to occur.

While the invasive tumours occurred across 3 different genotypes, they were all remarkably similar. The tumours also resembled the human disease on a number of levels. Histologically the tumours demonstrated variation in depth of invasion, with tumours ranging from superficially invasive, through to tumours that resulted in bowel perforation and extension into surrounding organs. These tumours also demonstrated the capacity to metastasise, although the fact that only one metastasis was identified in the study, indicates that other somatic events are necessary before this occurs.

At the molecular level the tumours also demonstrated similarities. Firstly we saw evidence of increased CIN in the tumours arising from the $APC^{fl/+}$ $p53^{R172H/+}$, $APC^{fl/+}$ $p53^{fl/fl}$ and $APC^{fl/+}$ $p53^{R172H/fl}$ cohorts, compared to those from the $APC^{fl/+}$ $p53^{+/+}$ and $APC^{fl/+}$ $p53^{fl/+}$cohorts. As it has been shown that CIN can contribute to tumour development (Weaver and Cleveland 2007), this would suggest that this increase in CIN may contribute to the increase in invasiveness seen. We went on to look at ZEB1 and E-cadherin, 2 known markers of EMT and showed that as with human tumours, there was an increase in ZEB1, with a reciprocal decrease in E-cadherin levels at the invasive front of the tumour. This suggests that many of the same processes which promote invasion in human CRC are also active in the invasive tumours from this model.

As well as increases in EMT markers at the invasive edge of tumours, we also saw a robust increase in nuclear β-catenin staining. This is similar to previous reports demonstrating high nuclear β-catenin at the invasive front of human CRC (Brabletz, Jung et al. 2001). However in that study the authors did not see nuclear β-catenin in the middle portion of tumours, whereas we saw staining throughout, with a marked increase at the invasive front. We also showed that the Wnt targets c-Myc and Sox9 were present throughout the tumours with the most intense staining seen at the invasive front, mirroring β-catenin. This may go some way to addressing the "β-catenin paradox" and indicates 2 thresholds of Wnt activity in tumours, the first which initiates adenoma formation and a second stronger Wnt activation, which promotes invasion. To support this we have subsequently stained human CRC for β-catenin and have been able to demonstrate nuclear staining throughout the tumour, with increased intensity at the invasive front. This also fits with previous studies from our laboratory, where we see Wnt targets such as *c-Myc*, *CD44* and *Cyclin D2* deregulated immediately

following APC loss (Sansom, Meniel et al. 2007) and with microarray studies from human tumours showing increased levels of Wnt target gene expression (Sabates-Bellver, Van der Flier et al. 2007; Van der Flier, Sabates-Bellver et al. 2007).

Two pro-invasive proteins were also found to be upregulated at invasive fronts. These were fascin, which is a key actin regulator and has been implicated in invasion (Vignjevic, Schoumacher et al. 2007), and pERK, which is known to play a pro-migratory and invasive role (Pollock, Shirasawa et al. 2005), but interestingly is not upregulated following APC loss, even in the presence of oncogenic KRas (Sansom, Meniel et al. 2006; Haigis, Kendall et al. 2008). This is interesting given that fascin has been shown to be upregulated at the invasive front of human CRC (Vignjevic, Schoumacher et al. 2007). The presence of pERK specifically at the invasive front of the tumour indicates activation of the MAPK pathway. It has been shown *in vitro* that *APC* deletion can activate ERK (Jeon, Yoon et al. 2007; Kim, Rath et al. 2007), however as mentioned above studies *in vivo* have shown that that ERK is not activated following *APC* deletion and activation of *KRas* (Sansom, Meniel et al. 2006; Haigis, Kendall et al. 2008). This suggests that the additional loss of p53 function permits activation of the MAPK pathway at the invasive front, allowing it to exert its pro-invasive effect.

The final protein that we found upregulated at the invasive front was ARF. At first glance this was somewhat surprising, given that ARF is generally considered a tumour suppressor. However it makes sense when one considers the pathways that we have shown to be active at the invasive front. It is known that high levels of c-Myc will activate ARF (Zindy, Eischen et al. 1998). This is an important tumour suppressor mechanism as in a normal cell this increase in ARF

promotes the stabilisation of p53 and a selective survival disadvantage (Zindy, Eischen et al. 1998), preventing the establishment of an invasive front. Indeed, this has been shown to occur in a mouse model of lymphomagenesis (Zindy, Williams et al. 2003). However in the absence of functional p53, this cannot happen and the cell derives a selective growth advantage through upregulated β-catenin and c-Myc, allowing the development of the invasive front.

We then went on to demonstrate that this increased level of ARF is not just marking aberrant activation of c-Myc but instead appears to have a functional role, promoting both cell survival and invasion in the absence of p53. To our knowledge this is the first report of ARF acting in a pro-invasive role in a solid tumour. However, there are some recent data supporting a tumourigenic role for ARF. Humbey *et al* reported that ARF can support tumour development in a mouse model of B-cell lymphoma, possibly by promoting autophagy in the tumour cells (Humbey, Pimkina et al. 2008). Intriguingly, this B-cell lymphoma model is driven by overexpression of Myc and mutation of *p53* (events reflected in our model), however, the authors did not observe the same effect of ARF when studying different oncogenic drivers (Humbey, Pimkina et al. 2008). The mechanism by which ARF promotes invasion is not clear; however, data from Herkert *et al* suggest a possibility. They have shown that in response to oncogenic stress ARF can promote formation of a Myc/Miz complex. This complex represses transcription of genes involved in adhesion. Functionally this resulted in cell detachment and subsequent apoptosis in p53 proficient cells. However, when apoptosis was inhibited the reduction in cell adhesion persisted (Herkert, Dwertmann et al. 2010). In our system this loss of the cell adhesion signature could promote invasion and the formation of "tumour buds" (which we witnessed both *in vitro* and *in vivo*), with the absence of p53 abrogating

subsequent apoptosis and allowing survival of the detached cells. The fact that we see EMT markers (including decreased E-cadherin expression) at the invasive front is consistent with this hypothesis.

A small number of mouse models of invasive intestinal carcinoma exist in the literature (Hung, Maricevich et al.; Sansom, Meniel et al. 2006; Marsh, Winton et al. 2008; Trobridge, Knoblaugh et al. 2009), so it is important to place the current model in context. Sansom *et al* reported superficial invasion upon mutation of *Apc* and expression of oncogenic *KRas* (Sansom, Meniel et al. 2006). Subsequently, full thickness invasion has been seen in tumours possessing *Apc* and *Pten* mutations (Marsh, Winton et al. 2008). Metastasis did not occur in either of these models. A recent model has been published which is capable of metastasising. In this model the tumours developed on a background of *TGFβR* mutation, in a Wnt independent fashion (Trobridge, Knoblaugh et al. 2009). This probably best represents a model of progression along the "serrated pathway", in which *TGFβR* mutation is common and *APC* mutation less common than in CRC as a whole. Tumours that arise along this route frequently occur on a background of microsatellite instability. Another, very recent study has overcome many of these issues, by combining *Apc* and *KRas* mutations in the distal colon. Tumours from this model showed invasion and occasional metastasis (Hung, Maricevich et al. 2010). Consistent with the activation of KRas the authors saw an upregulation of pERK in the tumours. The current model also generates deeply invasive tumours and metastasis. It is interesting to note that although we have deleted p53 rather than activating KRas we also see pERK upregulation, suggesting that this is an important event in the development of invasive carcinoma.

In summary, in this study we have demonstrated that *APC* and *p53* mutation, which are two of the most common genetic events in human CRC, can co-operate to strongly promote an invasive phenotype. This yields a novel mouse model of CRC which resembles the human disease at both the histological and molecular levels. We show that heterozygous point mutation is a more potent inducer of tumour invasion than heterozygous *p53* deletion. Finally, we present evidence for a mechanism for the increase in invasion seen in the setting of *p53* loss, based on a novel pro-invasive role for ARF.

Chapter 6 *p53* mutation and knockout have differing effects in a novel model of rhabdomyosarcoma

6.1 Introduction

The results of chapter 5, demonstrating a clear difference in the effect of *p53* deletion and mutation led us to study this effect in another system. We used a mouse model of pleomorphic rhabdomyosarcoma in which an oncogenic *KRas* mutant is co-expressed with either *p53* deletion or mutation.

Rhabdomyosarcoma is the most common soft tissue malignancy of childhood (Xia, Pressey et al. 2002), but is rarer in adults (Cormier and Pollock 2004). Three histological subtypes are recognised; Embryonal, Alveolar and Pleomorphic. The former 2 are most commonly associated with paediatric cases and have a relatively favourable prognosis, with overall survival approaching 80% (Ruymann and Grovas 2000). Pleomorphic rhabdomyosarcoma is seen largely in adults and the incidence increases with age (Simon, Paulino et al. 2003). Rhabdomyosarcoma in adults is associated with a worse prognosis, with a 5-year survival approximately 27-52% (Stock, Chibon et al. 2009; Sultan, Qaddoumi et al. 2009). Moreover, among adults the pleomorphic variant is associated with the worst prognosis (Sultan, Qaddoumi et al. 2009). Animal models of this variant would therefore be valuable, both in understanding the basic biology of the disease, and in the development of novel approaches to management.

The *KRas* oncogene has been implicated in a wide variety of human neoplasms, with up to one third of rhabdomyosarcomas displaying activation of one of the three Ras isoforms, including KRas (Stratton, Fisher et al. 1989; Wilke, Maillet et al. 1993; Garcia, Gonzalez et al. 2000). Similarly, the loss of function of the *p53* tumour suppressor protein has been shown to be a vital event in the progression of many human malignancies, including rhabdomyosarcoma (Felix, Kappel et al.

1992; Wexler and Helman 1994; Naini, Etheridge et al. 2008). Indeed this was the tumour that was initially investigated by Li and Fraumeni in studies which led to the characterisation of the eponymous syndrome (Li and Fraumeni 1969).

It has been shown that mutation and activation of Ras alone may not be enough to induce tumour formation, as the growth promoting effects can be counter-balanced by protective elements in the cell (Serrano, Lin et al. 1997). These mechanisms, which are activated following the activation of Ras, can send the cell into a state of growth arrest and thus prevent tumour formation and are dependent upon the activities of tumour suppressor proteins such as RB and p53 (Courtois-Cox, Jones et al. 2008).

In this study we demonstrate, through a novel mouse model of pleomorphic rhabdomyosarcoma, that oncogenic *KRas*^{G12V} can co-operate with *p53* loss in tumour development. Moreover, we show that a point mutation in the DNA binding domain of *p53* is a more potent activator of tumourigenesis and promoter of metastasis than simple *p53* loss. This supports data showing a role for mutant p53 over and above simple loss of its normal function.

6.2 Results

6.2.1 AhCre is active in skeletal muscle

The use of Cre-LoxP technology has allowed for a much more tissue specific approach to animal model studies. This is achieved by the use of tissue specific promoters to control Cre expression. *AhCre*, under the control of the *Cyp1A1* promoter was originally described as showing inducible expression in the small intestine, liver and colon (Ireland, Kemp et al. 2004). However sporadic expression (in the absence of Cyp1A1) has been seen in other organs, including the kidney (Sansom, Griffiths et al. 2005). In order to examine other potential sites of *AhCre* activity, mice expressing AhCre were crossed to mice expressing the conditional Z/EG GFP transgene. The GFP is preceded by a LoxP flanked β-geo insert, which is excised in the presence of Cre-recombinase. Thus the GFP is expressed only in tissues in which the Cre-recombinase is active (Novak, Guo et al. 2000). Mice that expressed both the *AhCre* and the GFP protein were euthanized and imaged using the OV-100 imaging system. GFP expression was seen throughout skeletal muscle and also in the cerebellum (Figure 6.1). Given this expression profile, *AhCre* is a useful tool for studying tumourigenesis in the skeletal muscle.

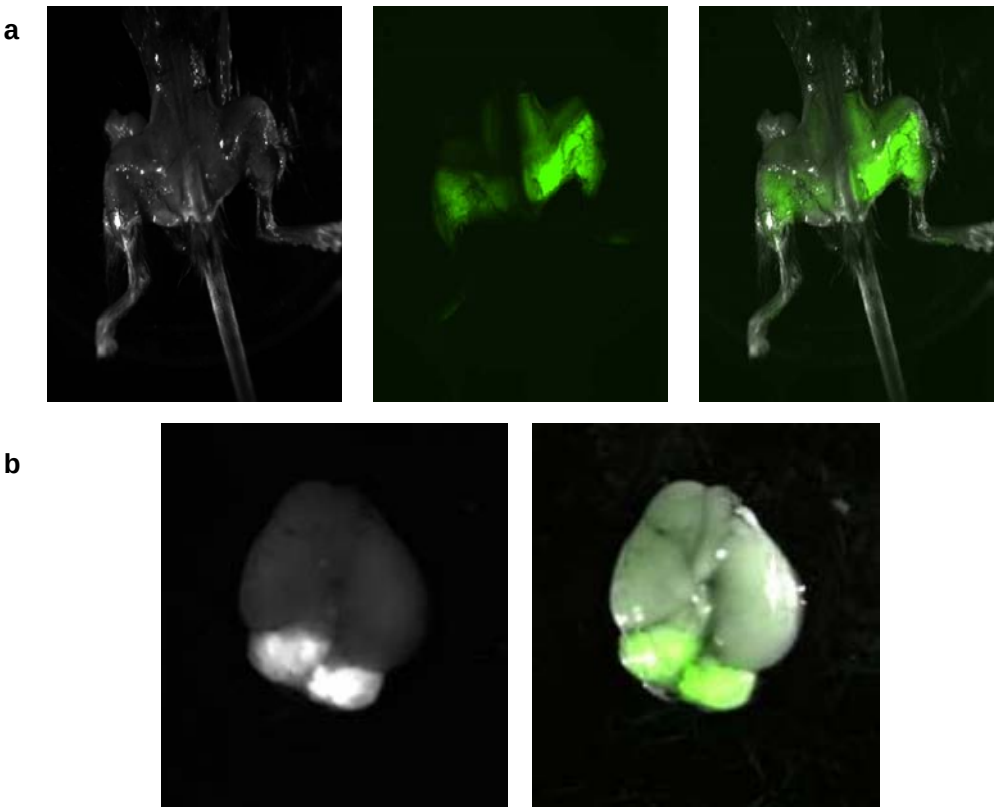


Figure 6.1: AhCre is active in skeletal muscle and in the cerebellum

Mice expressing the AhCre and a Z/EG GFP transgene were imaged using the Olympus OV100. As the GFP is preceded by a STOP cassette, signal will only be seen in organs in which the AhCre is active. Clear GFP positivity was seen in both the skeletal muscle (a) and in the cerebellum (b).

6.2.2 The combination of oncogenic *KRas^{G12V}* and loss of p53 activity accelerates tumour formation

Mice expressing *AhCre* were crossed to mice expressing the *KRas^{G12V}* allele under the control of the endogenous *KRas* promoter (See chapter 4.2.5). The *KRas^{G12V}* allele is linked bicistronically to a β-galactosidase reporter, which allows for identification of those tissues which express the oncogenic *KRas* allele. These mice were crossed to either *p53$^{fl/+}$* mice or to *p53$^{R172H/+}$* mice (see chapter 5.2.1). The breeding strategy yielded four experimental cohorts; all of which expressed the mutant *KRas^{G12V}* allele and the four cohorts were made up of mice expressing one wild type *p53* allele with loss of the second allele (*KRas$^{G12V/+}$ p53$^{fl/+}$*), one wild type *p53* allele and one mutant *p53* allele (*KRas$^{G12V/+}$ p53$^{R172H/+}$*), loss of both *p53* alleles (*KRas$^{G12V/+}$ p53$^{fl/fl}$*) and one mutant *p53* allele, with loss of the second allele (*KRas$^{G12V/+}$ p53$^{R172H/fl}$*).

There was a marked difference in lifespan between the four cohorts (Figure 6.2). The shortest lifespan was in the *KRas^{G12V} p53$^{fl/fl}$* mice and the *KRas^{G12V} p53$^{R172H/fl}$* groups, with a median lifespan of 48 and 51 days respectively. The *KRas^{G12V} p53$^{R172H/+}$* group had a statistically significantly longer lifespan, with a median of 62 days (Log-Rank p=0.001). The *KRas^{G12V} p53$^{fl/+}$* had the longest lifespan with a median of 112 days, which was statistically significantly different to all of the other groups (Log-Rank p<0.001).

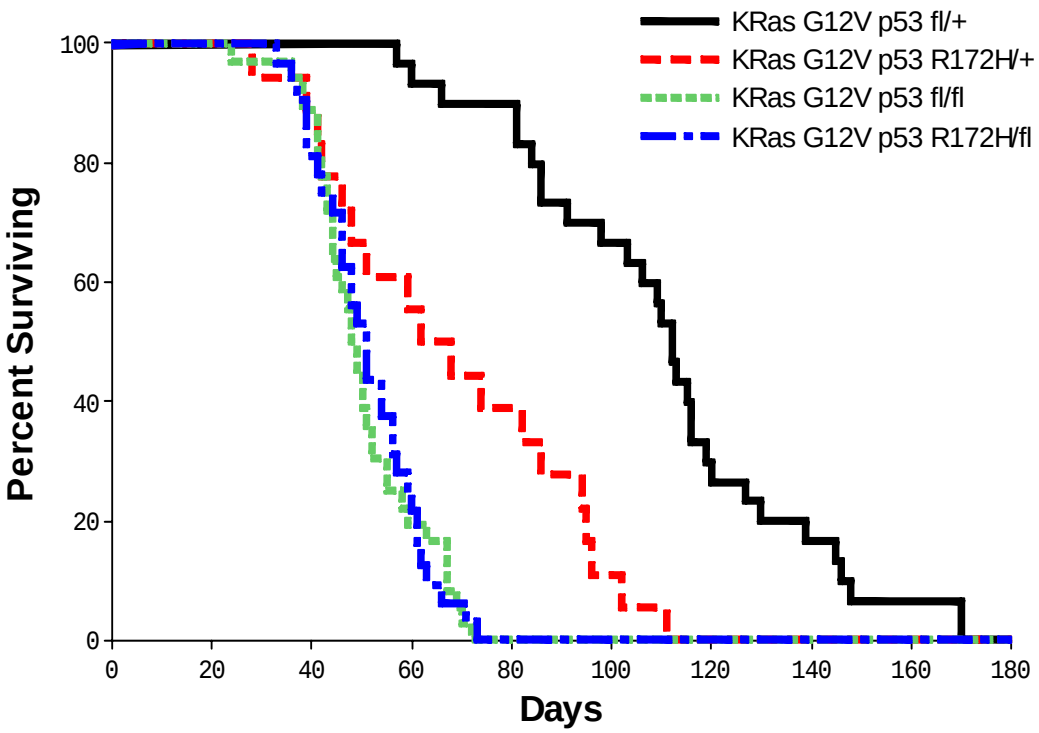


Figure 6.2: Kaplan-Meier survival analysis, stratified by genotype

There was a significant difference in survival between the $KRas^{G12V}$ $p53^{fl/+}$ (Black line) and the remaining 3 cohorts (Log-Rank p<0.001). The $KRas^{G12V}$ $p53^{R172H/+}$ (Red line) cohort also showed a significantly longer survival than either the $KRas^{G12V}$ $p53^{fl/fl}$ (Green line) or $KRas^{G12V}$ $p53^{R172H/fl}$ (Blue Line) cohorts (Log-Rank p=0.001).

The tumours that developed were predominantly on the limbs, with occasional tumours seen within the abdominal and thoracic cavity. On gross inspection the tumours were firm, well circumscribed masses (Figure 6.3). Areas of necrosis and haemorrhage were often seen. Histologically, the tumours were composed of a mixture of spindle shaped cells and bizarre, giant pleomorphic cells (Figure 6.3). A high mitotic rate was seen in all of the tumours. The tumour cells were seen to intermingle with normal skeletal muscle cells and appeared to originate from the skeletal muscle. Detailed inspection revealed the presence of cross striations and myotubes (Figure 6.3). This histological pattern was seen in the tumours from all three genotypes. IHC analysis of the tumour cells revealed positivity for both desmin and myogenin (Figure 6.3). The tumours were therefore classified as pleomorphic rhabdomyosarcoma.

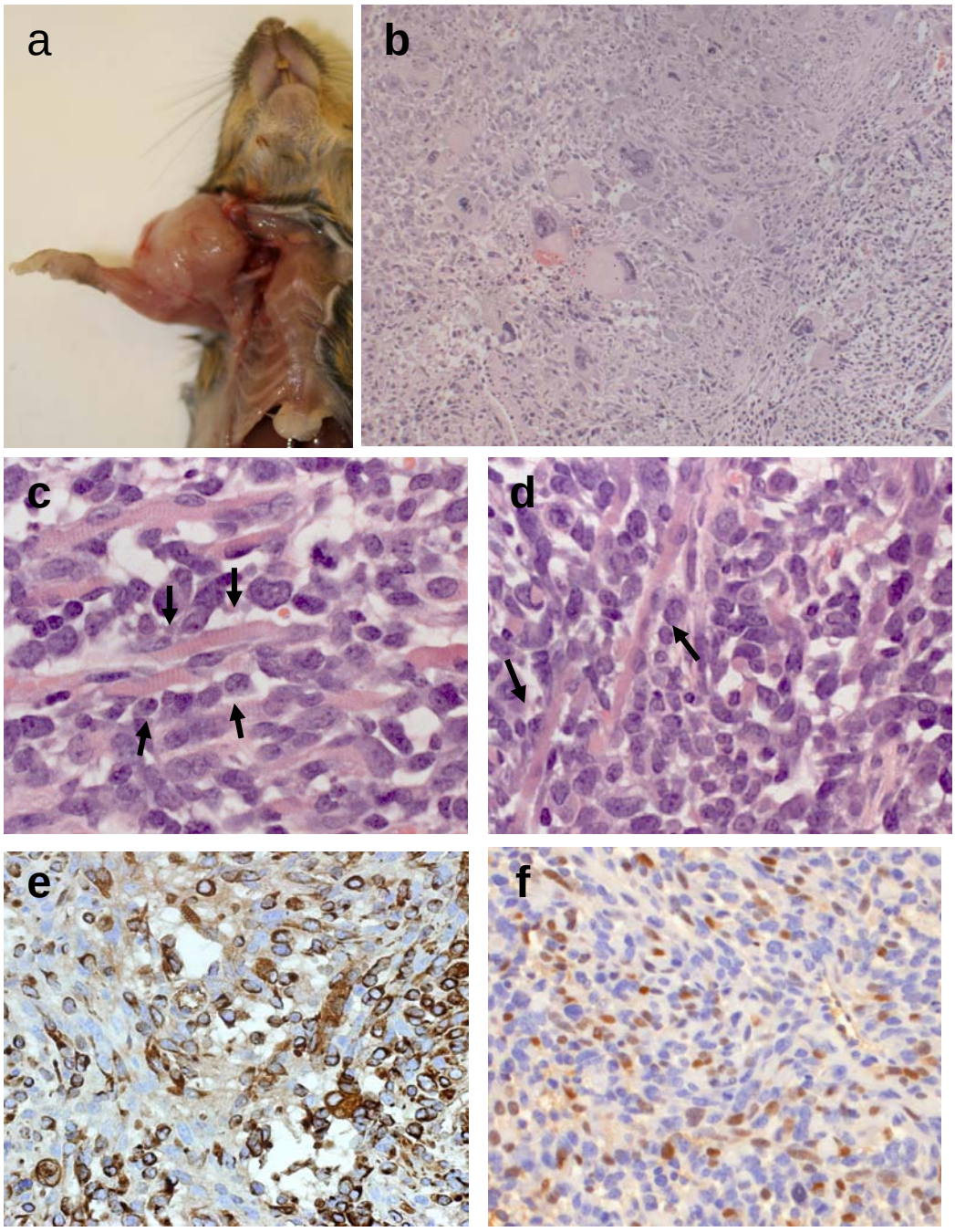


Figure 6.3: Point mutation or loss of function mutation results in the formation of pleomorphic rhabdomyosarcoma

The majority of the tumours developed from the muscles of the upper (a) or lower limbs, with occasional tumours developing in the abdominal or thoracic cavity. The tumours consisted of interlacing sheets of round to spindle shaped cells interspersed with large bizarre pleomorphic cells (b). Evidence of rhabdoid origin was seen in the presence of cross striations (c) and myotubes (d). Tumours from the $KRas^{G12V}$ $p53^{R172H/+}$, $KRas^{G12V}$ $p53^{fl/fl}$ and $KRas^{G12V}$ $p53^{R172H/fl}$ cohorts all showed this pleomorphic histology. Positive immunohistochemical staining for desmin (e) and myogenin (f) further supports the myogenic origin of the tumour.

6.2.3 Mutation but not loss of a single p53 allele is sufficient for tumour development

As well as having an almost identical lifespan, mice in the *KRas^{G12V} p53$^{fl/fl}$* and *KRas^{G12V} p53$^{R172H/fl}$* cohorts consistently developed rhabdomyosarcoma (Table 6.1). This indicates that deletion of both *p53* alleles (*p53$^{fl/fl}$*) has a similar effect on tumourigenesis as the mutation of a single allele, with concomitant deletion of the second *p53* allele (*p53$^{R172H/fl}$*). In contrast, there was a statistically significant difference in lifespan between mice in the *KRas^{G12V} p53$^{R172H/+}$* cohort and those in the *KRas^{G12V} p53$^{fl/+}$* cohort. This difference in lifespan coincided with a marked difference in tumour profile between the 2 groups (Table 6.1). 88% of mice in the *KRas^{G12V} p53$^{R172H/+}$* cohort developed rhabdomyosarcoma, compared to 6% in the *KRas^{G12V} p53$^{fl/+}$* cohort (Chi square $p<0.001$). The vast majority of mice in the *KRas^{G12V} p53$^{fl/+}$* cohort did not develop any tumours, despite their longer lifespan, and instead had to be euthanized due to ataxia. It has previously been demonstrated that ataxia may result from cerebellar defects in mice (Vogel, Caston et al. 2007). To test for cerebellar recombination, the brains of these animals were stained for β-galactosidase, which acted as a reporter for the presence of the mutant *KRas^{G12V}* allele. The staining was positive, specifically in the cerebellum, without staining in the rest of the brain. Brains from mice which had developed rhabdomyosarcoma did not show any staining (Figure 6.4). This is also in keeping with the GFP positivity previously seen in the cerebellum (Figure 6.1). Given that no cerebellar tumours developed in these mice, the exact cause for the ataxia is unclear. However, they do express the mutant KRas, which is constitutively active. In our previous studies we have aged *AhCre* positive *KRas^{G12V} p53$^{+/+}$* mice for up to a year without them

developing ataxia, this would suggest that both activation of Ras and p53 heterozygocity contribute to this phenotype.

Genotype	Number of Mice Developing Rhabdomyosarcoma (%)
KRas$^{G12V/+}$ *p53*$^{fl/+}$	2/31 (6%)
KRas$^{G12V/+}$ *p53*$^{R172H/+}$	14/16 (88%)
KRas$^{G12V/+}$ *p53*$^{fl/fl}$	15/16 (94%)
KRas$^{G12V/+}$ *p53*$^{R172H/fl}$	19/19 (100%)

Table 6.1: The incidence of rhabdomyosarcoma formation in the different genotypes studied.

Similar incidences were seen in the *KRas*$^{G12V/+}$ *p53*$^{R172H/+}$, *KRas*$^{G12V/+}$ *p53*$^{fl/fl}$ and *KRas*$^{G12V/+}$ *p53*$^{R172H/fl}$ cohorts. Mice in the *KRas*$^{G12V/+}$ *p53*$^{fl/+}$ cohort developed significantly fewer tumours than in any of the other cohorts (Chi square $p<0.001$).

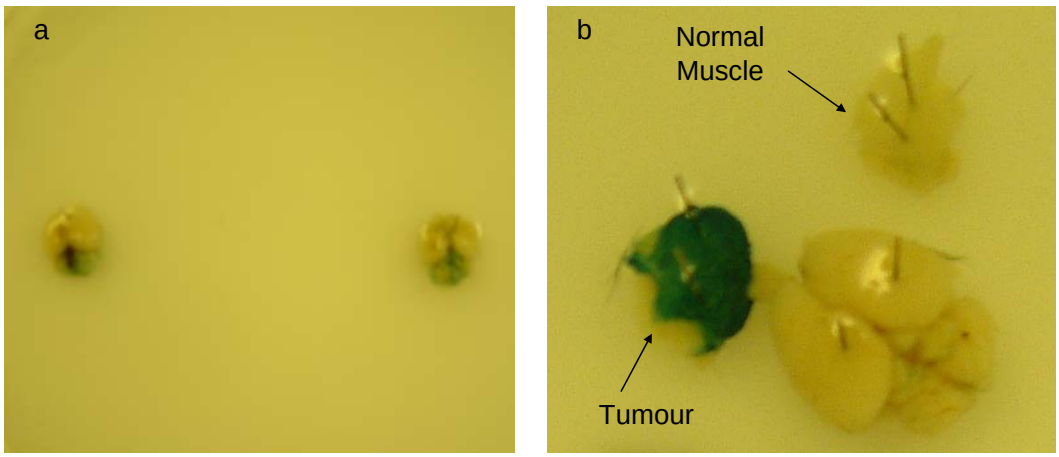
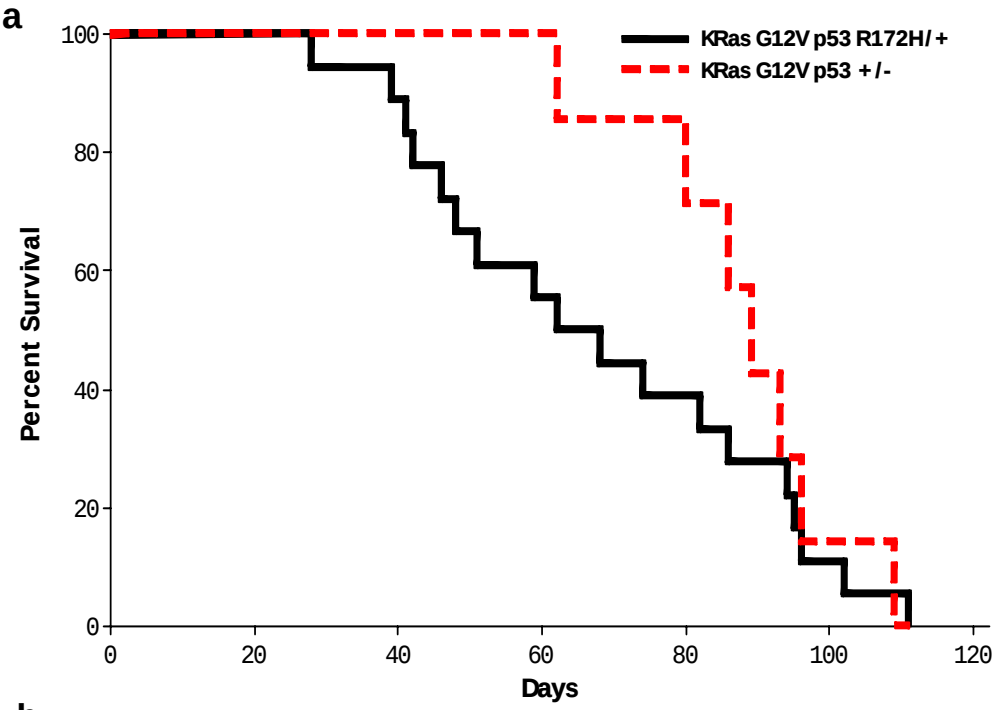


Figure 6.4: Mutant KRas is active in the cerebella of KRas^{G12V} p53$^{fl/+}$ mice

The great majority of mice from the KRas^{G12V} p53$^{fl/+}$ cohort did not develop tumours, instead they developed ataxia. Although no tumours could be detected in the cerebella of these mice, the positive LacZ staining (a) indicates that there is expression of the mutant Ras allele specifically in this area of the brain, while the cerebral hemispheres did not show any staining. No staining could be detected in the brains of the mice that developed tumours (b). A rhabdomyosarcoma and normal muscle are included as positive and negative controls respectively.

Given that the mutant *p53* allele is preceded by a STOP cassette and engineered into the endogenous locus, the mice in the *KRas*^{G12V} *p53*$^{R172H/+}$ cohort are essentially rendered *p53* heterozygote in the tissues where Cre is not expressed. To ensure that the phenotype that we observed was not due simply to this, we crossed *p53*$^{fl/+}$ mice to mice expressing a general Deletor Cre (Nagy 2000), which results in Cre recombinase activity in all tissues, thus generating *p53*$^{+/-}$ mice. These were then crossed to AhCre positive *KRas*^{G12V} mice to yield a *KRas*^{G12V} *p53*$^{+/-}$ cohort. These mice were aged until they developed any signs of ill-health. Despite having a longer median survival than the *KRas*^{G12V} *p53*$^{R172H/+}$ mice (Figure 6.5), only 1/7 mice from this cohort developed rhabdomyosarcoma. This difference in the incidence of rhabdomyosarcoma between the *KRas*^{G12V} *p53*$^{+/-}$ and *KRas*^{G12V} *p53*$^{R172H/+}$ cohorts was statistically significant (Chi-square p=0.002). The remainder of the mice died of various causes, including lymphoma and renal tumours (Figure 6.5). This data indicates that the increased incidence of rhabdomyosarcoma in the *KRas*^{G12V} *p53*$^{R172H/+}$ cohort is as a result of the mutant *p53* activity rather than simple *p53* heterozygosity.

a



b

	Age at Death	Cause of Death
1	80	Lymphoma
2	93	Rhabdomyosarcoma
3	109	Ataxia
4	62	Kidney Tumour
5	96	Ataxia
6	89	Lymphoma
7	74	Lymphoma

Figure 6.5: KRas^{G12V} p53$^{+/-}$ mice only rarely develop pleomorphic rhabdomyosarcoma

Although survival in the KRas$^{G12V/+}$ p53$^{+/-}$ cohort (a, Red line, median survival 93 days) was longer than that of the Kaplan-Meier curve KRas$^{G12V/+}$ p53$^{R172H/+}$ (a, Black line, median survival 62 days). Only 1/7 mice in the KRas$^{G12V/+}$ p53$^{+/-}$ cohort (b) developed rhabdomyosarcoma, compared with 14/16 (88%) in the KRas$^{G12V/+}$ p53$^{R172H/+}$ cohort. This difference in incidence was statistically significant (Chi-square p=0.002).

6.2.4 Tumours from KRas^{G12V} p53$^{R172H/+}$ mice undergo loss of heterozygosity (LOH)

Although the mice in the *KRas^{G12V} p53$^{R172H/+}$* cohort developed rhabdomyosarcoma at a similar incidence to the mice in the *KRas^{G12V} p53$^{fl/fl}$* and *KRas^{G12V} p53$^{R172H/fl}$* cohorts, they did so at a longer latency. One possible explanation for this is that in order for a tumour to develop it is necessary to lose the wild-type *p53* allele. In order to investigate this possibility, we used *p21* activation as a functional assay of p53 activity. *p21* is a recognised downstream target of p53. However, the mutant *p53^{R172H}* used in this study is not capable of activating *p21* transcription. Therefore, we stained the tumours for p53 and p21. Unsurprisingly, tumours from the *KRas^{G12V} p53$^{fl/fl}$* cohort showed no p53 staining. p21 staining in these tumours revealed occasional positive cells (Figure 6.6). This is presumably due to p53 independent mechanisms of p21 upregulation. It was interesting to note that the majority of cells that stained positive for p21 were the very large bizarre cells. These cells are likely to be aneuploid and it has been shown that p21 is a marker of aneuploidy, independent of p53 (Dikovskaya, Schiffmann et al. 2007). Tumours from the *KRas^{G12V} p53$^{R172H/fl}$* cohort showed high levels of p53 staining, in keeping with the accumulation of mutant p53 often seen in human tumours with *p53* mutations. However, low levels of p21 staining (similar to that seen in the tumours from the *KRas^{G12V} p53$^{fl/fl}$* cohort) were seen, confirming the inability of the mutant p53 to activate *p21* (Figure 6.6). In tumours from the *KRas^{G12V} p53$^{R172H/+}$* cohort, high levels of p53 staining were seen, consistent with the accumulation of the mutant p53. p21 staining did not match p53 staining, however it was slightly increased when compared with the 2 cohorts which lacked functional p53 (Figure 6.6). In order to clarify

whether this increase was due to the presence of functional p53, we irradiated a subset of the mice with 14Gy and euthanized them after 6 hours. The results for the *KRas*^{G12V} *p53*$^{fl/fl}$ and *KRas*^{G12V} *p53*$^{R172H/fl}$ mice revealed no p21 induction, similar to the staining patterns seen in the unirradiated animals. Crucially, in the irradiated *KRas*^{G12V} *p53*$^{R172H/+}$ mice no upregulation of p21 was seen (Figure 6.6). These results indicate that loss of the wild-type copy of p53 had occurred in the tumour.

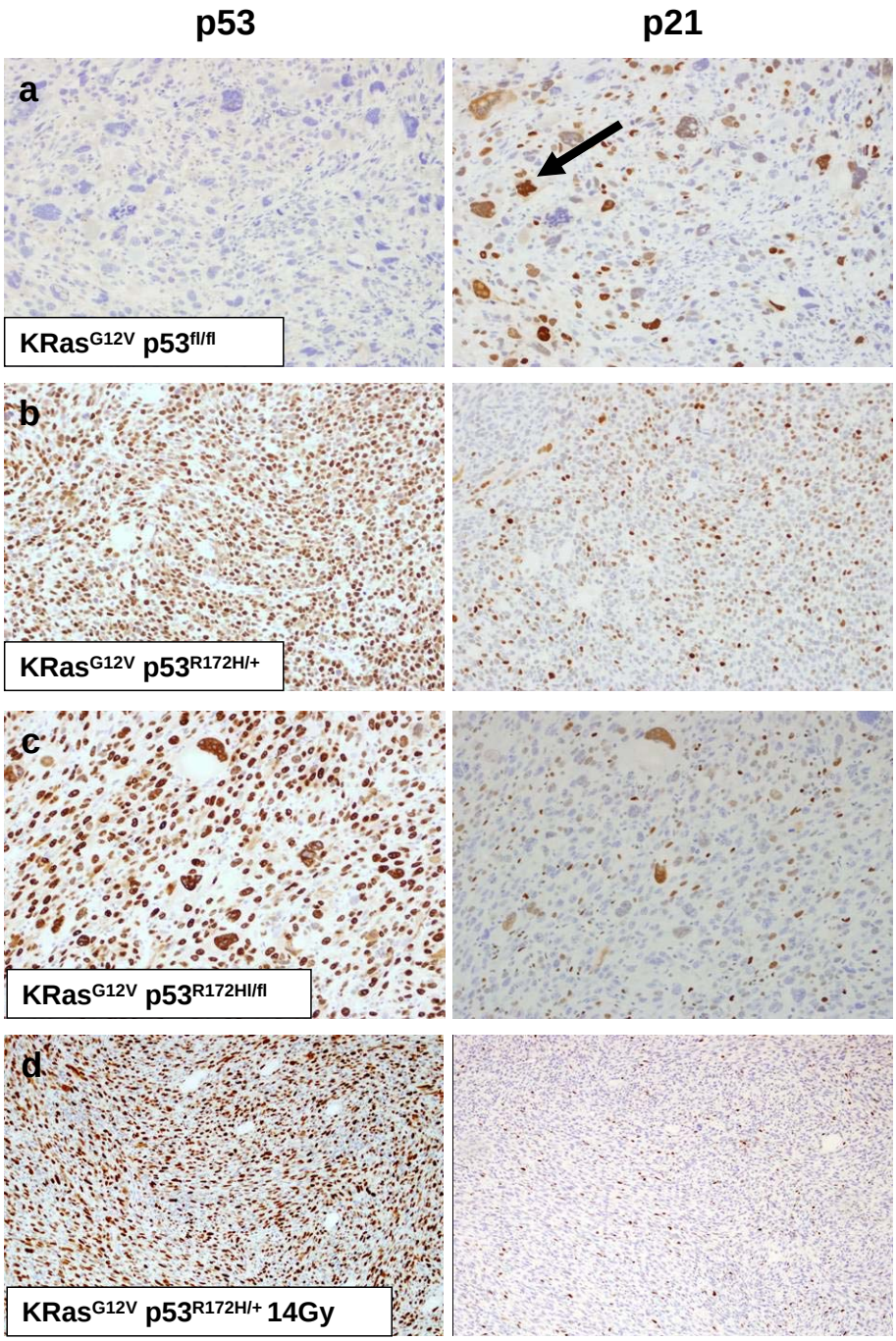


Figure 6.6: p21 is not upregulated with p53 in tumours form KRas^{G12V} p53$^{R172H/+}$ mice

In tumours from the KRas^{G12V} p53$^{fl/fl}$ cohort there was no p53 staining (a, left panel) and little p21 staining (a, right panel). Those cells which did stain positive tended to be large pleomorphic cells (arrow). In tumours from the KRas^{G12V} p53$^{R172H/fl}$ cohort there was widespread staining of p53 (c. left panel), however the lack of corresponding p21 staining (c, right panel) highlights that this is detecting the mutant, non-functional form of p53. There was similar p53 staining in the tumours from the KRas^{G12V} p53$^{R172H/+}$ cohort (b, left panel), and this was associated with low levels of p21 in the majority of tumours (b, right panel), even following irradiation (d).

These results suggested that LOH had occurred in the $KRas^{G12V}$ $p53^{R172H/+}$ tumours, however this is not the only possibility. It has been shown that mutant p53 can have a dominant negative effect, which could prevent activation of p21 by any remaining wild-type p53 (Ohnishi, Wang et al. 1998; Tang, Zhao et al. 1998). Therefore, in order to confirm these findings we assessed LOH using a different method. We performed pyrosequencing on DNA extracted from a subset of tumours from the $KRas^{G12V}$ $p53^{R172H/+}$ mice. The R172H mutation is the result of a G to A point mutation in exon 5 of the *p53* gene. Pyrosequencing allows for quantification of allele frequency and therefore we were able to accurately measure the percentage of wild type (G) and mutant (A) alleles present in a given tumour. 4/5 tumours analysed showed evidence of loss of the wild-type *p53* allele, with overrepresentation of the mutant allele (Figure 6.7). In one case the mutant and wild-type alleles were present at equal frequency.

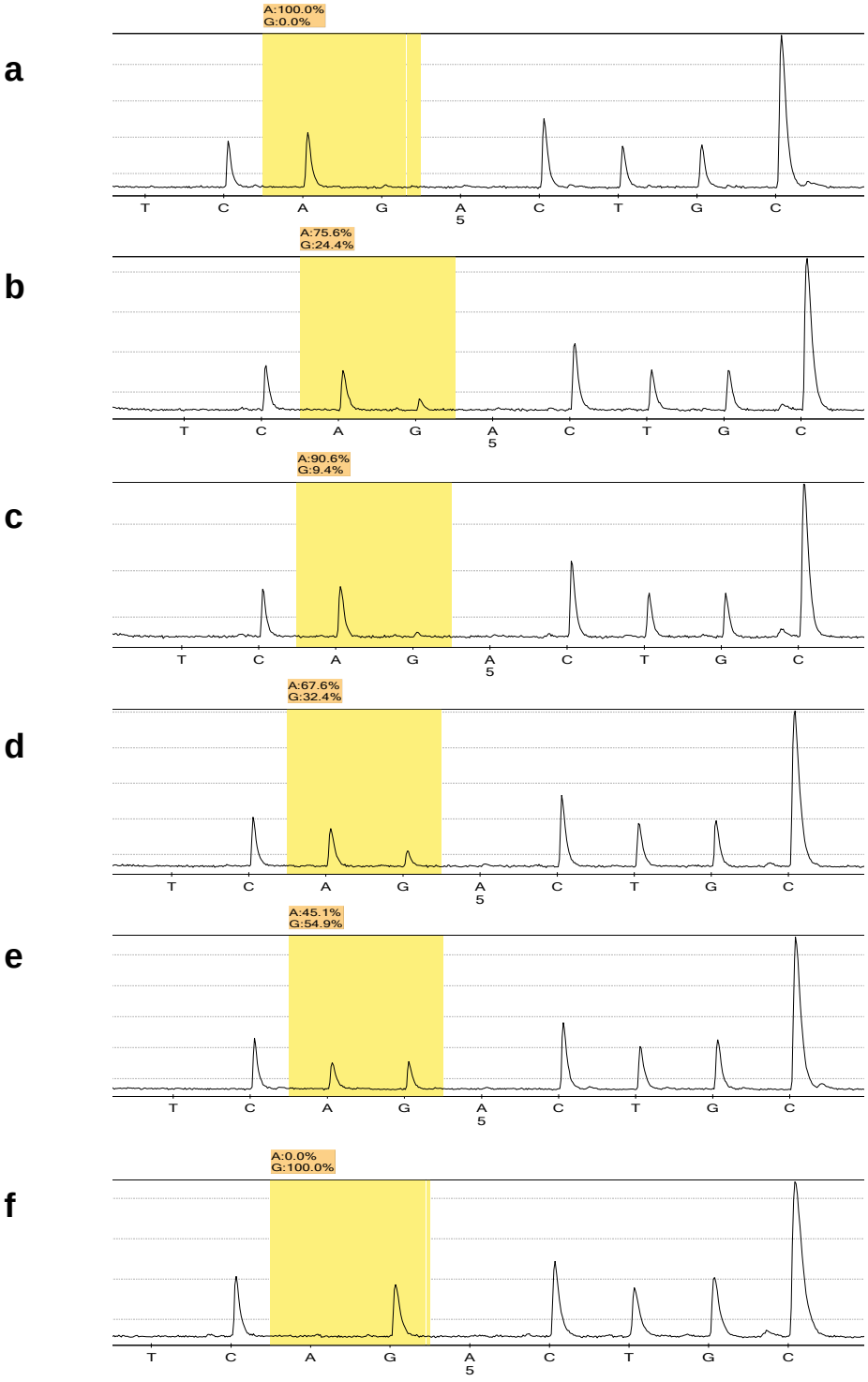


Figure 6.7: LOH occurs in the majority of KRas^{G12V} p53$^{R172H/+}$ tumours

Pyrograms demonstrating the LOH analysis performed on 5 tumours from the KRas^{G12V} p53$^{R172H/+}$ cohort. There was evidence of LOH in 4/5 tumours (a-d) with overrepresentation of the mutant (A) allele. In one tumour (e) the alleles were present at almost exactly equal frequency, indicating that LOH had not occurred in this tumour. 100% of the wild-type (G) allele can be seen in the normal muscle control (f).

6.2.5 Metastases developed only in mice expressing mutant p53

It has been shown previously in animal models that *p53* mutation can co-operate with activating *KRas* mutation to promote metastasis (Caulin, Nguyen et al. 2007; Morton, Timpson et al. 2010). We therefore investigated whether metastasis occurred in our model and in which groups. We examined lungs of the mice from the different tumour cohorts by histology. The rate of metastasis was low, with only 4 of the 31 mice examined developing lung metastases. All of the lesions detected were microscopic intravascular metastases that could not be seen grossly (Figure 6.8). Desmin IHC was performed to confirm that these were indeed metastases from a rhabdomyosarcoma (Figure 6.8). Interestingly, all of the mice that developed lung metastases were from either the $KRas^{G12V}$ $p53^{R172H/+}$ (1/4) or $KRas^{G12V}$ $p53^{R172H/fl}$ (3/4) cohorts, with no metastases seen in the $KRas^{G12V}$ $p53^{fl/fl}$ cohort. Even with the low overall rate of metastasis there was a statistically significant difference in metastasis rate between the $KRas^{G12V}$ $p53^{fl/fl}$ cohort and the two cohorts with the *p53* mutation (Chi-square p=0.038).

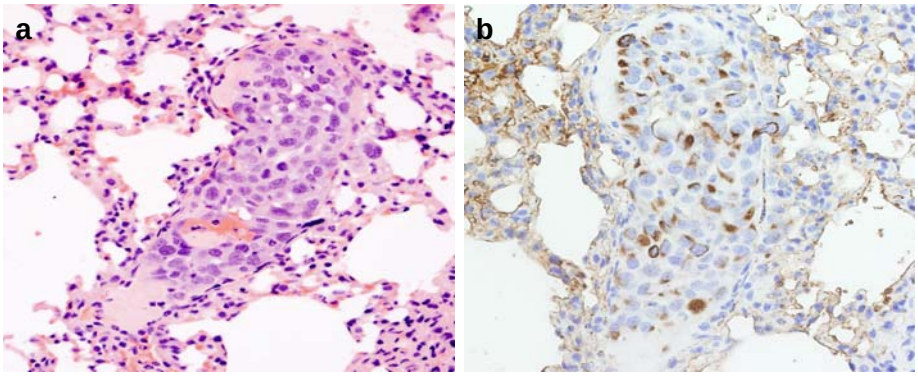


Figure 6.8: Metastases only occurred in mice expressing the mutant form of p53

An example of a metastasis from one of the $KRas^{G12V}$ $p53^{R172H/fl}$ tumours. The intravascular lung metastasis can be seen in the H&E image (a), the cells were positive for the muscle marker desmin (b).

6.3 Discussion

Pleomorphic rhabdomyosarcoma is an aggressive histological variant of rhabdomyosarcoma, which occurs predominantly in adults. Although there has been significant progress in improving patient outcome in paediatric rhabdomyosarcoma, the outcome in adults remains poor (Stock, Chibon et al. 2009; Sultan, Qaddoumi et al. 2009). Novel mouse models of this disease are therefore desirable to improve understanding of the basic biology and potentially improve outcome. In this study we demonstrate that mutation of *KRas* and *p53* can co-operate in a novel mouse model of pleomorphic rhabdomyosarcoma, which shows high penetrance and short tumour latency.

A number of authors have described models of pleomorphic rhabdomyosarcoma. Generally, these have occurred at a low incidence in studies of germline loss of tumour suppressor genes, for example, Jacks *et al* described pleomorphic rhabdomyosarcoma formation in a small percentage of mice in their model of Li-Fraumeni syndrome (Jacks, Remington et al. 1994). More recently, Tsumura *et al* described a more specific model based on the expression of oncogenic $KRas^{G12V}$ and either heterozygous or homozygous knockout of *p53* (Tsumura, Yoshida et al. 2006). The authors reported rhabdomyosarcomagenesis in all of the mice in the $KRas^{G12V}$ $p53^{-/-}$ group and a significantly smaller percentage in the $KRas^{G12V}$ $p53^{+/-}$ group. In the current study we have been able to replicate these results but we have also gone further by adding cohorts of mice which express a *p53* point mutation. This has allowed us to examine potential additional effects of the p53 mutant protein, bearing in mind that the point mutation is the more commonly occurring scenario in human tumours.

It has been shown that in animal models, that replacing *p53* knockout with a point mutation can alter the phenotype. Lang *et al* and Olive *et al* both demonstrated that *p53* knockout resulted in a different tumour spectrum to mutant *p53* expression in mouse models of Li-Fraumeni syndrome (Lang, Iwakuma et al. 2004; Olive, Tuveson et al. 2004). Moreover, they showed that the mutant *p53* more closely modelled the human syndrome. In this study, we have also seen a different and more aggressive phenotype associated with *p53* point mutation. In our model, mutation of a single allele of *p53* was enough to promote tumourigenesis in co-operation with oncogenic *KRas*. In contrast rhabdomyosarcoma was extremely rare in those animals which were heterozygous for *p53* loss even though they also carried the *KRas* mutation.

Moreover, although there was no difference seen in tumourigenesis between $KRas^{G12V}$ $p53^{R172H/fl}$ and $KRas^{G12V}$ $p53^{fl/fl}$ mice, it is interesting to note that metastases only developed in mice expressing the mutant form of *p53*. Similar results have been reported in different tumour models. Morton *et al* have shown that expression of mutant *p53* results in an increase in metastatic behaviour in a mouse model of pancreatic ductal adenocarcinoma (Morton, Timpson et al. 2010), and similar results were seen in a mouse model of squamous cell carcinoma (Caulin, Nguyen et al. 2007). Our results fit well with these and support, for the first time in a sarcoma model, the important role of *p53* mutation in both the early and late stages of tumour development.

LOH following an initial mutation of one allele of a tumour suppressor gene is an important event in the development of a tumour. In the case of *p53* it has been shown that such an LOH event occurs, at least in a proportion of both human (Varley, Thorncroft et al. 1997; Sedlacek, Kodet et al. 1998) and mouse tumours

(Lang, Iwakuma et al. 2004; Olive, Tuveson et al. 2004). We have postulated that the longer tumour latency seen in the *KRas*^{G12V} *p53*$^{R172H/+}$ cohort may have been due to the need to lose the wild-type *p53* allele prior to tumour initiation. We have taken two approaches to evaluate this. The first being a functional approach, relying on the fact that while the mutant form of p53 is known to accumulate in cells and is detectable by IHC, it is non-functional and therefore cannot activate transcription of target genes such as *p21*. However, since it is possible that other mechanisms (such as a dominant negative effect of the mutant p53) could explain the absence of p21 staining in the KRas^{G12V} p53$^{R172H/+}$ tumours, we also performed this analysis by another method. The second method relied on allele quantification by pyrosequencing. Both methods indicated that LOH occurred in the majority of these tumours. The speed at which these tumours develop would suggest that this LOH is quite an early event in tumour development.

In summary we have shown that the *AhCre* model can be used to study gene expression effects in the muscle of transgenic mice. We have used this model to demonstrate co-operation between an activating *KRas* mutation and *p53* mutation. Moreover, we show that this co-operation is stronger, both in terms of tumour development and tumour dissemination, when a *p53* null allele is replaced by a *p53* point mutant, thus strengthening the argument for an oncogenic role for mutant *p53* beyond simple loss of normal function.

Chapter 7 Summary

This section will give a brief overview of the major conclusions reached in this thesis:

The first aim of the thesis was to demonstrate that RKIP is a prognostic marker in Dukes B CRC. We have shown in a large, well characterised cohort of over 200 Dukes B patients that low levels of RKIP do indeed correlate with a poor prognosis. Moreover, we have shown in multivariate analysis that low RKIP level along with the presence of peritoneal invasion and LVI were the only independent prognostic markers in this cohort. Using these independent markers we have been able to construct a simple prognostic index. This allows for the selection of a poor prognosis group, comprising ~25% of patients whose 5-year survival is almost identical to patients presenting with lymph node metastases. It is likely that these are the patients who would derive the most benefit from close post-operative monitoring and therapy. The clinical relevance of this relates to the current controversy regarding adjuvant therapy in patients with Dukes B CRC. There is agreement that patients with “high risk” tumours should receive adjuvant treatment and we would propose that this prognostic index could form a framework for future trials to better define this high-risk group.

Following on from this the second aim of this thesis was to investigate the potential of RKIP to function as a predictive marker (i.e. to predict the response to therapy). What we saw was that patients with low levels of RKIP derived a significant survival benefit from chemotherapy while those patients with high levels of RKIP did not. Importantly, this result held true in Dukes B tumours. This again indicates the potential utility of RKIP as a marker in this group of patients. Although this result is interesting, this was a retrospective study without a

standardised chemotherapy regime and therefore the results would need to be replicated in further prospective studies.

The next aim was to analyse the effect of *RKIP* knockout *in vivo*, by studying its effect in established mouse models of CRC. Although *RKIP* knockout did not have the expected effect of increasing the invasive and metastatic properties of these tumours a number of interesting phenotypes were observed. These effects appeared to be linked by the known role for RKIP in promoting the stability of the spindle checkpoint and in preventing chromosomal instability. We observed that *RKIP* knockout can co-operate with *Apc* deletion to promote chromosomal instability in the liver. We also saw that in the presence of heterozygous deletion of *Apc* and an activating *KRas* mutation there was a shift in tumour location from the small intestine to the colon, a phenotype that has been seen previously to be associated with loss of spindle checkpoint regulators such as *BubR1* and *Bub1*.

Although it is not yet clear why *RKIP* deletion does not result in an increase in invasive and metastatic behaviour in the models studied it does not appear to be due to a compensatory increase in RKIP2, as this could not be detected in the tumours. Another possible explanation is that since the main role of RKIP appears to be in the prevention of metastasis it may be that the effect of its deletion will only be revealed in a more aggressive model of CRC. To this end the next aim of this thesis was to develop such a model.

A novel mouse model of CRC would be useful both to study the effect of RKIP deletion in the later stages of the disease but would also aid in developing new insights into the biology of CRC and potentially in pre-clinical testing of novel therapies. We took a rational approach to this by conditionally mutating both

Apc and *p53* in the intestine. These are two of the most commonly mutated genes in human CRC and mutation occurs at different points in the adenoma to carcinoma progression. As *p53* loss of function and point mutations have been shown to have different effects we also used the opportunity to study this potential difference in the setting of intestinal tumours *in vivo*.

We showed that *p53* and *Apc* mutations can co-operate to promote a dramatic phenotype in the intestine, with a rapid increase in tumourigenesis seen. This was accompanied by a marked increase in invasion and metastasis. Moreover, we saw that *p53* point mutation was a more potent driver of invasion than the loss of function mutation. This was demonstrated by the fact that over 90% of mice heterozygote for the point mutation developed invasive carcinoma, compared to ~20% of mice that were heterozygous for the loss of function mutation. Our results also suggest that LOH does not occur in these tumours, although this will need to be confirmed by further studies.

We have shown that both at the histological and molecular levels this model bares a close resemblance to human CRC. The tumours showed local invasion, with perforation of the bowel wall in many cases and therefore, probably best represent a model of Dukes B CRC. Therefore, it should be an ideal model in which to test the effect of RKIP deletion.

One of the aims of developing this model was to gain some insight into the process of invasion in CRC. We have been able to show *in vivo* that although Wnt signalling is active throughout the tumour there is a massive increase in this signal at the invasive front. This results in an increase in Wnt targets and pro-invasive proteins such as c-Myc, fascin and pERK. We also demonstrated an upregulation of ARF, specifically at the invasive front. We have gone on to show

that this increase in ARF is playing a functional role in promoting invasion in a p53 null environment. This is the first time that ARF has been shown to play such a pro-invasive role and further studies will be useful to determine both its downstream effectors and how it is interacting with the other pro-invasive proteins identified.

The final aim of the thesis was to build on the finding of the differing effects of *p53* loss of function and point mutation by studying this effect in another model, namely pleomorphic rhabdomyosarcoma. We showed that tumours developed in almost 100% of mice heterozygous for the point mutation but only rarely in those mice that were heterozygous for the loss of function mutation. Moreover metastases only developed in those mice expressing the *p53* point mutation. This demonstrated that *p53* point mutation promotes both tumour formation and progression in this system.

In the course of these studies we have relied heavily on the use of conditional animal models and on IHC. These techniques are powerful and associated with significant advantages; however it is also important to recognise the limitations associated with them. Below I will discuss the main advantages and disadvantages associated with these methods.

The use of conditional mouse models has allowed us to examine the role of p53 loss and mutation *in vivo* and allowed us for the first time to show the important role played by p53 mutation in CRC. This has been facilitated by the use of conditional expression of mutant genes in adult animals and in the specific tissue of interest. This is important as, in order to be relevant, a model system should closely recapitulate the events that occur in the native disease. In this setting conditional knockout has some advantages over germ line knockout in modelling

sporadic disease. While germ line knockouts are excellent models of familial cancer syndromes in which mutations are present from birth, there is the possibility that compensatory mechanisms arise which give result in a different phenotype than would occur in the case of a sporadic knockout. The differences that we have seen between our studies of the conditional APC p53 intestinal knockout mouse with previous studies using APC p53 germ line knockouts would seem to bare this point out. It also must be acknowledged that the model we have used is not a perfect model of sporadic CRC. Firstly, the majority of the tumours arise in the small bowel (human small bowel tumours are rare) rather than in the colon. Although, the fact that they are histologically similar to the human tumours and the previous studies from our laboratory showing similarities between tumours from the mouse small bowel, mouse colon and human colon supports their validity as a model system. A second disadvantage of this model is the temporal relation of the mutations. We know that in human CRC mutation of *APC* is an early event with *p53* mutation occurring at a later stage in the progression to carcinoma. However, in our model these two events occur simultaneously. This is a technical limitation of the system that we have used and further study will be required to develop novel systems, allowing different genetic events to occur at different time points.

The major advantage of using IHC in the initial studies showing the potential utility of RKIP as a prognostic marker is the fact that is a technique that is almost universally employed in all diagnostic pathology laboratories, thus making the results more easily transferable from the research to the clinical environment. The other great advantage of IHC is that it combines data regarding protein expression with morphology, thus allowing for the kind of analysis seen in Chapter 5 where differential protein expression was seen in

different areas of the tumour. The potential weakness of IHC is that it is not truly quantitative and the staining intensity depends not only on the amount of the protein being tested in the sample but also on a number of other factors such as chromogen incubation time. Therefore, when interpreting results it is important to have appropriate positive and negative controls with known staining intensities with which one can compare the test specimen. If possible these should be tissues on the same microscopic slide as the test tissue (for example we use normal colonic epithelium as a positive control for RKIP staining). In addition to the above measures we have, where possible, used automated staining to further minimise variability.

Overall, these studies have shown the important role for RKIP as a prognostic and potentially as a predictive marker in Dukes B CRC. Although we did not see the expected effect of *RKIP* deletion in established mouse models of CRC, we have been able to develop an ideal model in which to test its effect in the later stages of tumour development. In this model we have demonstrated the important role of Wnt signalling and its downstream targets in the establishment of an invasive front and have demonstrated a novel pro-invasive role for ARF. Finally, we have used both this and a novel model of rhabdomyosarcoma to demonstrate the differing effects of *p53* point mutation and loss of function mutations in vivo.

Future Work

Given the opportunity to bring this work forward there are a number of areas that I believe warrant further study. Firstly, I would like to further examine the role of RKIP as a prognostic and predictive marker in Dukes B CRC. It is important to validate the prognostic index developed in these studies. Ideally I would perform this analysis on a cohort of Dukes B patients who had been part of a well designed clinical trial, examining the effect of adjuvant therapy in Dukes B CRC. This would allow not only validation of the index, but also further investigation of the potential role of RKIP as a predictive marker in this patient cohort.

The second area from these studies that I would like to pursue relates to the findings on the differential expression of certain proteins at the invasive front of the mouse intestinal tumours compared to the superficial component. Of particular interest is the finding of increased ß-catenin and ARF levels at the invasive fronts of tumours and the fact that ARF appears to contribute to the invasive phenotype in the setting of p53 deficiency. The finding of increased ß-catenin at the invasive front suggests a possible solution to the ß-catenin paradox. I would like to do more work to confirm this (particularly in human cancers) and also try to establish the mechanistic link between p53 mutation and elevated ß-catenin. Following confirmation of the potentially exciting role for ARF in an independent system the next step in these studies would be to try to understand the mechanism of this novel function. In particular I would like to go on and investigate the downstream pathways that are involved in this shift from a tumour suppressive to pro-invasive function.

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