Investigating the mechanisms of pathogenesis underlying inherited colorectal adenomatous polyposis

Submitted for the degree of Doctor of Philosophy at Cardiff University

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Summary

In order to investigate the role of nonsynonymous variants of the *APC* gene in inherited predisposition to colorectal adenomas (CRAs) the entire *APC* ORF was sequenced in 691 unrelated North American patients with CRAs and 969 healthy controls. There was significant over representation of rare inherited nonsynonymous variants in patients who did not carry conventional pathogenic mutations in *APC* or *MUTYH* ($P = 0.0113$) when compared to patients with familial adenomatous polyposis (FAP) and *MUTYH* associated polyposis (MAP). The over representation was highest in non-FAP non-MAP patients with 11 to 99 CRAs ($P = 0.0103$). More non-FAP non-MAP patients carried rare nonsynonymous variants within the functionally important β-catenin down-regulating domain compared with healthy controls ($P = 0.0166$). *In silico* analyses predicted that approximately 46% of the variants identified were expected to affect function. Functional characterisation *in vitro* showed that 7 of 16 nonsynonymous variants altered β-catenin-regulated transcription consistent with a role in predisposition to CRAs.

An optimum level of β-catenin signalling is proposed to drive colorectal tumourigenesis, mediated by selection for *APC* genotypes retaining one, or rarely two, 20 amino acid β-catenin down regulating repeats (20AARs). We investigated the mechanism through which the *APC* variant E1317Q contributes to colorectal tumourigenesis. We compared the somatic mutation spectra of *APC* in tumours from AFAP patients that did (Family B) or did not (Family S) co-inherit E1317Q. Significant differences were identified between these tumours, 8.2% of tumours carrying E1317Q had somatic mutations predicted to result in mutant polypeptides retaining a single 20AAR, as compared to 62.1% of those which did not carry this variant ($P=5.64\times10^{-9}$). *In vitro* assays showed that E1317Q significantly impaired β-catenin regulated transcription when expressed with 'weak' truncating mutations ($P<0.05$) suggesting that E1317Q relaxes the target for tumourigenic somatic *APC* mutations through its own effects on β-catenin-associated signalling.

Inherited mutations in the *MUTYH* gene predispose to an autosomal recessive disorder characterise by multiple CRAs and carcinomas (MAP). MUTYH is a DNA glycosylase which removes adenines that are misincorporated opposite 8-oxo-7,8-

dihydro2'deoxyguanosine (8-oxoG), one of the most stable products of oxidative DNA damage. Tumours from patients with MAP display a high proportion of somatic G:C→T:A mutations due to a failure to repair these mismatches, and it is presumed that this mutator phenotype drives tumourigenesis. We studied the response of primary *MUTYH*-deficient fibroblasts to oxidative stress and found that significantly more of these cells survived exposure to hydrogen peroxide and *t*-butyl-hydroperoxide as compared to wild type cells. We found that MUTYH-deficient cells failed to enter apoptosis and showed that this may be mediated via an inability to recruit Rad9 to the correct nuclear position, indicating a failure to engage the 9-1-1 DNA damage sensor complex. Consistent with this, we found that MUTYH-deficient cells failed to activate the downstream checkpoint protein Chk1, after exposure to oxidative stress. We propose that MAP-associated tumourigenesis is driven by failure to undergo apoptosis in conjunction with an underlying mutator phenotype.

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FAP	Familial adenomatous polyposis
FBS	Foetal bovine serum
Fpg	Formamidopyrimidine DNA glycosylase
G	Guanine
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GG-NER	Global genome nucleotide excision repair
GST	Glutathione-S-transferase
GpX	Glutathione peroxidase
GTP	Guanosine triphosphate
HhH	Helix-harpin-helix
HNPCC	Hereditary non-polyposis colorectal cancer
HRR	Homologous recombination repair
IBD	Inflammatory bowel disease
IPTG	Isopropyl-β-D-thiogalactopyranoside
IVS	Intervening sequence
LB	Luria-Bertani
LD-PCR	Long distance PCR
LEF	Lymphoid enhancer factor
LM/LPC	Laser microdissection and laser pressure catapulting
LOH	Loss of heterozygosity
MAF	Minor allele frequency
MAP	*MUTYH* associated polyposis
MAPK	Mitogen activated protein kinase
MCR	Mutation cluster region
ME	β-mercaptoethanol
MIN	Microsatellite instability
MLPA	Multiplex ligation-dependent probe amplification
MLS	Mitochondrial localisation signal
MMR	Mismatch repair
mRNA	Messenger RNA
MSI	Microsatellite instability
MYH	Human MutY homologue (MUTYH)

NEIL	Nei like glycosylase
NER	Nucleotide excision repair
NES	Nuclear export signal
NLS	Nuclear localisation signal
NMD	Nonsense mediated decay
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PNK	Polynucleotide kinase
PUMA	p53 upregulated mediator of apoptosis
RNA	Ribonucleic acid
RFC	Replication factor C
ROS	Reactive oxygen species
RPA	Replication protein A
SAMP	Ser-Ala-Met-Pro
SOD	Superoxide scavenger superoxide dismutase
SDM	Site directed mutagenesis
SDS	Sodium dodecyl sulphate
T	Thymine
TBS	*t*-butyl hydroperoxide
TCF	T cell factor
TC – NER	Transcription coupled nucleotide excision repair
TGF	Transforming growth factor
TGFβRII	TGFbeta receptor type II
tm	Melting temperature
UTR	Untranslated region
UV	Ultraviolet
VEGF	Vascular endothelial derived growth factor
VHL	von Hippel-Lindau
X-gal	5 bromo-4-bromo-chloro-3-indoyl-D-galactoside
XRCC1	X-ray repair cross complementing 1
8-oxoG	8-oxo-7, 8 dihydro2'deoxyguanosine

Codon Table

First Position		Second position of Codon: T		C		A		G		Third Position
	T	TTT	(F)	TCT	(S)	TAT	(Y)	TGT	(C)	T
		TTC	(F)	TCC	(S)	TAC	(Y)	TGC	(C)	C
		TTA	(L)	TCA	(S)	TAA	(stop)	TGA	(stop)	A
		TTG	(L)	TCG	(S)	TAG	(stop)	TGG	(W)	G
	C	CTT	(L)	CCT	(P)	CAT	(H)	CGT	(R)	T
		CTC	(L)	CCC	(P)	CAC	(H)	CGC	(R)	C
		CTA	(L)	CCA	(P)	CAA	(Q)	CGA	(R)	A
		CTG	(L)	CCG	(P)	CAG	(Q)	CGG	(R)	G
	A	ATT	(I)	ACT	(T)	AAT	(N)	AGT	(S)	T
		ATC	(I)	ACC	(T)	AAC	(N)	AGC	(S)	C
		ATA	(I)	ACA	(T)	AAA	(K)	AGA	(R)	A
		ATG	(M)	ACG	(T)	AAG	(K)	AGG	(R)	G
	G	GTT	(V)	GCT	(A)	GAT	(D)	GGT	(G)	T
		GTC	(V)	GCC	(A)	GAC	(D)	GGC	(G)	C
		GTA	(V)	GCA	(A)	GAA	(E)	GGA	(G)	A
		GTG	(V)	GCG	(A)	GAG	(E)	GGG	(G)	G

Key:

G - Glycine (Gly)

P - Proline (Pro)

A - Alanine (Ala)

V - Valine (Val)

L - Leucine (Leu)

I - Isoleucine (Ile)

M - Methionine (Met)

C - Cysteine (Cys)

F - Phenylalanine (Phe)

Y - Tyrosine (Tyr)

W - Tryptophan (Trp)

H - Histidine (His)

K - Lysine (Lys)

R - Arginine (Arg)

Q - Glutamine (Gln)

N - Asparagine (Asn)

E - Glutamic Acid (Glu)

D - Aspartic Acid (Asp)

S - Serine (Ser)

T - Threonine (Thr)

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Chapter One: General Introduction

1.1 Colorectal Cancer

In developed countries, colorectal cancer (CRC) is the most common non-tobacco related cancer in both sexes combined and the fourth commonest type of cancer worldwide (Parkin *et al*, 2001; Boyle *et al*, 2000). CRC is the second most common cause of death by cancer in the United Kingdom (Office of National Statistics, 2006) and although mortality rates are now decreasing due to the implementation of improved screening and treatment, it is still estimated that 492,000 CRC deaths occur globally each year (Weitz *et al*, 2005).

Both environmental and genetic factors contribute to development of CRC and either can be the predominant cause of the disease whilst also being able to interact with each other thereby compounding risk. Environmental or non-genetic risk factors include age, since approximately 90% of all CRCs are found in individuals over the age of 50. So-called 'lifestyle choices' such as lack of exercise, smoking, alcohol intake and poor diet may somewhat increase the risk of developing CRC. Epidemiological studies have shown that poor diet is a significant factor influencing the incidence of CRC as a diet high in fat, red meat consumption and low vegetable intake has been associated with an increased risk (World Cancer Research Fund, 1997) and evidence exists supporting the theory that differences in diet account for the geographical difference in CRC incidence (Flood *et al,* 2002). Inflammatory bowel disease (IBD), specifically ulcerative colitis, is a precancerous condition predisposing to cancers of both the colon and rectum; an individual with IBD is estimated to have a lifetime CRC risk of 15 to 40% (Zisman and Rubin, 2008)

1.2 Colorectal Tumourigenesis: the adenoma-carcinoma sequence

Independent of the initiating factor of CRC, the development of malignancy requires multiple mutations. The stages of tumourigenesis have been relatively well defined and usually occur over several years (Figure 1.1) (Kinzler and Vogelstein, 1996). Each stage results from mutations in genes

involved in specific cell cycle control and signalling pathways. Different genes involved in the same pathway can be mutated in different CRCs but these mutations are generally mutually exclusive (Kinzler and Vogelstein, 1996). Mutation within genes involved in the transforming growth factor-β (TGF- β) pathway is a good example of this, since mutations in *TGFβRII* and *SMAD4* are found separately in CRC, but not together in the same tumour (Woodford-Richens *et al*, 2000).

Most CRCs develop from normal epithelium through sequentially worsening degrees of adenomatous dysplasia. CRC appears to be the best studied cancer in terms of a putative sequence of genetic events in its pathogenesis (Luebeck and Moolgavkar, 2002) and the genetic pathway model for the pathogenesis of sporadic CRC proposed by Fearon and Vogelstein (1990) is based upon this concept of an adenoma to carcinoma sequence. Although the total accumulation of mutations is the principal factor, the model proposes that causative mutations in tumour suppressor genes and oncogenes occur in a specific order in most CRCs and are implicated in both hereditary and sporadic cancers in humans (Kennedy and Hamilton, 1998).

Genes that are mutated in CRC and directly contribute to the adenoma to carcinoma sequence fall into two categories: oncogenes and tumour suppressor genes (Vogelstein and Kinzler 2002). Mutations in oncogenes cause them to become constitutively active or active when the wild type gene would not be. Such activation can be caused by chromosomal translocations, gene amplifications or subtle mutations within the gene that affect residues important for the activity of the protein (Vogelstein and Kinzler, 2004). Notably the gene mutated in a particular pathway is influenced by the type of genomic instability present in the adenoma or carcinoma. An oncogene often mutated in CRC is the *k-ras* gene. The k-ras protein is involved in signal transduction and couples growth factors

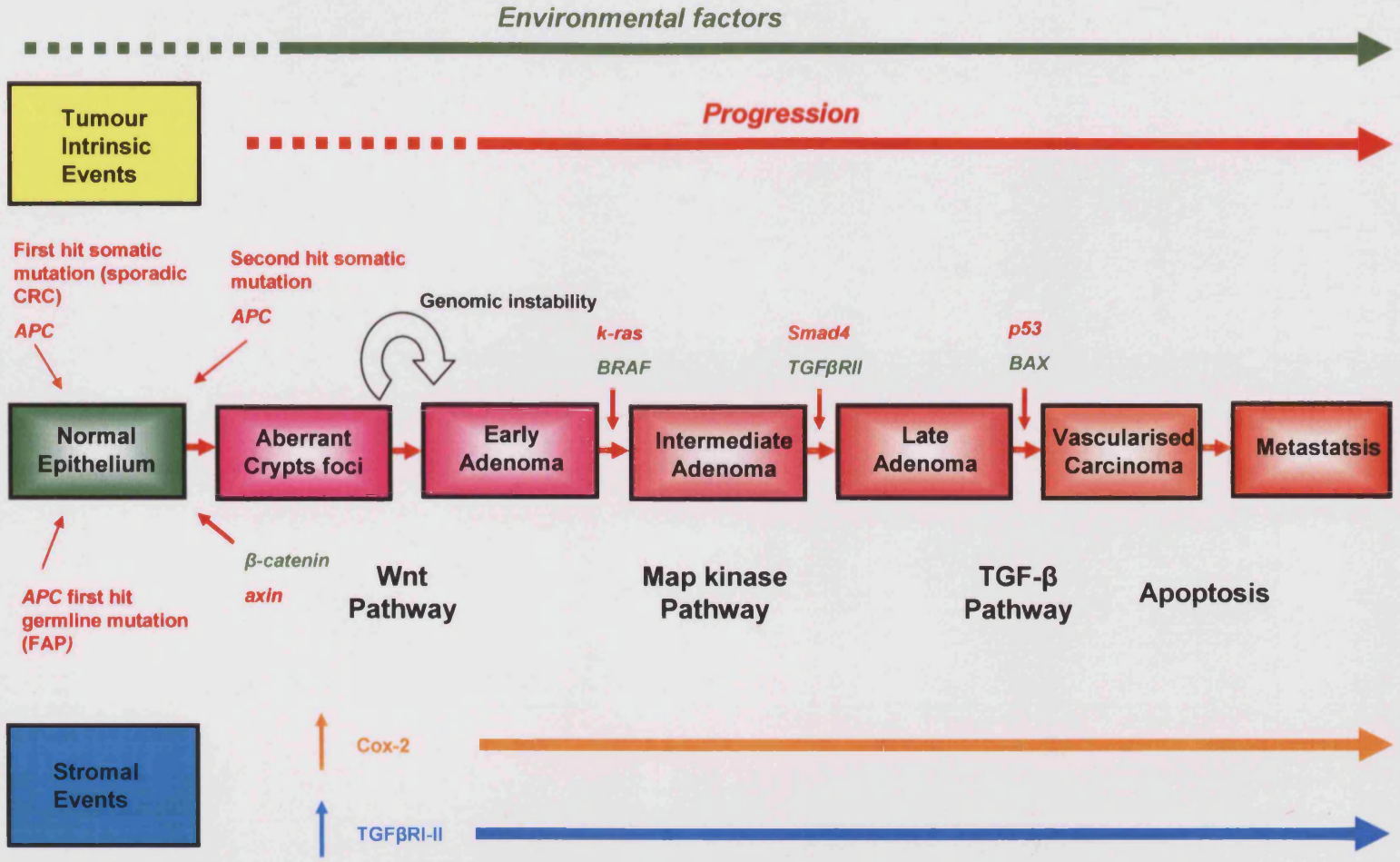


Figure 1.1. Multi-step genetic model for adenoma to carcinoma progression in CRC. Schematic diagram showing the sequence of events driving colorectal tumourigenesis. Genes shown in red are generally mutated in tumours with CIN and those in green are generally mutated in MSI tumours. Genetic instability plays a key role in tumourigenesis, contributing to the accumulation of mutations in genes involved in CRC. Stromal defects, shown in orange and blue, include the over-expression of key genes, occur in a number of hereditary gastrointestinal cancers such as juvenile polyposis (Adapted from Mishra, L. *et al*, 2005).

to the mitogen activated protein kinase (MAPK) cascade which results in expression of genes involved with cell proliferation. K-ras is a monomeric guanosine triphosphate (GTP)ase which is inactive when bound to guanosine diphosphate (GDP), but when GTP is bound, k-ras is activated and the MAPK cascade is stimulated. Normally, the GTPase activity of k-ras would then hydrolyse the bound GTP thus inactivating itself. The most common mutations in the *k-ras* gene affect codons 12, 13 and 61, all of which result in disrupted GTPase activity. This enables k-ras to remain active and stimulate cell proliferation (Grady and Markowitz, 2002). Only one allele of an oncogene needs to be somatically mutated to elicit a tumourigeneic effect. Other oncogenes involved in CRC tumourigenesis include *CTNNB1* (Morin *et al*, 1997), *BRAF* (Davies *et al*, 2002) and *PIK3CA* (Samuels *et al*, 2006).

Loss of function in tumour suppresser genes results from mutations that cause a truncated protein or lead to nonsense mediated decay (NMD), nonsynonymous mutations at functionally important residues, insertions, deletions or gene silencing by promoter hypermethylation (Vogelstein and Kinzler, 2004). A tumour suppressor gene mutated in the majority of CRCs is the adenomatous polyposis coli (*APC*) gene. The APC protein has multiple cellular functions including an inhibitory role preventing over-activation of the Wnt signalling pathway. This pathway stimulates cell growth when activated. *APC* mutations are often protein-truncating mutations and chromosomal deletions. These inactivate the APC protein so it can no longer regulate intracellular β-catenin turnover.

Loss of *APC* either by mutation or allelic loss is detected in ~75% of sporadic CRCs (Miyaki *et al*, 1994) and is often cited as an early event in tumourigenesis having been observed in early adenomas (Powell *et al*, 1992; Groden *et al*, 1991; Kinzler *et al*, 1991). Several genes are implicated in the progression of early adenoma to early carcinoma and initiating mutations alone appear insufficient for adenomas to progress (Giardello, 1994). Activation of the *k-ras* oncogene is detected in more than 40% of CRCs, with an increasing frequency in larger and more advanced tumours (Smith *et al*, 1994). Inactivating mutations in both *APC* and *k-ras* are thought to provide a

selective growth advantage to the nascent tumour cell as lone *k-ras* mutations are observed in histologically normal mucosa but are only present in dysplastic mucosa in-conjunction with *APC* mutations (Minamoto *et al*, 1995; Pretlow 1995; Bird 1995).

The *DCC* gene, is another putative tumour suppressor gene, located on chromosome 18 along with *SMAD2* and *SMAD4*, and is often deleted in CRCs (Thiagalingam *et al*, 1996). In addition, *p53* is mutated or deleted in many colorectal neoplasms (Fearon and Vogelstein, 1990). It has been suggested that chromosome 3p at the von Hippel-Lindau (VHL) disease gene locus may be implicated in the development or progression of sporadic colorectal carcinoma (Zhuang *et al,* 1996), since *VHL* disease gene deletion frequently occurs in sporadic colon carcinoma but not present in adenomas. Therefore, *VHL* may play a role in colonic carcinogenesis and represent a relatively late event in colonic neoplasia progression.

1.2.1 Genomic Instability

Contributing to the accumulation of mutations in genes involved in CRC, genomic instability plays a crucial role in tumourigenesis, (Grady and Markowitz, 2002). Loss of genomic stability can arise via chromosomal instability (CIN), microsatellite instability (MSI) or epigenetic silencing of gene promoters. CIN leads to genetic alterations through chromosomal loss and gains whereas MSI causes genetic alterations through defective DNA mismatch repair (MMR) proteins (Söreide *et al*, 2006).

CIN results in gross changes in DNA by increasing the rate of chromosomal gains or losses (Rajagopalan *et al*, 2003). This arises because of errors during mitotic recombination or aberrant segregation of chromosomes at mitosis lead to aneuploidy, a condition often observed in cancers with CIN (Kinzler and Vogelstein, 1996). Whilst the molecular mechanism responsible for CIN is not yet fully understood it is thought to occur at an early stage of colorectal tumourigenesis (Shih *et al*, 2001). Genes involved in accurate mitotic segregation of chromosomes have long been implicated as a causative factor of CIN and mutations in the genes *hBUB1* (Cahill *et al*, 1998), *APC*

(Fodde *et al,* 2001) and *hCDC4* (Rajagopalan *et al,* 2004), have all been suggested as possible cause of CIN. Chromosomal arms that are frequently lost in tumours with CIN are the loci of genes important in tumourigenesis, suggesting that the chromosomal changes are not random but are driven by natural selection based on the growth advantage conferred to the nascent tumour. Allelic loss often occurs at 17p, 18q and 5q, the loci of *p53, SMAD4* and *APC* respectively (Teridman 2002).

MSI induces smaller changes in DNA than CIN and affects short repetitive sequences called microsatellites that are prone to misalignment errors during replication. Such errors are repaired by the MMR proteins in normal cells but defects in these proteins result in a germline microsatellite allele undergoing a somatic change in length by gaining or losing tandem repeats. MSI influences which genes are mutated in colorectal tumours since genes containing microsatellites are susceptible to mutation (Söreide *et al*, 2006).

Whilst CIN is the mode of genetic instability observed in the majority of CRCs (approximately 85%) (Kinzler and Vogelstein, 1996), aCpG island methylator phenotype (CIMP) has been identified in some CRCs. The hypermethylation of CpG islands often found within the promoter region of genes. This epigenetic instability results in tumourigenesis because of the transcriptional silencing tumour suppressor genes (Toyota *et al*, 1999). Genetic and epigenetic instability are not mutually exclusive; Samowitz *et al,* (2005) identified colorectal tumours with CIMP and MSI, or less frequently, CIN. In some CRCs, promoter hypermethylation of the MMR gene *hMLH1* has lead to defects in the MMR pathway (Herman *et al*, 1998).

Whilst the majority of CRC cases are sporadic with no known genetic cause, the remaining cases arise as a result of inherited genetic factors. Twin studies have often been used to elucidate genetic mechanisms of human disease and have been successfully applied to show that genetic factors also play an important role in causing CRC. Lichtenstein *et al* (2000) estimated that genetic factors could account for up to 20% of CRC by comparing concordance for CRC in monozygotic twins with that in dizygotic twins.

Approximately 2-6% of all CRCs are the result of known genetic predisposition syndromes accounted for by established CRC predisposition genes (Fearnhead *et al*, 2002, Kemp *et al*, 2004) (Figure 1.1).

1.3 Inherited CRC Syndromes

Of the several autosomal dominant syndromes, the two commonest familial forms are familial adenomatous polyposis (FAP) (MIM17500), which historically accounted for <1% (Hodgson and Maher, 1999; Lynch and de la Chappelle, 1999) and hereditary non-polyposis colorectal cancer (HNPCC) (MIM 114500) which accounts for up to 5% (Lynch and de la Chappellle, 1999). The importance of a correct diagnosis of an inherited CRC syndrome is essential to provide long term clinical management of the disease and associated surveillance and genetic counselling of the immediate family.

1.3.1 Familial adenomatous polyposis

FAP is a rare, autosomal dominant inherited disorder caused by mutations in the *APC* gene (Kinzler and Vogelstein, 1996).It is characterised by the development of hundreds to thousands of colorectal adenomas (CRAs) within the colon and the rectum (Wallis and MacDonald, 1996) (Figure 1.3). These polyps usually develop during the second and third decade of life (Kinzler and Vogelstein, 1996) and if left untreated, one or more will progress to carcinoma often by the fourth decade of life (Hodgson and Maher, 1999; Kinzler and Vogelstein, 1996). Whilst predominantly manifesting within the colon and rectum, cancer is not limited to these areas since malignant tumours may occur in the duodenum, liver, brain and thyroid (Hodgson and Maher, 1999). Extracolonic manifestations of FAP and include adenoma and adenocarcinoma of the stomach, periampullary region, pancreas and thyroid, osteomas, desmoids, dental abnormalities, epidermal cysts, congenital hypertrophy of the retinal pigmented epithelium (CHRPE) and central nervous system (CNS) tumours (Kinzler and Vogelstein, 1996). A variant of FAP known as attenuated FAP (AFAP) is characterised by the development of a

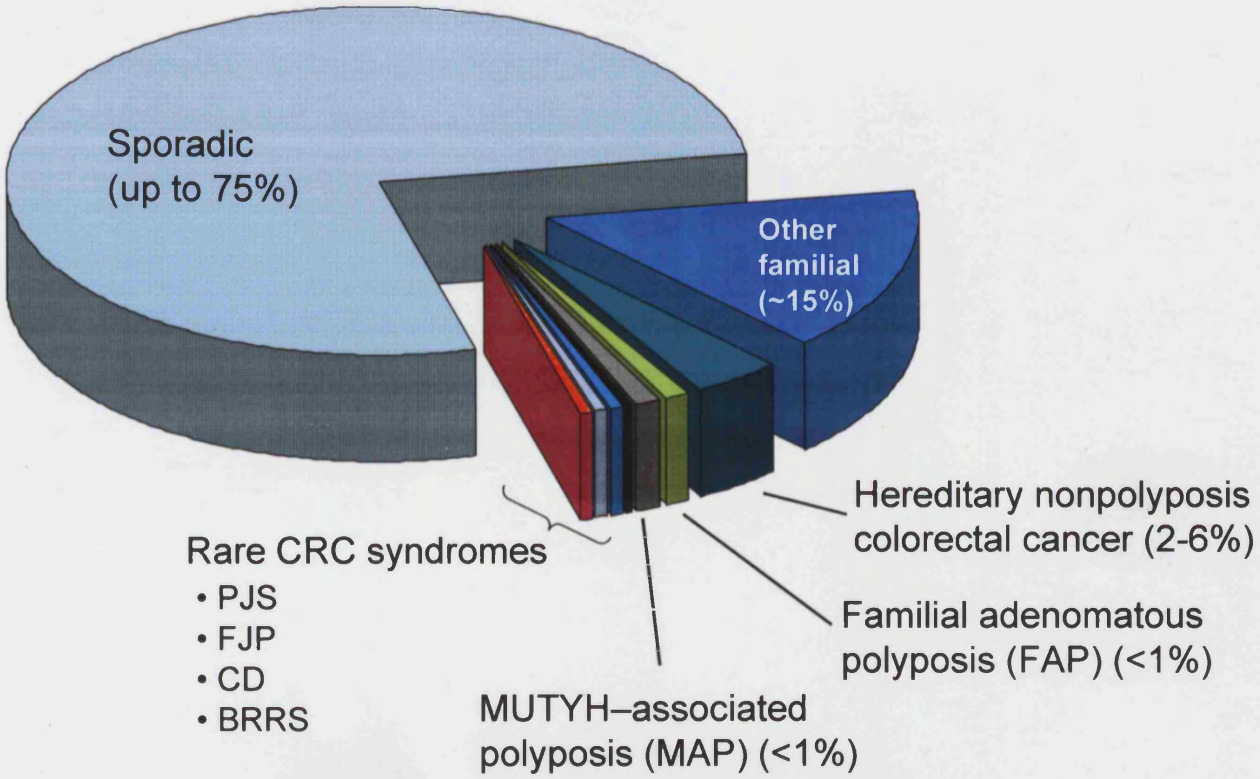


Figure 1. 2. Predisposition to CRC that are considered either sporadic, familial or due to a recognised hereditary cancer syndrome. PJS:Peutz-Jeghers syndrome; FJP: Familial juvenile polyposis; CD Cowden's disease; BRRS:Bannayan-Ruvalcaba-Riley syndrome

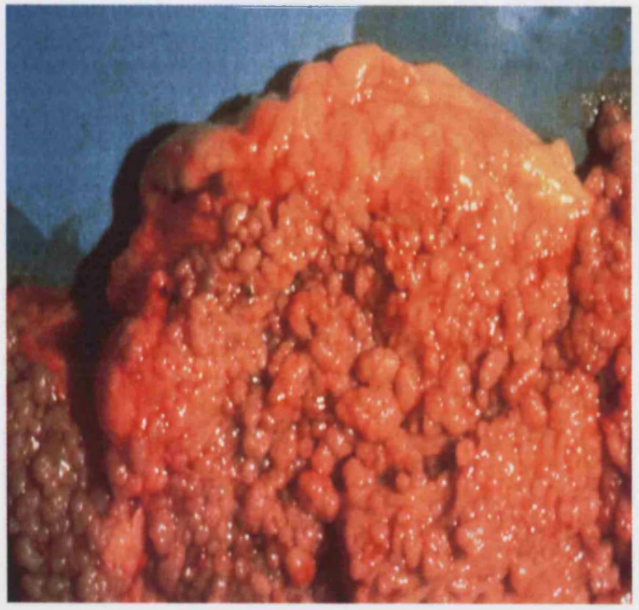
lower number (<100) of CRAs (Figure 1.3) by the fourth decade, a later age of onset and is associated with germline mutations in specific regions of the *APC* gene.

1.3.2 The *APC* gene

The *APC* gene is located on chromosome 5q21 and consists of 21 exons (Thliveris 1996). The initial link between FAP and chromosome 5 came with the identification of a deletion in the long arm of this chromosome in a patient with CRC (Herrera *et al*, 1986). Subsequent linkage analysis in FAP families narrowed the region containing the gene to 5q21-5q22 (Bodmer *et al*, 1987; Leppert *et al*, 1987), from which the APC gene was subsequently cloned and sequenced (Groden *et al*, 1991; Joslyn *et al*, 1991; Kinzler *et al*, 1991).

The *APC* gene includes 21 exons contained within a 98-kilobase locus (Thliveris, 1996). Different combinations of splice donor and acceptor sites are utilised, resulting in a range of alternative transcripts and tissue specific expression patterns. In addition to the conventional form of APC encoded by exons 1 to 15, alternatively expressed exons of the gene (0.3, brain specific [BS], 0.1, 0.2, 1, 9, and 10A) (Thliveris *et al*, 1994; Horii *et al* 1993; Sulekova and Balhausen, 1995) encode alternate protein isoforms (Pyles *et al*, 1998). Exon 15 comprises more than 75% of the coding sequence (Figure 1.4) and is the target of most germline mutations in FAP patients and somatic mutations in tumours. Although the tissue-specific expression patterns of these isoforms differ from conventional APC (Pyles *et al*, 1998), their function is not yet completely understood. They are found generally in post mitotic tissues and terminally differentiated cell lines (Pyles *et al*, 1998) and differ most significantly from conventional APC in their amino-termini and predicted ability to homo- or heterodimerize.

A) Classic FAP



B) Attenuated FAP (AFAP)

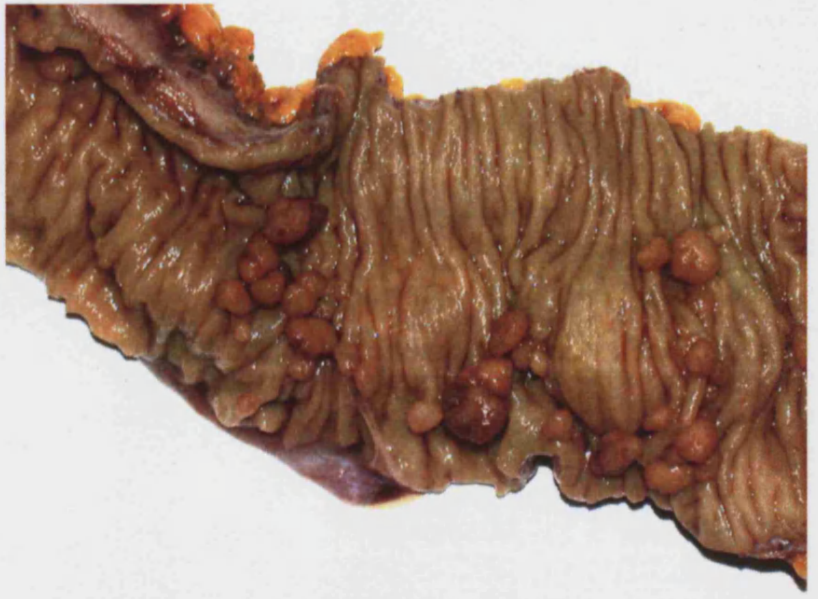


Figure 1.3. Gross appearance of colon from A) a patient with classical FAP showing a profuse carpet of polyps and b) a patient with AFAP displaying the attenuated phenotype (<100 colorectal adenomas). Pictures courtesy of Prof. J. Sampson, Cardiff University, UK.

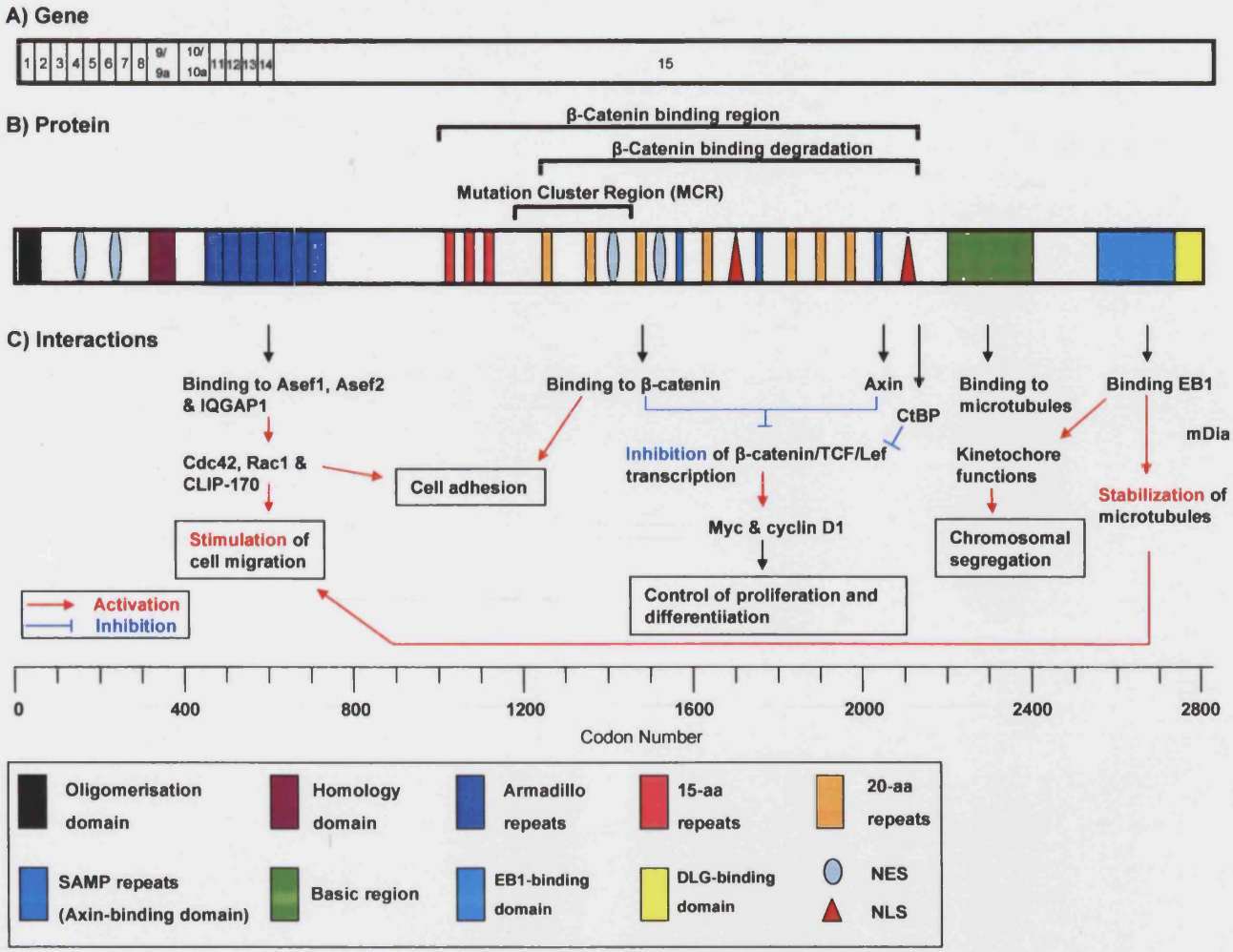


Figure 1. 4 Schematics of: A) the conventional form of the APC gene showing 15 exons, with exon 15 containing over 75% of the coding sequence. The alternatively spliced exons 1, 9 and 10a are also shown. B) Full length APC protein illustrating multiple domains and their functions. C) APC stimulates cell migration through interactions with Asef, IQGAP1 or mDia. APC is involved in cell adhesion through controlling β-catenin distribution between the nucleus/cytoplasm and the plasma membrane. APC inhibits β -catenin/TCF/Lef transcription through interactions with β -catenin or CtBP. APC regulates chromosome segregation through kinetochore binding and through suppression.

1.3.3 Mutations in *APC*: Knudson's two-hit hypothesis

Knudson's two-hit hypothesis states that tumour formation is initiated by biallelic inactivation of a tumour suppressor gene and that both inherited and sporadic cancers can arise as a result of mutations in the same gene (Knudson 1971; Figure 1.5). Inherited predisposition to tumourigenesis results from a germline mutation in a tumour suppressor gene e.g. *APC* (the 'first hit'), but that this alone is not sufficient for tumour development; inactivation of the wildtype allele by a somatic mutation (second hit) is required. Development of sporadic tumours requires somatic mutational events to inactivate both wild type *APC* alleles (Ichii *et al*, 1992; Powell *et al* 1992) consistent with the lower number of tumours and late age of onset in sporadic cases (Knudson 1996).

1.3.3.1 Distribution of germline mutations in *APC*

The vast majority of germline *APC* mutations are predicted to result in the synthesis of a C- terminal truncated protein (Beroud and Soussi, 1996; Laken *et al*, 1999; Powell *et al*, 1992). Nonsense point mutations and frame-shifts (due to small insertions and deletions) that result in a truncated protein account for greater than 98% of APC mutations (Giardello *et al* 1994). Most germline mutations are scattered throughout the 5' half of the gene with two common hotspots for mutations occurring at codons 1309 (Beroud and Soussi, 1996; Miyoshi *et al* 1992) and 1062 (Beroud and Soussi, 1996; Mandl *et al*, 1994) (Figure 1.5). Recurrent mutations at these two codons account for 10 - 30% of germline mutations (Beroud and Soussi, 1996; Miyoshi *et al*, 199a). Mutations throughout the 3' end of the gene are less frequent and rarely occur downstream of codon 1600 (Friedl *et al*, 1996; Miyoshi *et al*, 1992).

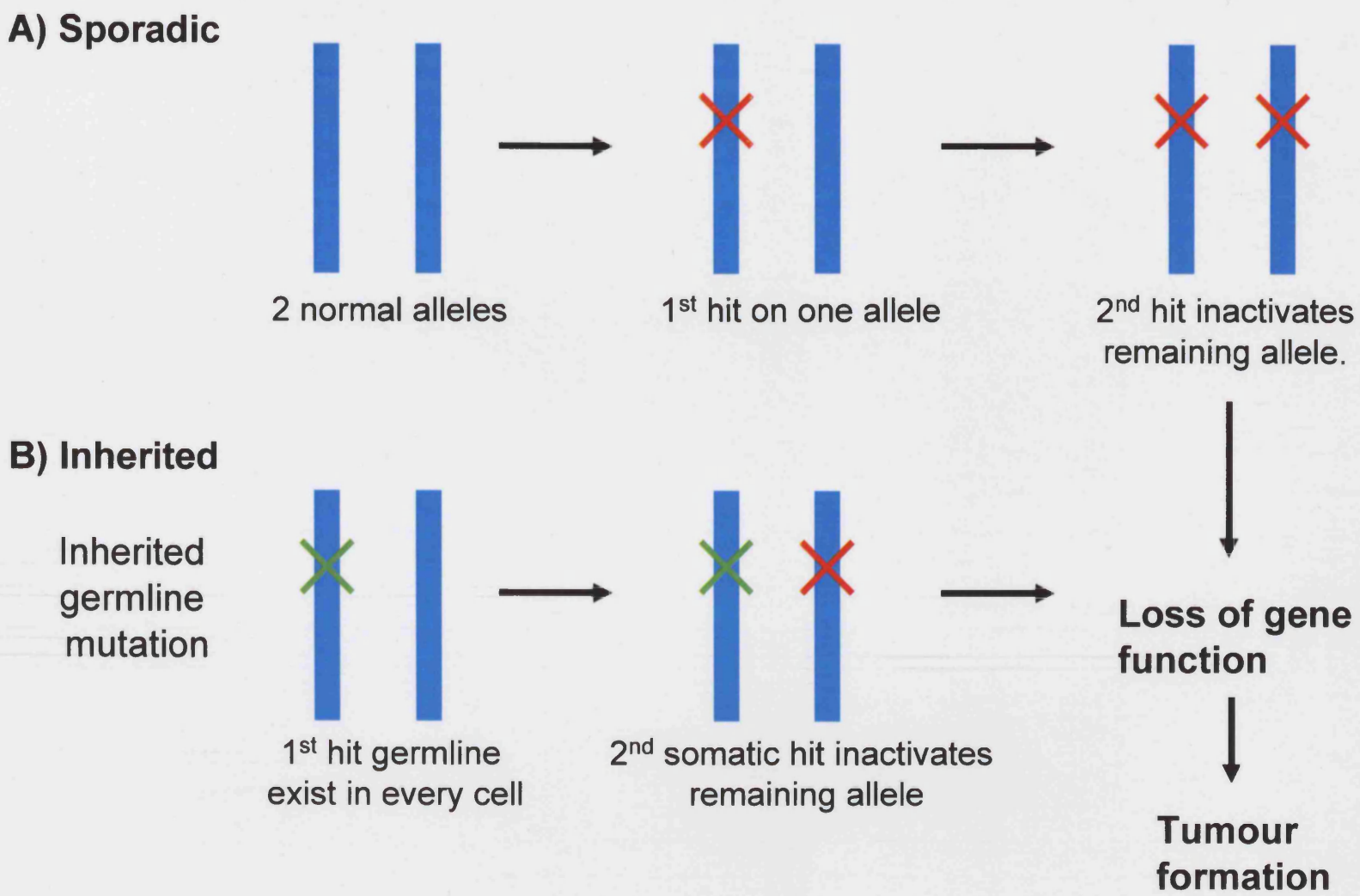


Figure 1.5 Knudson's 2-hit hypothesis of tumourigenesis in A) sporadic and B) inherited forms of cancer.

1.3.3.2 Distribution of somatic mutations in *APC*

Somatic *APC* mutations are detected in over 75% of sporadic CRC cases as well as in tumours from FAP patients (Ichii *et al*, 1993: Rowan *et al*, 2000). Over 60% of somatic mutations are concentrated between codons 1281 and 1556, a region referred to as the mutation cluster region (MCR) (Beroud and Soussi, 1996). (Figure 1.6) There are three somatic mutation hotspots located at codons 1309, 1450 and 1556 (Miyoshi *et al*, 1992; Cheadle *et al*, 2002). Somatic mutations are rare in the terminal third of the gene (Laurent-Puig *et al*, 1998, Beroud and Soussi, 1996).

The majority of somatic point mutations are cytosine (C) to thymine (T) transitions at CGA codons resulting in a stop signal, indicating a selective pressure to truncate APC (Beroud and Soussi, 1996; Miyoshi *et al*, 1992; Nagase *et al*, 1992). Higher proportions of single base pair (bp) insertions and deletions (≤ 2bps) are more common in somatic cells than in the germline (Marshall *et al*, 1997) suggesting possible mechanistic differences for mutation in somatic cells (Laurent-Puig *et al*, 1998). Loss of heterozygosity (LOH) at 5q21-22 is observed in FAP-associated (Okamoto *et al*, 1994; Miyaki *et al*, 1994) and sporadic tumours, but is more frequent in sporadic tumours (Solomon *et al*, 1987; Rowan *et al*, 2000). Hypermethylation of the 1A promoter region of APC has been detected in CRCs (Hiltunen *et al*, 1997). This mechanism prevents expression of the APC protein (Esteller *et al*, 2000), suggesting that this is an alternative, although infrequent mechanism for *APC* inactivation.

A significant observation in colorectal adenomas and carcinomas due to inactivation of both *APC* alleles is the interdependence between the so called 'first' and 'second hit' (Lamlum *et al*, 1999; Rowan *et al*, 2000; Albuquerque *et al*, 2002) (Figure 1.6). In familial tumours, 'first hit' mutations between codons 1194-1392 within the MCR are associated with loss of the other allele as a second hit and these patients tend to display more severe phenotype, whereas mutations outside this region are associated with an MCR truncating mutation (Lamlum *et al*, 1999; Rowan *et al*, 2000). This suggests that mutations in the *APC* gene are not selected for simple loss of protein function

(Lamlum *et al*, 1999; Rowan *et al* 2000) but that there is in-fact, a strong selection for the retention of at least one truncated *APC* product containing the first 20-amino acid β-catenin down-regulating domain. Smits *et al* (1999), showed that truncated *APC* is required for maximuml cell proliferation and that there exists an almost systematic selection for truncating mutations occurring before the first Ser-Ala-Met-Pro (SAMP) repeat implying that such mutant proteins are selected to eliminate the binding sites for axin/conductin on APC, thereby inactivating this function of the protein. The idea that truncated mutants are selected for based on the provision of growth advantages for the nascent tumour cell is reinforced by findings of the Cheadle group (2002), which showed that different combinations of *APC* mutations confer different growth advantages. Using a statistical model based on the data from sporadic tumours it was found that biallelic truncating mutations within in the MCR were 3.9 times more likely to be selected than one truncating mutation in the MCR combined with either a truncating mutation outside the MCR or an incidence of LOH, which in turn were 27.8 times more likely to be selected than two truncating mutations outside the MCR or a truncating mutation outside the MCR and an LOH.

1.3.3.3 Genotype - phenotype correlations in FAP

The correlation between genotype and phenotype of FAP has been the subject of much investigation: the clinical manifestation of FAP varies greatly between individuals; Giardello *et al* (1994) were able to show that patients with identical mutations could develop different clinical features. However, mutational analyses indicate an association between the FAP phenotype and the position of the germline *APC* mutation (Table 1.1 and Figure 1.7). These relationships involve both the severity of the polyposis phenotype as well as several other features that are subject to phenotypic variability such as the age of onset of CRC and the development of extracolonic manifestations.

Severe polyposis (> 5000 CRAs) is typically seen in individuals with germline mutations in the MCR (codons 1250 and 1464; Nagase *et al,* 1992), although a similar phenotype has been found in individuals with *APC* mutations at

codon 233 in exon 6 (Nugent *et al,* 1994) and at codons 486 and 499 in exon 11 (Eccles *et al,* 1997). AFAP is typified by mutations at the extreme 5' (Spirio *et al*, 1993; Dobbie *et al,* 1994; Wallis *et al,* 1994) or 3' (van der Luijt *et al,* 1996; Friedl *et al,* 1996) ends of the *APC* gene, or in the alternatively spliced region of exon 9 (Rozen *et al,* 1999; Soravia *et a.,* 1998).

Extracolonic manifestations (i.e. desmoids, osteomas, epidermoid cysts and upper gastrointestinal polyps) occur most commonly in FAP patients with mutations between codons 1445 and 1578 (Caspari *et al,* 1995) or between codons 1395 and 1493 (Wallis *et al,* 1996). CHRPE is only present in individuals with mutations between codons 457 and 1444 (Spirio *et al*, 1993; Wallis *et al*, 1994; Olschwang *et al,* 1993).

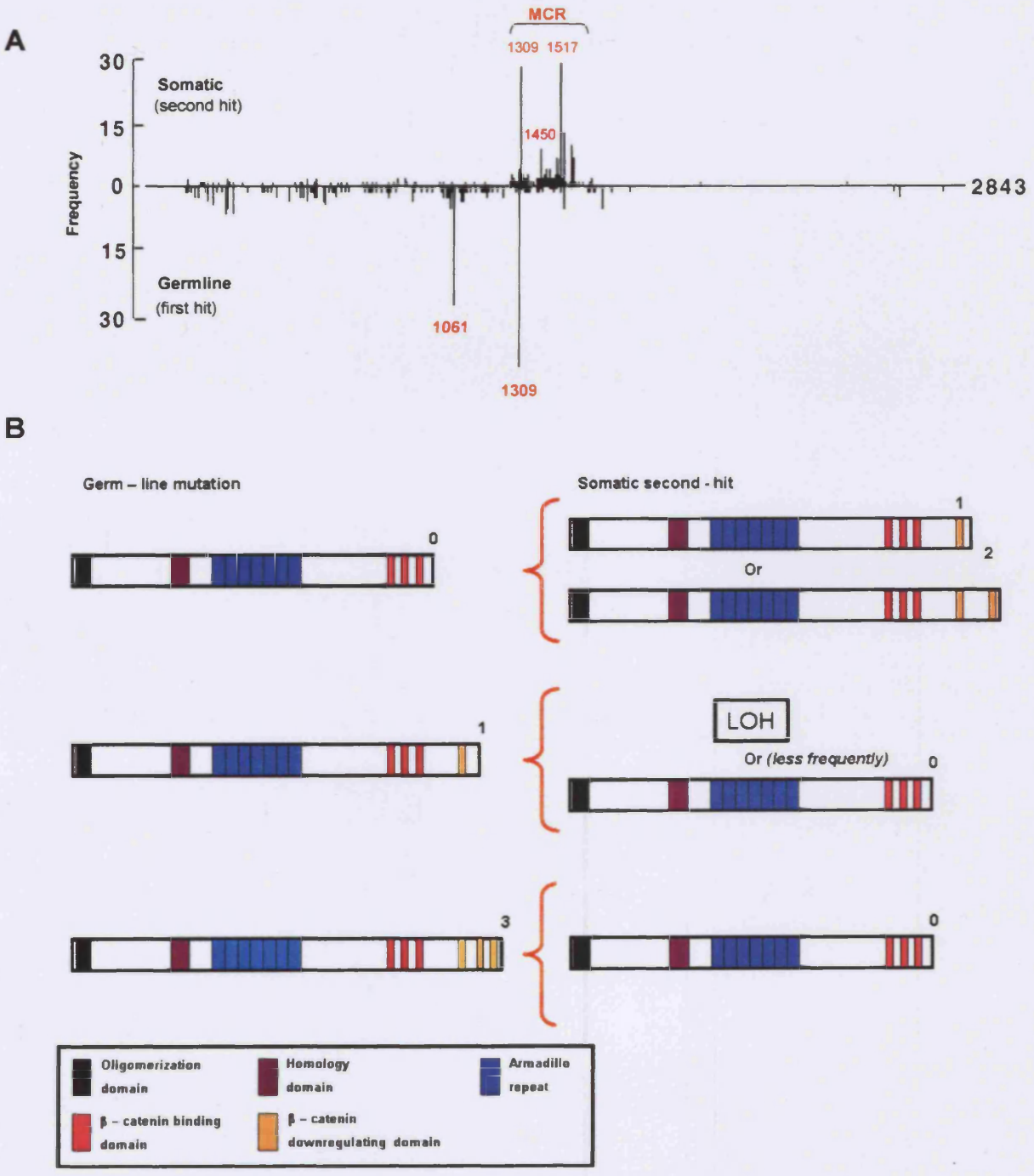


Figure 1. 6 A) Histogram indicating the frequency of somatic and germline mutations in APC. Germline mutations are distributed all over APC, with two 'hotspots' at codons 1061and 1309. Somatic mutations show a propensity to lie within the mutation cluster region (MCR). B) The interdependent association between the positions of the initial germline mutation and the somatic mutation. (Figure adapted from Klaus and Birchmeier 2008).

FAP Phenotype	No. of Polyps	Age of onset of polyps	Mean age of onset of CRC (Yr)	Site of CRC	Sites of mutation codon no.	Extracolonic manifestations
Severe (classical FAP)	>5000	I and II Decades	34	Left	1250 – 1464 (MCR)	Extreme
Intermediate (classical FAP)	100 – 5000	II and III decades	42	Left	157 – 311 and 412-1597 (excludes MCR)	Common
Attenuated (AFAP)	<100	IV and V decades	delayed	Right	1-57[a], 311 – 412[b] and 1596-2644[c]	Limited

Table 1.1 Classification of FAP illustrating the phenotype-genotype correlation between various type of colonic polyposis and site of germline mutation. FAP: familial adenomatous polyposis; CRC: colorectal cancer. a=5' end exons 4 and 5; b= within exon 9; c=3'distal end of *APC* gene. (Adapted from Cetta and Dhamo, 2007)

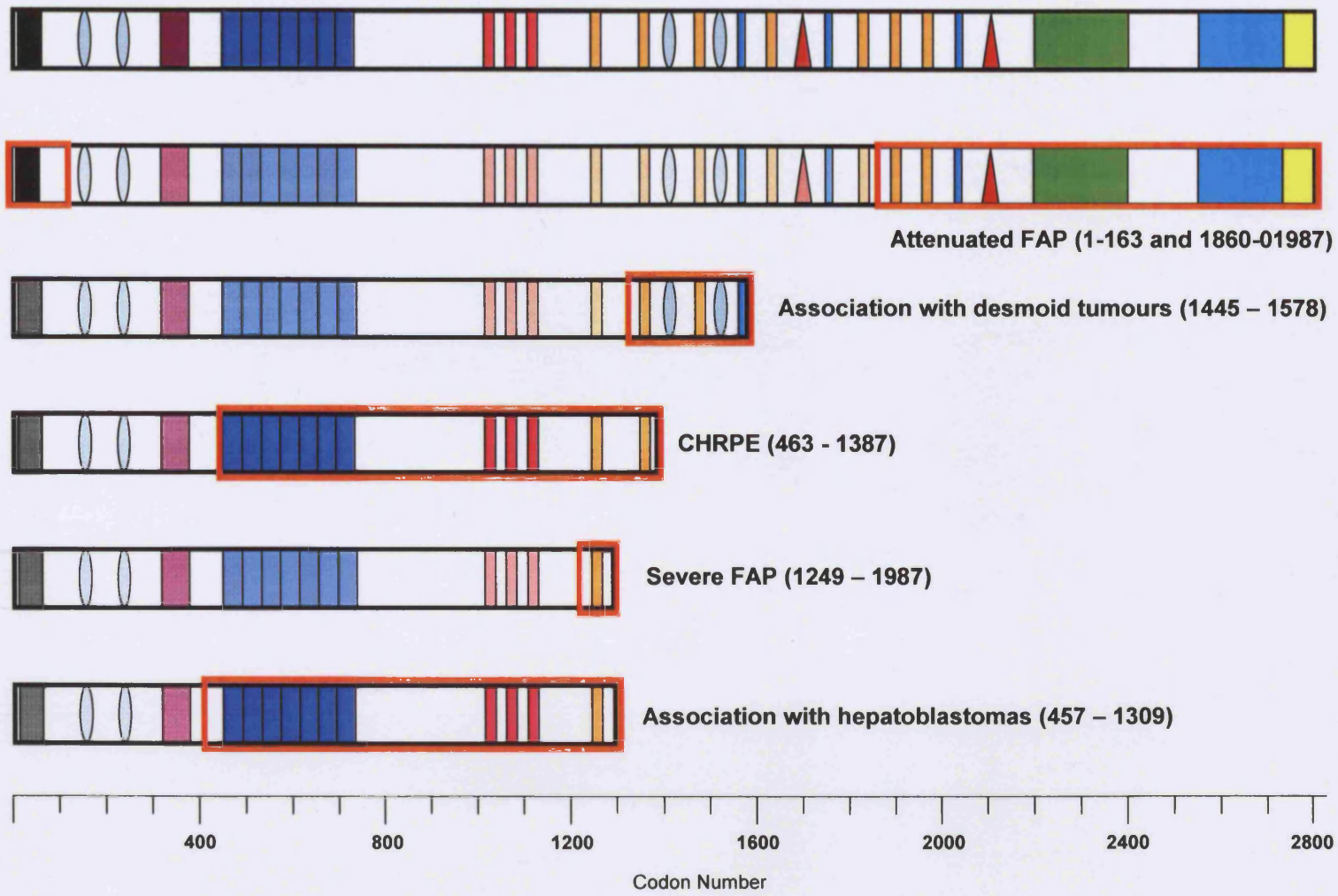


Figure 1.7 Schematic showing truncated APC proteins illustrating the association between position of germline *APC* mutation and FAP phenotype. Boxed area indicates position of mutation, codon range noted in figure. Adapted from Fearnhead *et al*, 2001

1.3.3.4 Low penetrance alleles in *APC*

Whether rare inherited nonsynonymous variants in *APC* act as low penetrance alleles that predispose to colorectal tumours remains highly speculative. The low penetrant nonsynonymous variant I1307K predisposes to multiple colorectal adenomas in the Ashkenazi Jewish population (Laken *et al*, 1997). This variant allele was observed in 6% of Ashkenazi controls, 10% of Ashkenazi CRC cases, and 28% of Ashkenazim CRC cases who also had a family history of CRC. It is rarely present in non-Ashkenazi individuals. An A-to-T transition at codon 1307 creates a hypermutable $[A]_8$ tract and appears to be associated with a 1.5 to 2 fold risk of colon cancer (Laken *et al*, 1997; Woodage *et al*, 1998; Rozen *et al*, 1999; Gryfe *et al* 1999).

The E1317Q nonsynonymous variant was first described by White *et al* (1996), in two of four siblings in an Ashkenazi Jewish CRC family. It is more common and widespread than I1307K and has been proposed to act as a CRA predisposition allele (Frayling *et al*, 1998) but the underlying mechanism is yet to be ascertained. Evidence exists to support the suggestion that E1317Q elicits a pathogenic effect because the amino acid substitution leads to a charge change in the critical 20 amino acid repeat (20AAR) domain responsible for β-catenin binding. E1317Q has been identified as i) a somatic mutation in sporadic CRA development (White *et al,* 1996), ii) a somatic mutation in a colorectal adenocarcinoma from an I1307K patient (Laken *et al*, 1997), iii) one of the two detected mutations in the colon cancer cell line SKCO1 (Rowan *et al*, 1997), iv) it is overrepresented in the germline of patients with multiple CRAs (Lamlum *et al*, 2000) and v) it has also been identified as a somatic change in sporadic CRC (Frayling *et al*, 1998).

The *APC* germline variant N1026S was identified in a Spanish AFAP family and characterised by Menendez *et al* (2008). It is located within the highly conserved region of the first 15AAR within the β-catenin binding domain. Menendez *et al* (2008) suggested a pathogenic role via a dominant-negative effect on APC function. Functional characterisation showed that *APC*-N1026S failed to reduce repression of β-catenin regulated transcription when

compared to wildtype APC (constitutively active β-catenin regulated transcription in colon cancer cell lines is greatly reduced upon the introduction of wild-type APC), strongly supporting the hypothesis of a pathogenic role for *APC* N1026S variant in the AFAP phenotype.

1.3.4 Domains of the APC protein

Mammalian *APC* is expressed in the majority of foetal tissues and in adult epithelial cells (Midgley *et al*, 1997) and occurs in several isoforms, arising through several mechanisms i.e. alternative splicing at the mRNA level, post-translational modification and nascent protein degradation. The most abundant isoform, APC is present as a 2843-amino acid (Horii *et al,* 1993). It is detected in both the nucleus and the cytoplasm (Neufeld and White, 1997) of cells in a variety of organs including the brain, lung aorta, spleen, heart, kidney, liver, stomach, placenta and colonic mucosa (Groden *et al*, 1991).

APC is a large multifunctional protein consisting of 2843-amino acids; is approximately 311kDa in size (Groden *et al*, 1991) and consists of several functional domains, each of which enables an interaction with several protein partners in numerous cellular processes (Figure 1.4); mammalian APC has an oligomerization domain, an armadillo repeat-domain, 15- and 20AAR domains important for binding to β-catenin, SAMP repeats for axin binding, a basic domain for microtubule binding and a C-terminal domain that binds to EB1 and hDLG proteins. Through these multiple domains APC performs functions in not only tumour suppression but also cell differentiation, adhesion, polarity formation, migration, development, apoptosis and neuronal functions (Senda *et al*, 2007; Figure 1.4). Table 1.2 lists known APC binding partners and summarises their proposed function.

1.3.4.1 Oligomerization domain

Heptad repeats enable APC to homodimerize (Groden *et al*, 1991, Su *et al*, 1993; Joslyn *et al*, 1991). Since this domain lies at the N-terminus of the protein it consequently facilitates homodimerisation of wildtype APC polypeptides with truncated APC mutant polypeptides (Polakis 1997).

1.3.4.2 Armadillo repeat domain

The armadillo domain at the N-terminus consists of seven repeats with a high degree of homology to *Drosophila* armadillo and the mammalian homologue β-catenin (Rubinfeld *et al,* 1993). It is the most highly conserved region of APC being 60% identical with the *Drosophila* APC as compared to an overall identity of less than 30% (Hayashi *et al*, 1997). The armadillo domain appears to mediate protein/protein interaction; it is able to bind the APC-stimulated guanine nucleotide exchange factor (ASEF), which is a Rac specific guanine nucleotide exchange factor (Kawasaki *et al*, 2000); Kap3, the kinesin super family associated protein 3, which is a mediator between APC and kinesin motor proteins (Jimbo *et al*, 2002); and IQGAP1, which is an effecter of Rac1 and Cdc42 (Watanabe *et al*, 2004). These binding partners suggest that the armadillo domain of APC may be involved in cell morphology, motility and migration through the actin and microtubule cytoskeleton.

1.3.4.3 15-amino acid and 20-amino acid repeat domain

Three highly conserved 15AARs and seven 20AARs are located within the central portion of APC and provide critical binding sites for β-catenin (Rubinfeld *et al*, 1993 Su *et al* 1993; Rubinfeld *et al*, 1995; Rubinfeld *et al*, 1997). The binding of β-catenin to the 15AARs does not lead to the down regulation of β -catenin. Rather, β -catenin down regulation requires the presence of at least three of the seven 20AARs (Rubinfeld *et al*, 1997).

1.3.4.4 Samp repeats

Three SAMP repeats lie between the third and fourth, the fourth and fifth, and after the seventh 20AARs (Behrens *et al*, 1998) respectively. They contain the conserved sequence (I/LxxxCLxSxMxK/R) and are binding sites for axin and its homologue, conductin.

1.3.4.5 NES and NLS domains

Highly conserved nuclear export signals (NES) that are essential for shuttling the nuclear β -catenin to the cytoplasm were identified at the N-terminus and within the 20 amino acid repeats (Henderson 2000). Each carried one of the following motifs LxxxLxxLxL or VxxxVxxVxV (Henderson 2000). Two nuclear localisation signals (NLS) necessary for nuclear import of APC were recognised between amino acids 1767 – 1772 and between amino acids 2048-2053 (Hang *et al*, 2000).

1.3.4.6 Basic domain

The basic domain is localized between amino acids 2200 and 2400 within the C-terminus of APC (Groden *et al*, 1991). This region is rich in arginine, lysine and proline. This type of basic region is also found in microtubule-associated proteins and confers the microtubule binding function of APC (Deka *et al*, 1998). Studies have shown that this domain stimulates polymerization of tubulin *in vitro*, and binds to microtubules when expressed in epithelial cells (Munemitsu*et al*, 1994; Smith *et al*, 1994; Deka *et al*, 1998).

Binding partner	APC domain	Function	References
Asef	Armadillo repeats	Rac GEF, cell migration, cell adhesion	Kawasaki et al, 2000 and Kawasaki et al, 2003
β-catenin	15 and 20 aa repeats	Cell adhesion, membrane attachment of mitotic spindle, Wnt signaling	Barth et al, 1997, Pollack et al, 1997, McCartney et al, 1999, Yu et al, 1999, Townsley and Bienz, 2000, Lu et al, 2001, McCartney et al, 2001, Hamada and Bienz, 2002, Klingelhofer et al, 2003 and Faux et al, 2004
Bub Kinases	Unknown	Chromosome segregation	Kaplan et al, 2001 and Green and Kaplan, 2003
EB1	EB1 binding domain	Cell migration, chromosome segregation, spindle assembly, neuronal differentiation	Askham et al, 2000, Barth et al, 2002, Lu et al, 2001, Nakamura et al, 2001, Rogers et al, 2002, Liakopoulos et al, 2003, Louie et al, 2004, Temburni et al, 2004 and Wen et al, 2004
hDLG	PDZ binding motif	Cell cycle, neuronal differentiation	Matsumine et al, 1996 and Ishidate et al, 2000
IQGAP1	Armadillo repeats	Rho GTPase regulation, cell migration, cell adhesion	Watanabe et al, 2004
KAP3	Armadillo repeats	Cell migration	Jimbo et al, 2002
Microtubules	Basic domain	Cell migration, cell adhesion, chromosome segregation, spindle assembly, neuronal differentiation	Matsumine et al, 1996, Smith et al, 1994, Nathke et al, 1996, Morrison et al, 1997, Fodde et al, 2001, Kaplan et al, 2001 and Zumbrunn et al, 2001
mDia	Basic domain	Rho GTPase regulation, cell migration, cell adhesion	Wen et al, 2004
Plakoglobin	15 and 20 aa repeats	Cell adhesion, Wnt signaling	Rubinfeld et al, 1995
PSD-93	Aa 2498–2844	Neuronal differentiation	Temburni et al, 2004
XMCAK	Basic domain	Chromosome segregation, spindle assembly	Banks and Heald, 2004

Table 1.2 APC interacts with multiple binding partners that contribute to its function outside the Wnt signalling pathway. APC binding partners summarised, the domains of APC they interact with and the function they participate in with APC

1.3.4.7 C-terminal domains

The C-terminal region of APC contains a 170 amino acid binding site for EB1 (Su *et al*, 1995). EB1 associates with the centromere, mitotic spindles and plus-end of microtubules throughout the cell cycle (Berrueta *et al*, 1998; Morrison *et al*, 1998; Juwana *et al*, 1999) and EB1 is required for microtubule integrity, the maintenance of cell form (Beinhauer *et al* 1997) and the cell cycle checkpoint mechanism (Muhua *et al*, 1998).

The most C-terminal portion of APC contains the Ser-Thr-X-Val (S/TXV) amino acid sequence, (where X is any amino acid). Two unique proteins bind to this site, the human homologue of *Drosophila* DSG tumour suppressor (Matsumine *et al*, 1996) and the protein tyrosine phosphatase PTP-BL (Erdmann *et al*, 2000). The co-localization of APC and PTP-BL was first observed in the nucleus and cellular protrusions of MDCK cells and this interaction may indirectly modulate the steady-state levels of tyrosine phosphorylations of associated proteins such as β -catenin (Erdmann *et al*, 2000).

1.3.5 APC functions

The most studied role of APC protein is an involvement in Wnt signalling and β -catenin degradation. It also plays roles in cell-cell adhesion, cell cycle regulation and chromosome stability.

1.3.5.1 Wnt Signalling

APC plays a critical role suppressing the Wnt signalling pathway that controls cell proliferation and differentiation. When this pathway is stimulated, β -catenin activates TCF/LEF-dependent transcription of Wnt-target genes such as *CCND1* (Tetsu and McCormick, 1999), *c-myc* (He *et al,* 1998) and *EphB* (Batlle *et al,* 2002) (Figure 1.8).

Wnt ligands are secreted glycoproteins (Polakis 2000) expressed in a tissue specific manner (Seidensticker and Behrens, 2000). They bind to a class of seven transmembrane receptors (eleven in vertebrates) encoded by the frizzled genes through their N-terminal cysteine rich extracellular domains and

activate them, leading to the phosphorylation of a protein called dishevelled (Polakis 2000; Seidensticker and Behrens, 2000). Dishevelled prevents phosphorylation of axin, APC and β-catenin by GSK3β (Kishida *et al*, 1999). Dishevelled binds and alters the conformation of axin via two domains (Kishida *et al*, 1999, Seidensticker and Behrens, 2000). This leads to an increase in mature unphosphorylated β -catenin, which cannot be degraded, causing intracellular accumulation in the cytoplasm and the nucleus (Seidensticker and Behrens, 2000). In the nucleus, β -catenin binds to members of T-cell factor (TCF) and lymphoid enhancer factor (LEF) family of transcriptional activators (Behrens *et al*, 1996). This leads to the formation of complexes that transmit the Wnt signal into the nucleus and activate Wnt target genes.

In the absence of exogenous Wnt signalling factors, APC provides a scaffold for a destruction complex together with GSK3β and axin that promotes phosphorylation and consequent ubiquitin-dependent degradation of β -catenin by the 26S proteosomes (Rubinfeld *et al,* 1996). APC inactivation, aberrantly switches on the Wnt pathway (Peifer and Polakis, 2000) since APC is a negative regulator of the Wnt signalling. Truncating mutations in *APC* have been reported in the majority of CRCs and the spectrum of these mutations suggests a selection against β -catenin regulating domains (reviewed in Polakis 2000). Activating mutations in β -catenin, which affect N-terminus serine and threonine residues, have been reported in ~50% of colon tumours without an *APC* mutation (Sparks *et al*, 1998). Biallelic inactivation of axin in human hepatocellular cancers and cell lines has also been reported (Satoh *et al*, 2000).

Several lines of evidence indicate that APC inhibits β -catenin/TCF/LEF-dependent transcription through other mechanisms: i) APC promotes export of β -catenin from the nucleus, which reduces the amount of nuclear β -catenin/TCF (Henderson and Fagotto, 2002; Neufeld *et al,* 2000; Rosin-Arbesfeld *et al,* 2003), ii) APC binds to β -catenin, blocking the interaction with TCF (Neufeld *et al,* 2000; Rosin-Arbesfeld *et al,* 2003) and iii) recent studies show that APC can inhibit β -catenin/TCF/LEF-dependent transcription

through a direct interaction with a repressor complex: APC binds to β transducin repeat containing protein (bTrCP) and transcriptional repressor C-terminal binding protein (CtBP) and forms a stable complex with additional co-repressors TLE-1 and HDAC1 (Hamada and Bienz, 2002; Sierra *et al,* 2006). Because APC interacts with bTrCP at the *MYC* enhancer, it may facilitate CtBP-mediated repression of Wnt-target genes (Hamada and Bienz, 2004; Sierra *et al,* 2006). Because the C-terminal half of APC mediates binding to CtBP, truncated APC mutants found in cancer cannot stimulate the CtBP-mediated transcriptional repression (Hamada and Bienz, 2004; Sierra *et al,* 2006). Such mutations thus activate β -catenin/TCF-dependent transcription by both increasing levels of β -catenin-TCF complexes in the nucleus and reducing CtBP-mediated inhibition of the complex.

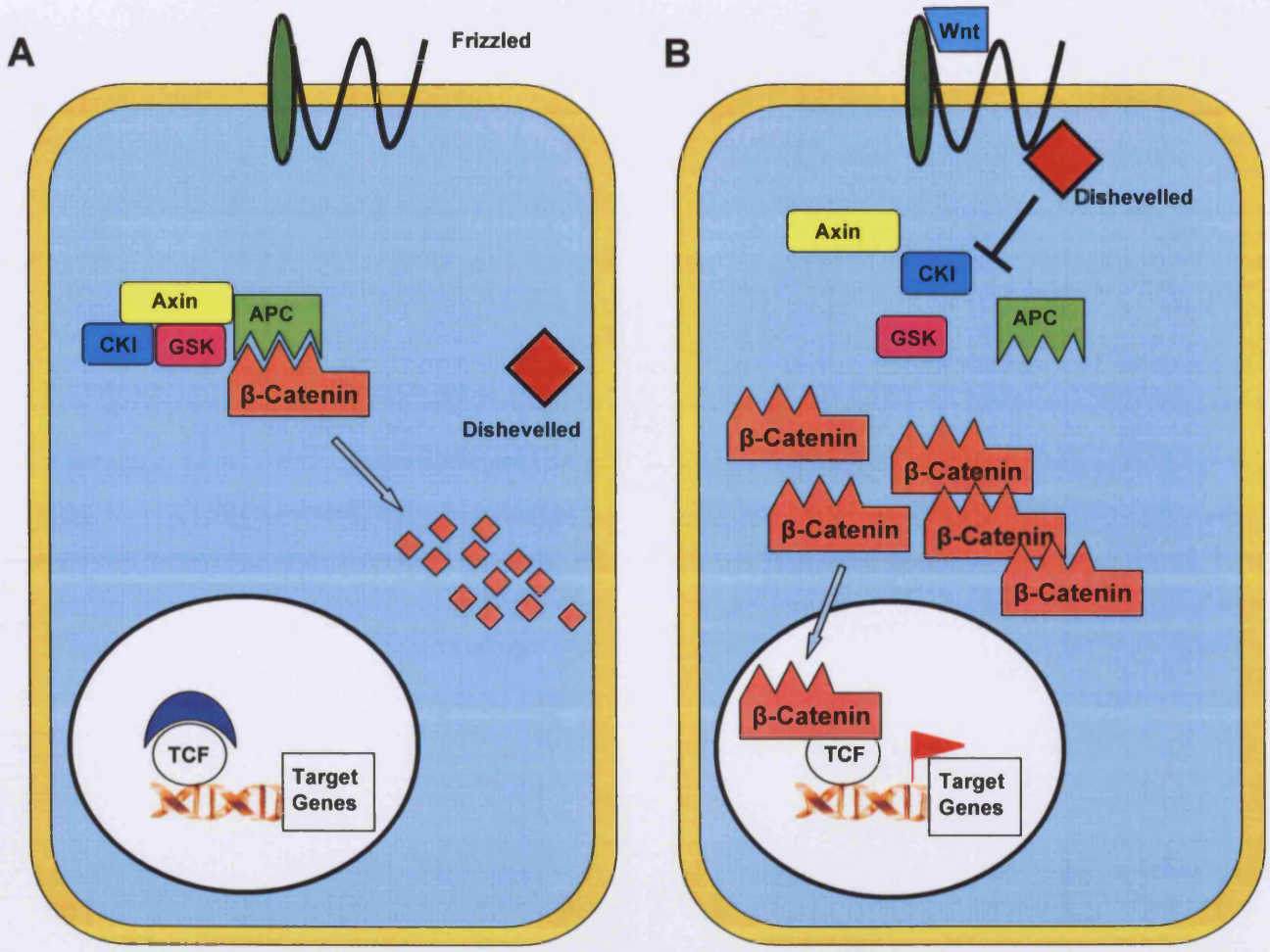


Figure 1.8.Wnt signalling in normal colonic epithelial cells. A) In the absence of Wnt signalling factors, a β-catenin-axin-APC complex is formed and phosphorylated by GSK3β. This promotes the degradation of intracellular β-catenin via the ubiquitin/proteasome pathway, resulting in the repression of Wnt target genes. B) Binding of exogenous Wnt ligand to it's transmembrnae receptor, results in the recruitment of dishevelled from the cytosol leading to the inhibition of the β-catenin destruction complex formation by dephosphorylating axin. β-catenin accumulates in the cytosol and translocates to the nucleus where it binds with TCF/LEF transcription factors and activates the transcription of target genes.

1.3.5.2 Role for APC in cell migration

APC binds with microtubules directly through the C-terminal microtubule binding domain or indirectly through association with EB1. The observation that APC localizes to the plus ends of microtubules in mammalian cells provided the first evidence that APC might play a role in cell migration. APC is prominently localized in puncta at the plus end tips of microtubules (Nathke *et al*, 1996; Mimori-Kiyosue *et al*, 2000; Etienne-Manneville and Hall, 2003; Zhou *et al*, 2004) and is also present diffusely along the length of microtubules (Nathke *et al,* 1996, Barth *et al,* 2002). Interaction of APC with the kinesin super family-associated protein (KAP3) facilitates the transport of APC along microtubules to their plus ends (Jimbo *et al*, 2002). Nathke *et al* (1996) observed that APC localisation occurred at a higher affinity at the tips of actively migrating cells during wound healing assays. APC has since been shown to act as a positive regulator of microtubule stability by binding to non-assembled tubulin and promotes the assembly of branched microtubule arrays (Munemitsu *et al*, 1994; Zumbrunn *et al*, 2001). Additionally, deletion of the microtubule-binding domain of APC results in a decrease in microtubule stability (Smith *et al*, 1994; Zumbrunn *et al*, 2001).

Binding with EB1 via the C-terminal EB1 binding domain at the plus end of microtubules is thought to regulate its role in cell migration (Su *et al*, 1995) since EB1 is known to localise to the plus ends of microtubules and targets APC to the proximal tips (Morrison *et al*, 1998; Barth *et al*, 2002) and EB1 promotes polymerisation of microtubules only when bound to APC (Nakamura *et al*, 2001). Phosphorylation of APC by protein kinase A and p34cdc2 inhibits microtubule polymerisation by reducing its association with EB1 (Nakamura *et al*, 2001) further modulating the role of APC in stabilising and promoting microtubule polymerisation. Cell migration is thought to be promoted via interactions with Rho and the formamin family protein mDia to facilitate microtubule stability at the leading edge of the plasma membrane (Wen *et al*, 2004).

Binding of APC to IQ containing GTPase activating protein 1 (IQGAP1) at the 'leading edge' of the microtubule also regulates cell migration (Noritake *et al*, 2005) by forming a scaffold structure complex with microtubule binding protein CLIP-170 and the activated forms of Rac1 and Cdc42 (Watanabe *et al*, 2004; Briggs and Sacks, 2003).

Cell migration is also promoted by the binding of APC to Asef via the Armadillo repeat domain, which leads to increased cell spreading and membrane ruffling (Kawasaki *et al*, 2000). This activity has been implicated by the ability of truncated APC proteins to stimulate inappropriate cell migration by constitutively activating the guanine nucleotide exchange factor activity of Asef. (Kawasaki *et al*, 2003).

Defects in cell migration due to a loss of APC function could lead to the accumulation of cells in an environment which might promote their proliferation. Moreover, impaired cell migration might increase the survival rate of cells with defective genetic material (Dikovskaya *et al*, 2001).

1.3.5.3 Role for APC in cell-cell adhesion

In addition to its function in gene regulation, β-catenin also participates in cell-cell adhesion via interactions with the members of the cadherin family of proteins (Gumbiner, 2000; Polakis *et al*, 2000). The C-terminal domain of E-cadherin is shown to interact with β- and γ -catenin, which associate with α-catenin and form an E-cadherin complex with the actin cytoskeleton. This complex is known to maintain stable cell-cell adhesion (Gumbiner, 2000). APC is also a part of the cell-cell adhesion complex linked with E-cadherin, since it directly binds with β-catenin, γ-catenin, and actin filament (Ben-Ze'ev and Geiger, 1998; Polakis 2000).

In cells, in addition to its association with Wnt signalling and cell-cell adhesion complexes, β-catenin exists as a free pool in the cytosol (Papkoff, 1997). Thus, the dynamic distribution of β-catenin in different pools may determine its role in different cellular functions adding another facet of importancet of

controlled rates of β-catenin turnover mediated by the axin/conduction/APC/GSK3β destruction complex.

1.3.5.4 Regulation of the cell cycle

Several studies involving the transfection of full length, wildtype APC into human colon adenocarcinoma cell lines such as NIH3T3 and SW480 (Groden *et al*, 1995; Baeg *et al*, 1995) have shown that similar to other tumour suppressor genes, such as Rb or p53, APC plays a role in controlling cell cycle progression. These experiments indicate that maintenance of the G_1-S checkpoint by APC is mediated through effecting components of the Rb pathway and is attributable, at least in part, to regulation of β-catenin/Tcf/Lef–mediated transcription of S-phase regulators such as *cyclin D1* and *c-myc*. A role for APC at the G_2-M transition is also likely given the observations that APC is hyperphosphorylated during M-phase (Bhattacharjee *et al*, 1996) and is a target of the M-phase kinase $p34^{cdc2}$(Trzepacz *et al*, 1997).

The binding of APC to hDLG also implicates a role for APC in control of cell cycle progression. Over expression of APC suppresses cell proliferation through blocking cell cycle progression from G_0/G_1 to the S phase (Baeg *et al*, 1995) and the APC-hDLG protein complex is a negative regulator of cell growth and is essential for cell cycle inhibition (Ishidate *et al*, 2000).

1.3.5.5 Chromosome stability

The localisation of APC to chromosomes during interphase and mitosis are consistent with and imply a role in maintaining chromosomal stability; furthermore, this role was shown not to involve β-catenin (Fodde *et al*, 2001, Kaplan *et al*, 2001), indicating that it occurs independently of APCs role in signal transduction and is mediated through the C-terminal end domain.

Throughout interphase APC is localised in cellular protrusions of microtubules (Nathke *et al*, 1996); during mitosis APC has been reported at kinetochores (Fodde *et al*, 2001; Kaplan *et al*, 2001) and at centrosomes (Banks and Heald, 2004; Louie *et al*, 2004). At kinetochores, it might regulate microtubule-kinetochore attachment or the spindle assembly checkpoint; at centrosomes it could influence centrosome duplication or nucleation of spindle/astral microtubules during mitosis.

By interacting with a complex set of proteins and pathways, APC contributes to differentiation, cell migration, proliferation and adhesion. High cell turnover in the gut lumen means that all cells other than stem cells have a short life span in this tissue. Active migration accompanies cell differentiation to ensure that epithelial cells in the gut are usually replenished within 3-5 days. As a consequence, normal gut maintenance requires that cell-cell and cell-substrate adhesion along with migration, proliferation and differentiation are balanced and maintained at all times. Mutations in *APC* are likely to affect all of these processes, which may explain why mutations in this single gene are sufficient to initiate the development of cancer.

1.3.6 *MUTYH* associated polyposis (MAP)

MAP (MIM 608456) is an autosomal recessive colorectal adenoma and carcinoma predisposition syndrome identified in 2002 by Al - Tassan *et al,* and is thought to be responsible for ~1% of all CRCs (Fleischmann *et al,* 2004). The MAP phenotype closely resembles the AFAP phenotype and presents as i) a variable number of polyps, ranging from a few to >100 (Figure 1.8), ii) early onset of CRC, iii) an absence of vertical transmission from parent to child and iv) sporadic or multiple-case presentations within one generation (Sieber *et al*, 2003; Wang *et al*, 2004; Croitoru *et al*, 2004; Gismondi *et al*, 2004; Farrington *et al* 2005; Balaguer *et al*, 2007). Clinically, differentiating between patients with *de novo* APC mutation (~20% of all FAP cases) and MAP patients can be difficult since there is no family history of polyposis in either case (Grady *et al*, 2003), indeed, Sampson *et al* (2003) reported that 9 of 25 patients with MAP had a phenotype indistinguishable from FAP. Duodenal adenomas that are frequently present in FAP also appear to be the

most common extracolonic feature of MAP (Aretz *et al*, 2006) although other extracolonic manifestations have been reported in MAP patients but not at significant frequencies.

The identification of MAP arose from investigating several siblings from a single family presenting with an AFAP-like phenotype, but in whom a germline truncating APC mutation was not identified. Somatic *APC* mutation analysis in colorectal tumours from the affected siblings revealed that the majority were G:C to T:A transversions resulting in stop codons. The pattern of *APC* somatic mutations was then linked to functionally compromising biallelic germline *MUTYH* mutations in the affected cases (Al-Tassan *et al,* 2002), an association that was subsequently confirmed in further unrelated MAP cases (Jones *et al*, 2002). A mouse model has also shown that homozygous *Mutyh* deficiency increases intestinal tumourigenesis in $Apc^{Min/+}$ mice and some polyps from these mice harboured G:C to T:A transversions resulting in termination codons (Sieber *et al*, 2004). A specific *k-ras* mutation has been identified in some MAP adenomas and is associated with increased dysplasia. The G12C activating mutation is the result of a G:C to T:A transversions (Lipton *et al*, 2003; Jones *et al*, 2004).

1.3.7 The *MUTYH* gene

The MUTYH gene is located on the short arm of chromosome 1, between p32.1 and p34.3 and consists of 16 exons and is 7.1kb in size (Slupska *et al*, 1999). Three major mRNA transcripts (α, β and γ) which differ in their 5' sequence have been observed, indicating that *MUTYH* has three independent transcription initiation sites (Ohtsubo *et al*, 2000). Each transcript can be alternatively spliced giving rise to at least ten different mature transcripts.

1.3.8 Domains of the *MUTYH* protein

In its most abundant isoform as encoded by transcript α3, *MUTYH* is a 59kDA protein (Slupska 1999; Tsai-Wu *et al,* 2000) and exhibits 41% similarity to the MutY homologue in *Eschericia coli* (*E. coli*). *MUTYH* consists of several functional domains enabling it to interact with several protein partners and to bind directly to DNA (Figure 1.10). Located at the N-terminus of the protein the replication protein A (RPA) binding domain facilitates binding of RPA via a conserved motif which includes the sequence K/RXK/R (Parker *et al,* 2001). Several other domains within the N-terminus have been identified as highly conserved motifs due of their homology to MutY. These include the DNA minor groove motif, the pseudo helix-hairpin-helix (HhH) motif, the HhH motif, the iron-sulphur cluster and the adenine recognition motif (Guan *et al*, 1998). The MSH6 binding domain is a 23 amino acid region at the centre of the protein that enables interaction with MutS homologue 6 (MSH6) (Gu *et al,* 2002). The apurinic/apyramidinic endonuclease 1 (APE1) binding site contains the SGXXDV motif (Parker *et al*, 2001) and overlaps with the recently identified Hus1 binding site (Shi *et al*, 2001). The N-terminus also contains the mitochondrial localisation signal (MLS) motif and a functional NLS motif is found at the C-terminus (Takao *et al,* 1999). Parker *et al* (2003) identified six putative MUTYH serine phosphorylation sites; three in the N-terminal region (codons 9, 49 and 85) and the remaining 3 within the C-terminal half of the protein (codons 349, 494 and 504).

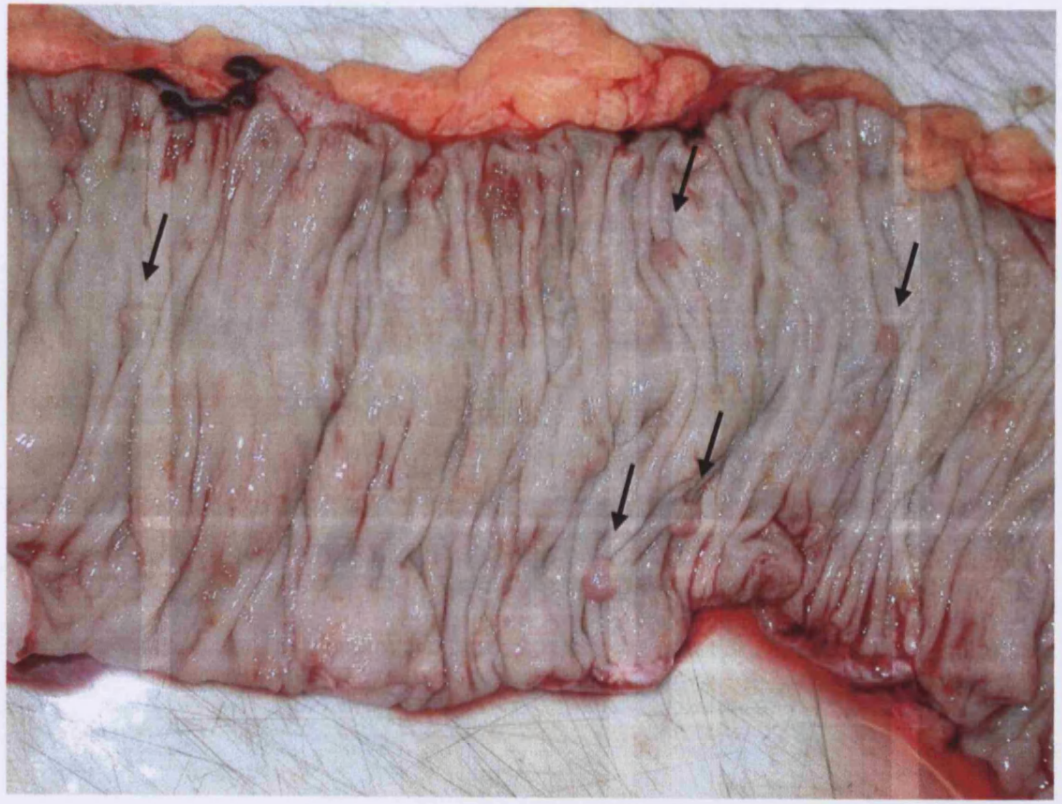


Figure 1. 9 Gross appearance of colon from a patient with MAP showing few colorectal adenomas (indicated by back arrows). Pictures courtesy of Prof. J. Sampson, Cardiff University, UK

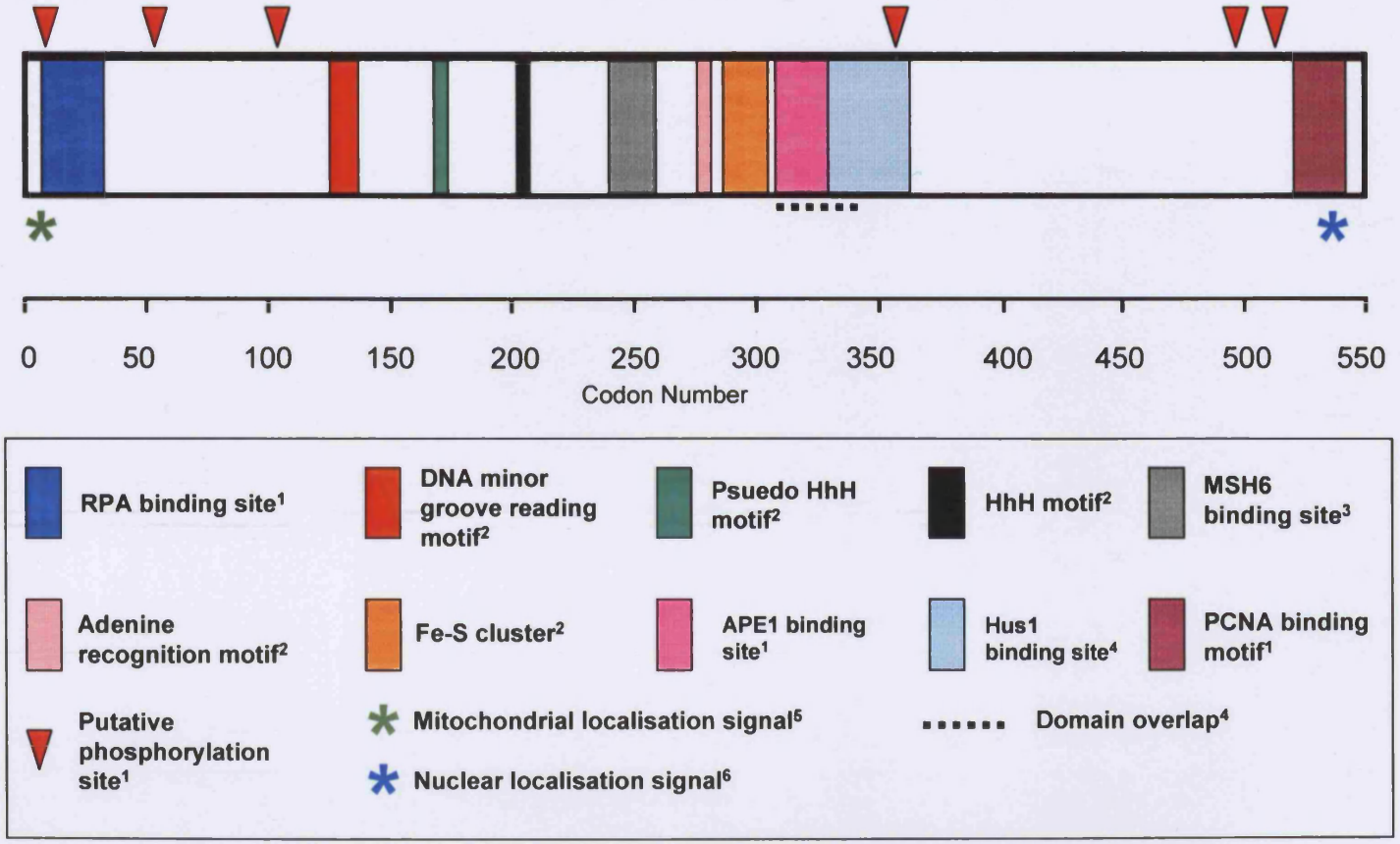


Figure 1.10 Functional domains of the MUTYH protein. As encoded by *MUTYH* transcript α1. [1]Parker *et al*, 2003; [2]Guan *et al*, 1998; [3]Gu *et al*, 2002; [4]Shi *et al*, 2006; [5]Ohtsubo *et al*, 2000; [6]Takao *et al*, 1999.

1.3.9 MUTYH Function

MUTYH is a DNA glycosylase that helps maintain genomic stability through its role in the base excision repair (BER) of oxidative DNA damage. Oxidative damage mediated by reactive oxygen species (ROS) has been linked to the initiation and progression of cancer (Klaunig and Kamendulis, 2004), implicated in neurodegenerative disorders and associated with the cellular deficiencies brought about by ageing. ROS such as hydrogen peroxide, superoxide and hydroxyl radicals are by-products of aerobic metabolism and endogenous inflammatory responses as well as products of environmental exposure to ionizing radiation, transition metals, chemical oxidants and free radicals. ROS are known to attack the lipids, proteins, and nucleic acids of cells and tissues (Lu *et al,* 2001; O'Reilly 2001). Oxidative DNA damage can cause numerous lesions because of the DNA base modification, apurinic/apyrimidinic sites, and DNA protein cross-links which can lead to single strand breaks.

In humans, the frequency of oxidative damage to DNA is estimated to be 10^4 lesions per cell, per day (Ames and Gold, 1991). The extent of the damage can be assessed through a number of intracellular mechanisms, leading to either programmed cell death if the damage is extensive or repair without consequence to the cell if the damage is minimal. If aberrant or modified bases are not repaired before DNA replication, they can subsequently be misread, resulting in mutations (Lu *et al,* 2001).

1.3.10 The BER process

BER is the main mechanism protecting the cell against oxidative DNA damage (Hazra *et al,* 2003). Lesions resulting from methylation, deamination and hydroxylation can also be repaired through this pathway (Hoeijmakers 2001). As shown in Figure 1.11, the damaged base is recognised by a DNA glycosylase and cleaved from the sugar-phosphate backbone, resulting in an apurinic/apyrimidinic (AP) site. Some glycosylases have an AP lyase activity and cleave the sugar-backbone 3' to the AP site. The APE1 endonuclease then processes the DNA in preparation for DNA synthesis. In the absence of a glycosylase lyase activity, APE1 prepares the DNA for synthesis by cleaving the sugar-phosphate backbone 5' to the AP site (Wilson and Bohr, 2007). Recently, Wiederhold *et al* (2004) described an APE1-independent BER pathway in which the NEIL1 or NEIL2 DNA glycosylase

cleaves the DNA at the AP site, leaving a 3' phosphate group which is then removed by polynucleotide kinase (PNK) enabling DNA synthesis (Wiederhold, L. *et al,* 2004). The repair pathway involving monofunctional glycosylases then diverges into either short-patch or long-patch BER (Wilson and Bohr, 2007). If long-patch repair is carried out, DNA polymerase β incorporates the first nucleotide but elongation is carried out by DNA polymerase δ/ε. A two or more nucleotide repair patch is synthesised from the damaged site, facilitated by proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). Flap endonuclease 1 (FEN1) then removes the displaced DNA 'flap' and the nick is ligated by DNA ligase I. In short-patch repair, DNA polymerase β cleaves the sugar-phosphate backbone 3' to the AP site through its phosphodiesterase activity and fills the one nucleotide gap. The remaining nick is sealed by DNA ligase III/XRCC1 (X-ray repair cross complementing 1) heterodimer. DNA synthesis in repair pathways initiated by glycosylases with AP lyase activity or the NEILs is also short-patch and carried out by DNA polymerase β, with the nick ligated by the DNA ligase III/XRCC1 complex. The choice of short patch or long patch repair is dependent on the DNA glycosylase recruited, type of DNA damage present and the cell cycle phase. Direct single strand breaks induced by ROS can also be repaired by either short-patch or long-patch BER once their termini have been prepared for DNA synthesis by the addition of a 3' hydroxyl group and a 5' phosphate group (Fortini and Dogliotti 2007).

1.3.10.1 8-oxoG repair

The DNA base guanine (G) is particularly susceptible to oxidative damage because of its low redox potential (Neeley and Essigmann, 2006). 8-oxo-7, 8 dihydro2'deoxyguanosine (8-oxoG) is one of the most stable deleterious products of oxidative DNA damage and is often used as a cellular biomarker to indicate the extent of oxidative stress in tissues (Klaunig and Kamendulis, 2004) and has been detected in high concentrations in breast, lung and kidney cancers (Okamoto *et al,* 1994; Olinski *et al,* 1992; Malins and Haimanot, 1991; Jaruga *et al,* 1994).

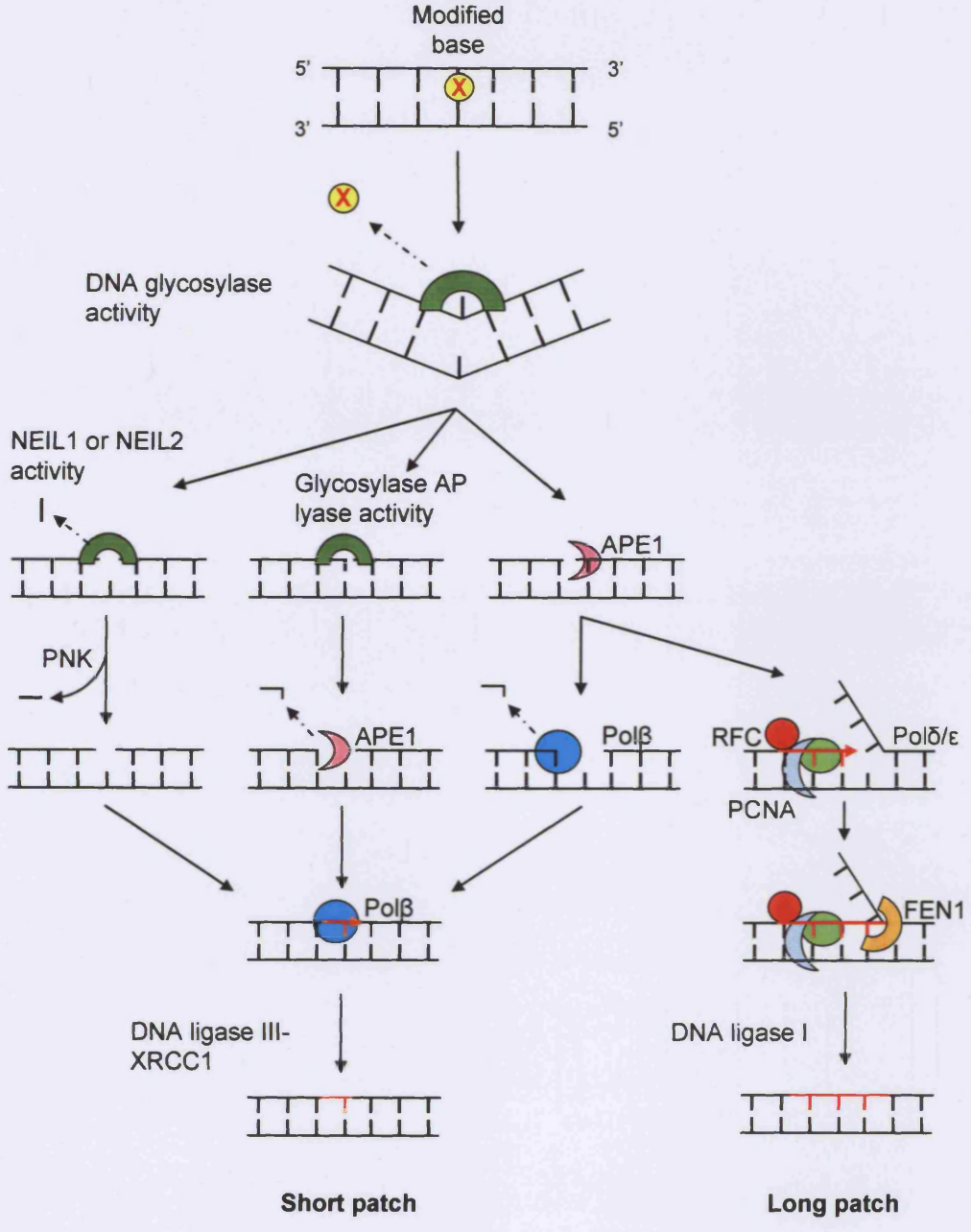


Figure 1.11 Base excision repair of a damaged DNA base. Implementation of short- or long-patch repair is determined by the DNA glycosylase recruited, type of DNA damage and cell-cycle phase. Polβ incorporates the first nucleotide but Polδ/ε carries performs the elongation in the DNA synthesis step for long-patch repair. (Adapted from Wilson and Bohr, 2007; Fortini and Dogliotti, 2007)

DNA replication with 8-oxoG lesions present in the template strand results in G:C→T:A transversion mutations in the DNA daughter strands (Neeley and Essigmann, 2006) since 8-oxoG functionally mimics thymine (T) and readily mispairs with adenine (A) residues during replication. This has been observed to occur at a rate of between five and 200 times greater than the incorporation of cytosine (C) opposite 8-oxoG lesions (Shibutani *et al*, 1991). The BER pathway is an important defence mechanism initiated by lesion-specific DNA glycosylases that repair the effects of oxidative damage (Swanson *et al* 1999) and was initially characterised in *E. coli.* The bacterial enzymes MutT, MutM and MutY were identified as having key roles (Michaels and Miller, 1992) (Figure 1.12). MutT is a nucleoside triphosphate which hydrolyses 8-oxoGTP in the nucleotide pool, preventing its incorporation into nascent DNA (Maki and Sekiguchi, 1992). The MutM DNA glycosylase recognises and excises 8-oxoG paired opposite cytosine (Boiteux *et al,* 1992) and MutY is a DNA glycosylase responsible for removing adenine residues misincorporated into the daughter strand opposite 8-oxoG following replication (Michaels and Miller, 1992). Cytosine is then inserted in place of the excised adenine, resulting in an 8-oxoG:C pair which can be repaired by MutM. The MutS DNA glycosylase is part of the mismatch repair (MMR) system and can perform the same function as MutY (Zhao and Winkler, 2000). Another DNA glycosylase, endonuclease VIII (or Nei) provides a backup pathway for 8-oxoG repair as it can excise this damaged base from 8-oxoG:C pairs (Blaisdell *et al,* 1999) from within the nascent strand when misincorporated opposite adenine during replication (Hazra *et al,* 2001). Human homologues of *mutT, mutM, MutY, MutS* and *nei* have been identified as *NUDT1* (Sakumi *et al,* 1993), *OGG1* (Roldan-Arjona *et al,* 1997), *MUTYH* (Slupska *et al,* 1996), the *MSHs* (Fishel *et al,* 1993) and the *NEILs* (Bandaru *et al,* 2002) respectively.

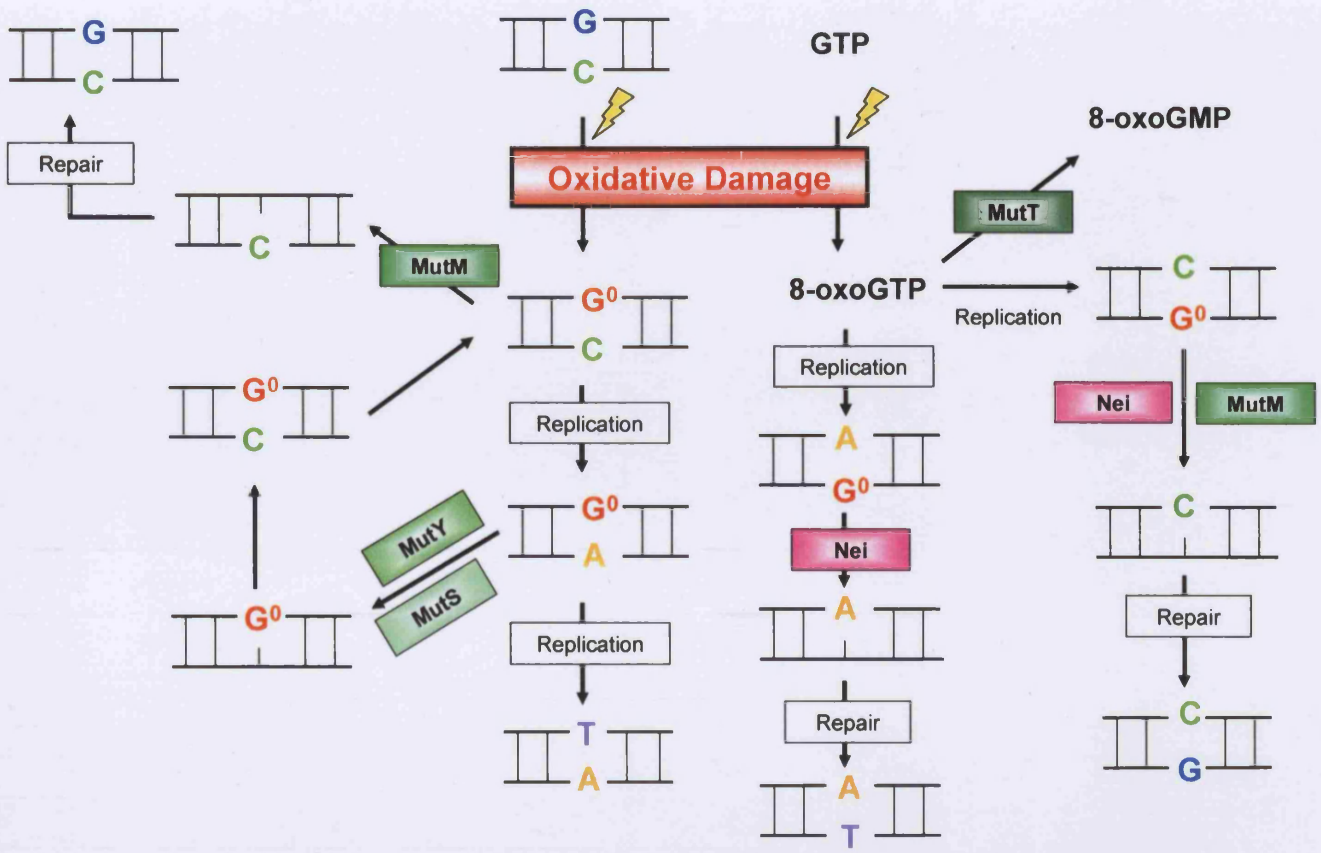


Figure 1.12 The role of bacterial BER enzymes. MutY, MutM, and MutT and Nei participate in the prevention and repair of 8-oxoG associated DNA damage. MutT is an 8-oxo-dGTPase that prevents incorporation into nascent DNA. MutM is a DNA glycosylase that excises the oxidised base from 8-oxoG:C pairs, MutY is a DNA glycosylase that excises adenine residues that have been misincorporated opposite 8-oxoG, MutS is a DNA glycosylase that performs a similar function to MutY within the MMR pathway, Nei is an endonuclease that can excise 8-oxoG when mispaired with cytosine.

1.3.10.2 MutY catalytic mechanism

Bacterial MutY has been characterised more than human MUTYH and as such, has provided the majority of the structural and biochemical data on this enzyme. It is a member of the helix-hairpin-helix (HhH) super family of proteins which all have a conserved catalytic domain containing an HhH motif proceeded by a glycine/proline-rich loop and a catalytic residue (Fromme *et al,* 2004). The N-terminal catalytic domain of MutY consists of two modules: a six-helix barrel containing the HhH motif and an iron-sulphur cluster (Guan *et al,* 1998). The C-terminal domain of MutY is unique among HhH proteins and is necessary for substrate specificity (Noll *et al,* 1999)

The mechanism by which MutY searches for mismatches within the vastness of the entire genome remains unknown, but several models have been proposed. Boon *et al* (2003) suggested a model of long range scanning for mismatches via DNA-mediated charge transport chemistry. Non-specific binding of MutY to DNA causes the oxidation of its iron-sulphur cluster and loss of an electron which is then transported along the DNA. This is eventually intercepted by a second MutY molecule, already non-specifically bound and its iron-sulphur cluster similarly reduced, promoting dissociation of the protein from the DNA. If a mismatch is encountered between the two MutY molecules, the DNA-mediated charge transport cannot occur and the protein remains bound to the DNA and progressively diffuses to the mismatch. The charge transport process is continuous in the absence of a mismatch and may explain why small numbers of MutY molecules can rapidly and effectively scan the genome (Boon *et al,* 2003). Lee *et al* (2004) proposed a model that also required co-operation between MutY molecules. Two MutY monomers simultaneously bind around the same region of DNA to form a dimer. The MutY dimer slides along the DNA until one of the molecules binds specifically to a mismatch resulting in a conformational change occurring in the active monomer and DNA (Lee *et al,* 2004). This is consistent with a report that upon DNA binding, MutY assembles into a dimer and that this is the functionally active state (Wong *et al,* 2003). However in Lee's model the dimer searches for a mismatch but only the active MutY molecule remains bound for catalysis (Lee *et al,* 2004). The six-helix barrel motif interacts with the backbone of the DNA strand containing the complementary 8-oxoG residue (Fromme *et al,* 2004). The C-terminal domain of

MutY confers specificity for 8-oxoG:A mispairs by recognising the 8-oxoG base (Noll *et al,* 1999; Li *et al*, 2000). Extensive contacts are made with the 8-oxoG strand of DNA and the oxidised base itself. 8-oxoG is hydrogen bonded to both the C-terminal and catalytic domains, ensuring that adenines correctly paired opposite thymines are not excised (Fromme *et al*, 2004). MutY mainly contacts the 8-oxoG strand and interacts with DNA via at least five phosphates and purine bases on either side of the mismatch (Lu *et al*, 1995). A double base flipping mechanism was proposed to facilitate recognition of the 8-oxoG:A mispair and aid adenine extrusion into the active site (Bernards *et al*, 2002) but crystal structures of MutY bound to DNA have shown that although the DNA is bent and the mispaired adenine is extruded, 8-oxoG remains within the helix (Fromme *et al*, 2004). The HhH and pseudo HhH motifs at either end of the DNA binding groove compress the DNA intra-strand phosphate diester either side of the mispaired adenine causing the DNA to bend and the target base to 'flip out' of the DNA helix, into the active pocket site (Guan *et al*, 1998). When MutY binds to an 8-oxoG:A mispair, the 8-oxoG nucleoside changes its glycosydic bound conformation from a *syn* to an *anti* orientation. The *anti* conformation of 8-oxoG would sterically clash with an adenine base opposite and so may also promote flipping of adenine into the active site (Fromme *et al*, 2004). 8-oxoG binding to the C-terminal domain facilitates base-flipping and thus accelerates the glycosylase reaction (Noll *et al*, 1999). The active site pocket is complementary to the extrahelical adenine residue, which is deeply sequestered and hydrogen bonded by the MutY protein. The catalytic and C-terminal domains interact and allow the enzyme to surround the DNA duplex (Fromme *et al*, 2004). The 'flipped-out' adenine leaves a gap in the DNA base stack which may be stabilised by the minor groove reading motif. MutY then cleaves the N-glycosylic bond through a hydrolytic mechanism (Guan *et al*, 1998).

1.3.10.3 MUTYH in human BER repair

In humans, the enzymes MTH1, OGG1 and MUTYH constitute the 8-oxoG repair pathway, often referred to as the GO repair pathway (Figure 1.13).The human MutY homologue, MUTYH, demonstrates glycosylase activity with DNA substrates containing the following mispairs: A:G, 2-hydroxyadenine (2-OH-A):8-oxoG; A:8-oxoG, 2-OH-A:G and 2-OH-A:A but exhibits little AP lyase activity (Ohtsubo, T. *et al*, 2000). 2-OH-A is another product of oxidative DNA damage (Lu *et al*, 2001). OGG1 is a purine oxidation glycosylase that recognizes and excises 8-oxoG lesions paired with cytosine. The repair enzyme MTH1 hydrolyzes oxidized purine nucleoside triphosphates such as 8-oxo-dGTP, 8-oxo-GTP, 8-oxo-dATP, and 2-hydroxy-dATP, effectively removing them from the nucleotide pool and preventing their incorporation into nascent DNA (Fujikawa *et al*, 1999).

Mutant MUTYH is defective in binding and repair of A:8-oxoG and 8-oxoA:G mispairs (Parker *et al*, 2005). Phosphorylation of serine residues enhances MUTYH repair of A:8-oxoG mispairs and may regulate protein-protein interactions (Parker *et al*, 2003). MUTYH interacts with APE1, PCNA and RPA1 suggesting that repair of DNA lesions by MUTYH occurs via the long-patch pathway of BER (Parker *et al*, 2005). Indeed, short-patch BER initiated by murine MUTYH is futile and the repair has to proceed to the long patch pathway of BER (Hashimoto *et al*, 2004). MUTYH can interact with Hus1 (part of the 9-1-1 DNA damage sensor complex) and may act as an adaptor for sensor checkpoint proteins following oxidative DNA damage (Shi *et al*, 2006). Nuclear MUTYH co-localises with PCNA at replication foci and levels of MUTYH are maximal during S-phase indicating that MUTYH repair may be coupled to replication (Boldogh *et al*, 2001). Consistent with this, DNA replication enhances murine MUTYH repair through its interaction with PCNA (Hayashi *et al*, 2002). The coupling of replication and MUTYH repair allows MUTYH to be directed to the daughter strand, thereby preventing the A:T to C:G transversions that would arise from adenine glycosylase activity on the template strand.

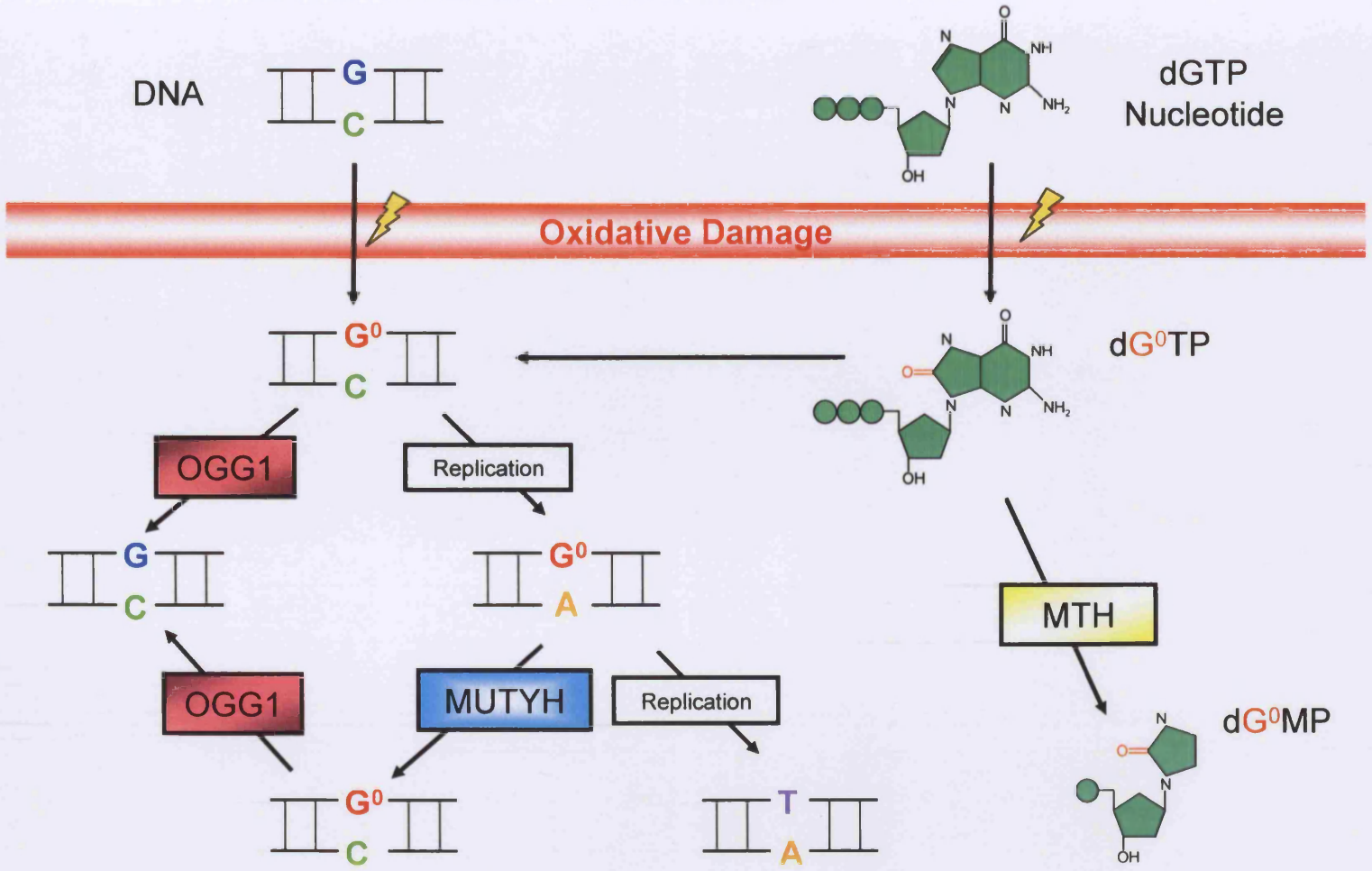


Figure 1.13 The role of mammalian BER enzymes. MUTYH excises adenine residues incorrectly mispaired with 8-oxoG post-replication. OGG1 excises the 8-oxoG lesion and MTH is an 8-oxo-dGTPase that prevents incorporation into nascent DNA.

MUTYH also interacts with the MMR protein MSH6 which forms a heterodimer with MSH2. The mammalian MSH2/MSH6 complex can repair 8-oxoG incorporated into the daughter strand during replication (Colussi *et al*, 2002) and A:8-oxoG mismatches where the adenine is on the template strand are mainly recognised by this complex (Gu *et al*, 2002). However, it has been reported that MSH2/MSH6 repair of 8-oxoG incorporated during replication opposite adenine or cytosine is inefficient (Larson *et al*, 2003). Recently Macpherson *et al* (2005) have shown MSH2/MSH6 can efficiently recognise 8-oxoG opposite adenine or cytosine when these mispairs lie within repetitive sequences that have undergone a slippage event (Macpherson *et al*, 2005). The MSH2/MSH6 complex binds 8-oxoG:G and 8-oxoG:T mispairs efficiently and although such mispairs are rare *in vivo*, when the oxidised base is on the template strand MMR can result in 8-oxoG:A mispairs which are recognised by MUTYH (Larson *et al*, 2003). The MSH2/MSH6 dimer can enhance binding of MUTYH to A:8-oxoG mismatches and interactions between these repair proteins may help target MUTYH to the daughter strands (Gu *et al*, 2002). Wong *et al* (2003) proposed a mechanism by which dimeric MutY could determine the level of oxidative damage present and remove adenines only present on the nascent strand. A:8-oxoG mispairs resulting from incorporation of adenine into the nascent strand opposite 8-oxoG are found in isolation and repaired by MutY. However, A:8-oxoG mispairs formed as a result of incorporation of 8-oxo-GTP into the nascent strand opposite adenine would arise as patches of damage. Wong *et al* (2003) suggested that concurrent binding of two of these mispairs by dimeric MutY would inhibit its glycosylase activity, thereby preventing A:T to C:G transversions and allow recruitment of more appropriate repair enzymes such as those involved in MMR (Wong *et al*, 2003). Mammalian MUTYH protects its DNA product from OGG1 as this DNA glycosylase could excise 8-oxoG opposite the AP site, resulting in a loss of informative bases on both strands and a double break strand. MUTYH may encircle and tightly bind the DNA duplex much like its bacterial homologue, thus physically preventing OGG1 from accessing the DNA (Tominaga *et al*, 2004).

1.3.11 Germline mutations in *MUTYH*

To date, approximately 85 mutations have been described in MAP patients from Western populations (Cheadle and Sampson; 2007). Around thirty percent are predicted result in a truncated protein and comprise of nonsense, small insertion/deletions and splice site variants (Fig. 1.14). Additionally, 52 missense variants and three small inframe insertion/deletions have been reported and are distributed throughout the gene. The Y165C (Tyr165Cys) and G382D (Gly382Asp) nonsynonymous mutations account for approximately 73% of all *MUTYH* mutations reported in Caucasian MAP patients but this is likely to be an overestimation as many groups search specifically for these mutations. The Y165C mutation is located in the pseudo HhH region that is involved in mismatch specificity and flipping of the adenine into the base specificity pocket whilst the G382D variant is located in the C-terminal domain involved in 8-oxoG recognition.

To-date, a limited amount of functional characterisation of a few *MUTYH* mutations has been carried out. Al-Tassan *et al* (2002) initially showed that the Y82C and G253D MutY proteins in *E. coli* (homologues of Y165C and G382D variants of human MUTYH, respectively) were partially defective in removing mispaired adenine bases. Wooden *et al* (2004) reported that recombinant Y165C and G382D MUTYH proteins were totally devoid of glycosylase activity. Parker *et al* (2005) confirmed these findings using lysates derived from lymphoblastoid cell lines from MAP patients who expressed either the Y165C and G382D variants as well as the 1103delC mutation to show they exhibited lowered DNA binding and adenine cleavage activities with heteroduplex oligonucleotides containing A:8-oxoG and 8-oxoA:G mispairs. Bai *et al* (2005) characterised the R227W and V232F MUTYH mutants known to lie within the putative hMSH6 binding domain and showed that they were also defective in both DNA substrate binding and glycosylase activity whilst observing that physical interaction with the hMutSα heterodimer was unaffected. Both of these mutants also failed to complement bacterial MutY deficiency when expressed in *E coli* cells *in vivo*. The R231L nonsynonymous variant has also been shown to be severely defective in DNA substrate binding and in adenine removal activity and whilst the variant showed normal binding activity with hMutSα, it did not complement MutY deficiency in *E coli*. (Bai *et al*, 2007).

Unlike other genes involved in inherited CRC predisposition, somatic mutations in *MUTYH* do not appear to be involved in sporadic colorectal tumourigenesis. No somatic *MUTYH* mutations were found in 75 sporadic CRCs and functional MUTYH mRNA and protein were detected in all of the 35 CRC cell lines examined, indicating that epigenetic silencing was not occurring (Halford *et al*, 2003).

1.3.12 Hereditary non-polyposis colorectal cancer (HNPCC)

HNPCC (MIM 114500) is the most common hereditary form of CRC, accounting for 2-5% of cases. HNPCC, also known as Lynch Syndrome, is an autosomal dominant inherited disease (Potter, 1999) predisposing to CRC and characterised by an early age at onset (average age is typically 44 years compared to an average age of 64 for sporadic CRC in the general population). HNPCC related CRCs have a propensity to develop in the right colon with the majority of carcinomas probably develop from pre-existing adenomatous polyps. Despite the development of pre-cancerous adenomas in HNPCC, individuals do not exhibit the same florid polyposis phenotype associated with FAP (Lynch *et al*, 1991).

HNPCC patients can exhibit synchronous and metachronous CRCs as well as other primary extracolonic malignancies, such as carcinomas of the endometrium, stomach, small intestine, liver and biliary tract, pancreas, ovary, brain and transitional cell carcinoma of the urethra and renal pelvis (Bellacosa *et al*; 1996, Mecklin and Jarvenin, 1991; Watson and Lynch, 1993); predisposed individuals have lifetime risk of 50–80% of developing colorectal carcinoma, 50–60% of endometrial carcinoma and below 15% risk for other tumours (Watson and Lynch, 1991; Aarnio *et al*, 1995). Muir Torre syndrome (MIM 158320) is a rare autosomal dominant condition considered to be a variant of HNPCC and is characterised by the development of primary malignancies and sebaceous gland tumours. (Lynch and de la Chapelle, 2003). Turcots Syndrome (MIM 276300) is also considered to be a variant of HNPCC despite the full molecular pathogenesis of the disease being undetermined. It is characterized by the development of colorectal adenomas and central nervous system tumours such as medulloblastoma and gliomas.

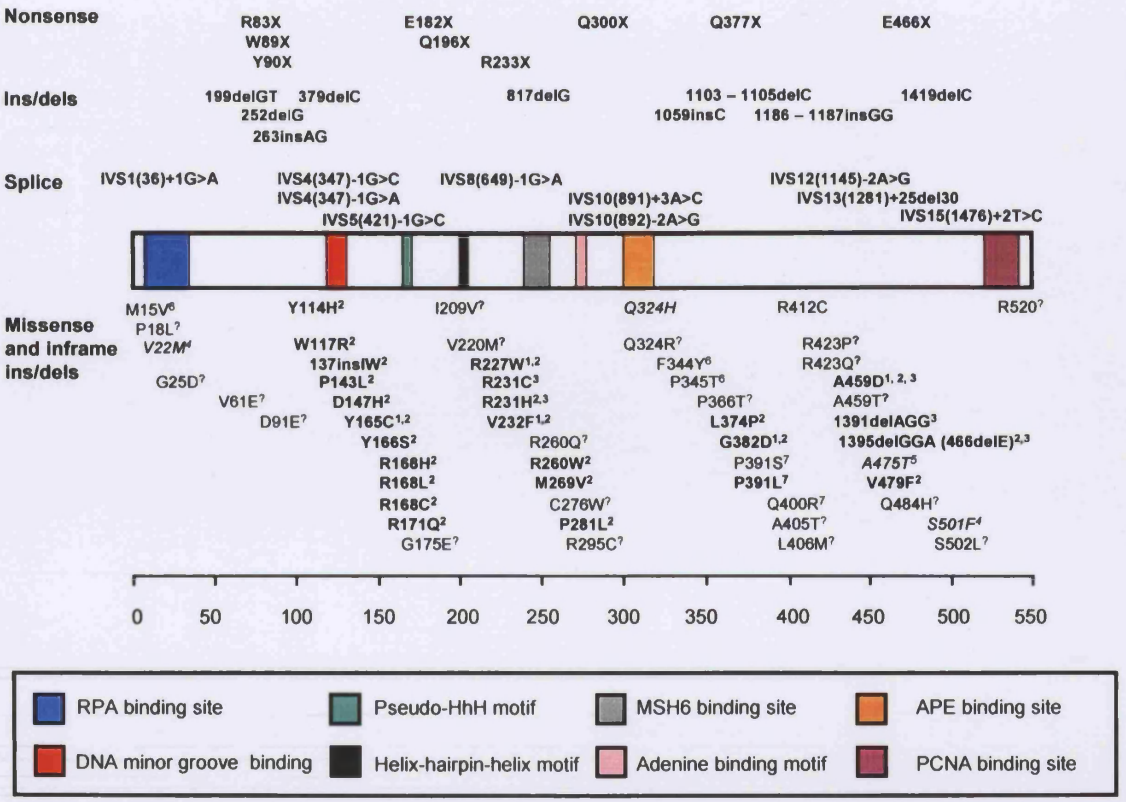


Figure 1.14 Spectrum and distribution of truncating mutations (shown above the locus) and missense/inframe insertion/deletion variants (shown below the locus) identified in *MUTYH*. Approximate positions of putative functional domains are indicated in relation to the *MUTYH* coding region. Missense variants proven or likely to be pathogenic are highlighted in bold (1: data demonstrating functionally compromised, 2: rare variant found in combination with a proven MUTYH mutation in a patient with CRAs, 3: rare variant found in a homozygous state in a patient with CRAs), those that are unlikely to pathogenic are italicised (4:common polymorphism, 5: identified in cases with biallelic *MUTYH* mutations) and those that are undefined are in regular font (6: rare variant found in combination with another *MUTYH* mutation in a patient with CRC under the age of 60 and with 2 or more affected family members, 7: somatic mutation in gastric cancer, ?: no clear evidence for pathogenicity). Adapted from Cheadle and Sampson, 2007.

1.3.13 DNA mismatch repair

HNPCC and its variants arise through germline mutations in one of several genes resulting in impaired DNA MMR function (Berends *et al*, 2002; Bronner *et al*, 1994; Leach *et al*, 1993; Liu *et al*, 1994; Nicolaides *et al*, 1994; Papadopolous *et al*, 1994) (Table 1.3). Each of these genes encodes a protein involved in MMR, a complex enzymatic proofreading system that corrects base pair mismatches and insertion–deletion loops that result in single base substitutions and microsatellite instability respectively. Such errors occur because of DNA polymerase slippage during DNA replication (Peltomaki, 2001).

Syndrome	Mode of Inheritance	Gene/locus
Hereditary non-polyposis colorectal cancer	AD	*MSH2*, 2p21 *MLH1*, 3p21-23 *MSH6*, 2p21 *PMS2*, 7p22 (*MLH3*, 14q24.3)
Muir-Torre Syndrome	AD	MSH1, MLH1
Turcot's syndrome	AD	MLH1, MSH2, PMS2, MSH6

Table 1.3. Genes causing predisposition to HNPCC and known variants. AD: autosomal dominant.

Detection of DNA damage is the initial process for MMR (Figure 1.13) and in humans this role is carried out by two heterodimeric complexes: 1) MSH2/ MSH6 which is responsible for recognising single base mismatches and small insertion deletion loops (<12 unpaired bases) and 2) MSH2/MSH3, which predominantly recognises insertion deletion loops (small or large) (Kolodner 1993; Marsischky *et al,* 1996; Greene *et al,* 1997). Subsequent to this, a MLH1/PMS2 heterodimer is recruited to the MSH2 that is already bound to mispaired bases (Jascur and Boland 2006; Plotz *et al*, 2006). The MLH1/MLH3 heterodimer may function in repair of MSH2/MSH6 substrates, but it is not thought to play a major role in MMR, *in vivo* (Cannavo *et al*, 2005). A number of other proteins are involved in MMR and these include DNA polymerase δ, RPA, PCNA, RFC, exonuclease I, FEN1 (RAD27), DNA polymerase ε ad associated exonucleases (Syngal *et al*, 1999). Repair of the mismatched DNA proceeds by activating exonulcease mediated degradation of DNA from a "nick" that is a distance of up to 1-2 kb from the mismatch (Sancar 1999). Degradation continues until the mismatched base is removed. The resulting long excision tract is filled in by DNA polymerase δ which inserts the correct nucleotide into the sequence and the resulting nick is ligated.

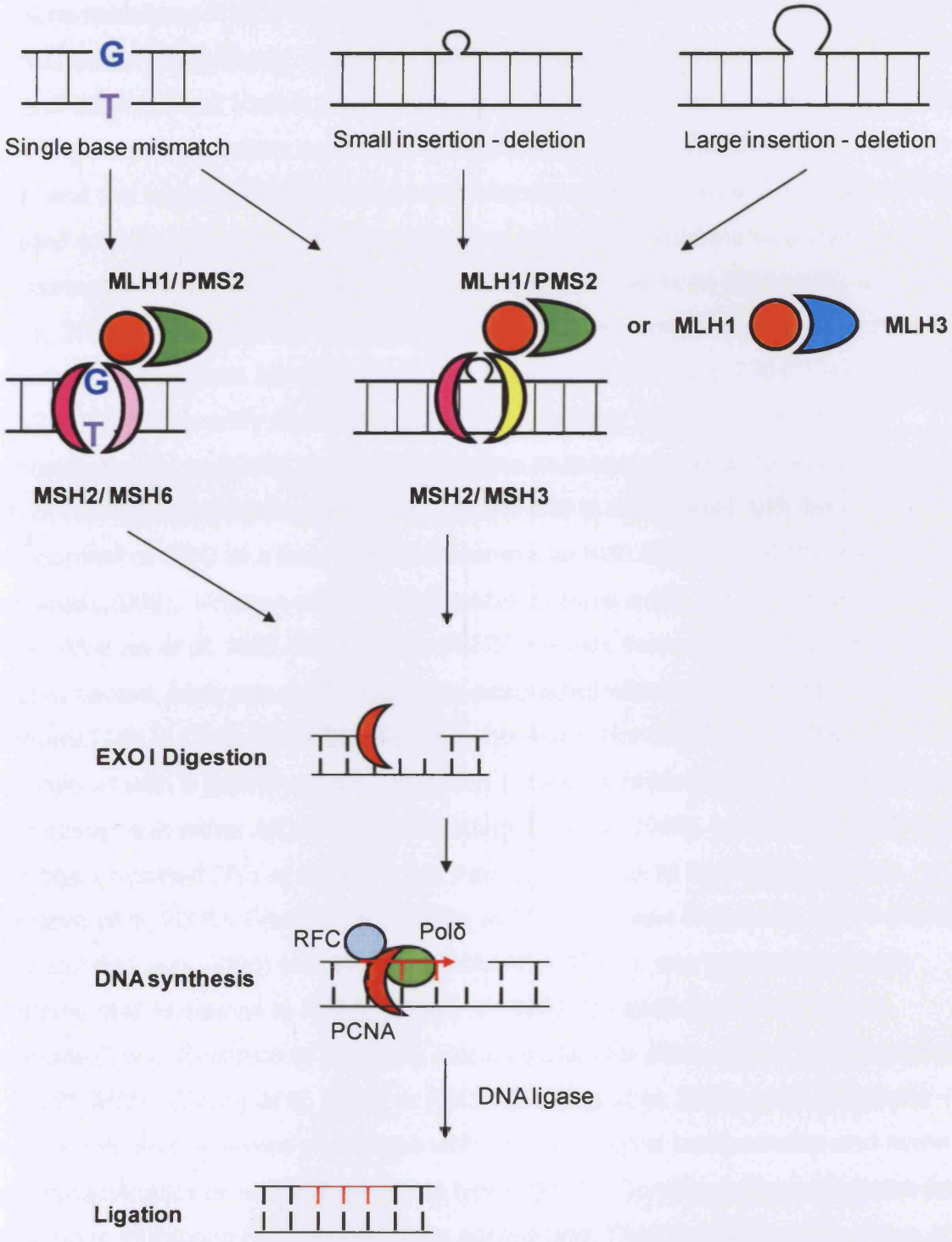


Figure 1.15. The human mismatch repair pathway.

1.3.13.1 Germline mutations in MMR genes

Germline mutations in *MSH2* and *MLH1* account for 38% and 49% of reported HNPCC cases respectively. The other four genes account for approximately 13% of cases (Peltomäki and Vasen, 2004; Peltomaki, 2001). Germline mutations identified in MMR genes do not show a propensity to occur within specific regions or 'hot spots' and the majority of mutations have been reported only once. The nature of the reported germline mutations is heterogeneous and includes nonsynonymous, splice site, nonsense, small deletions and large deletions or insertions (Peltomäki and Vasen, 2004; Papadopoulos and Lindblom, 1997). The intragenic position of the germline mutation does not affect the phenotype (de la Chapelle 2004) however *MSH2* is more frequently mutated in patients presenting with extracolonic tumours(Lynch *et al*, 2006). An MSH6 germline mutation accounts for approximately 10% of HNPCC associated germline mutations and is associated with the development of CRC at a later age when compared with *MSH2* or *MLH1* mutations (Peltomäki 2005). Women with a *MSH6* mutation have a high risk of endometrial cancer (Wijnen *et al*, 199). Mutations in *PMS2* are less frequent and were thought to result in severe, early onset HNPCC often associated with brain tumours (Turcot syndrome) (de la Chapelle 2004). Recently however, Hendriks *et al* (2006) reported that patients with a germline *PMS2* mutation showed a milder phenotype than those with mutations in either *MLH1* or *MSH2* (Hendriks *et al*, 2006). Mutations in *MLH3* have been reported (Wu *et al*, 2001) but their contribution to HNPCC is unclear (Cannavo *et al*, 2005). Germline mutations in *MSH2*, or less frequently *MLH1* (Ponti, and Ponz de Leon, 2005) and *MSH6* (Arnold *et al*, 2007), can cause Muir-Torre syndrome and mutations in *MSH2, MLH1 or PMS2* are associated with Turcot syndrome (Lucci-Cordisco *et al*, 2003). Homozygotes for either *MSH2* (Whiteside *et al*, 2002), *MLH1* (Wang *et al*, 1999) or *PMS2* (De Vos *et al*, 2004) point mutations are rare and have a severe phenotype with haematological malignancies and some skin characteristics of neurofibromatosis type 1 (NF1). Compound heterozygotes for *PMS2* point mutations have a phenotype consist with Turcot syndrome (De Rosa *et al*, 2000). Recently, Will *et al* (2007) described a large (400kb) homozygous *PMS2* deletion in a patient with 10 CRCs, 35 colorectal adenomas and duodenal cancer in his twenties. The patient had no brain tumour but had mental retardation and café au lait spots. This case shows that gross homozygous mutations in a MMR gene can result in a severe phenotype uncharacteristic of HNPCC (Will *et al*, 2007).

1.3.13.2 Somatic mutations in HNPCC

Somatic inactivation can occur via a deletion (LOH) (Parsons *et al*, 1993), mutation (Hemminki *et al,* 1994) or methylation of CpG islands in the MLH1 promoter (Kuismanen *et al,* 2000; Kuismanen *et al*, 1999). A somatic inactivating mutation in the inherited wildtype allele of the MMR gene results in defective MMR (Yuen *et al*, 2002). The MMR genes behave like tumour suppressor genes since cells heterozygous for MMR gene mutation function normally (Parsons *et al*, 1993) but somatic inactivation of the wild type allele leads to loss of tumour suppressor function and is required for tumourigenesis (in accordance with Knudson's hypothesis). Therefore, errors normally corrected by MMR are left unrepaired, leading to a mutator phenotype which increases the chance of mutations occurring in growth regulatory genes (Ionov *et al*, 1993, Fishel *et al*, 1993).

MMR deficiency increases mutation rates in tumour cells by 100–1000-fold compared to normal cells, so the development of CRC at an early age and accelerated tumour progression may be explained by the accumulation of replication errors over the first 4 decades of life. Also, de Jong *et al* (2004) identified that mutations in MMR genes also predisposed carriers to the development of CRAs effectively increasing the predisposition to CRC since adenomas may be considered carcinoma precursor lesions

1.3.14 Microsatellite instability (MSI)

MSI is a consequence of defective MMR (Thibodeau *et al*, 1993) and colorectal tumourigenesis in HNPCC. Microsatellites are short repetitive DNA motifs of 1 – 6 nucleotides in length that are repeated about 10 – 60 times (Beckmann and Weber, 1992). They are present throughout the genome but are more common in non-coding regions. They are highly polymorphic throughout the population, but uniform in the DNA of any one individual (Beckmann and Weber, 1992).

MSI is observed in the majority of tumours in HNPCC cases, whilst only being detected in approximately 10-15% of sporadic CRCs (Thibodeau *et al*, 1993; Ionov *et al* 1993). Tumours deficient in MLH1, MSH2 or PMS2 show MSI at no less than 40% of loci and are termed MSI-high (Peltomäki 2005; Umar 2004), whereas tumours from patients with *MSH6* mutations can have lower levels of instability

(Peltomäki 2005). Some sporadic tumours also display MSI but unlike HNPCC tumours, their MMR deficiency is most often the result of hypermethylation of the *MLH1* promoter (Kuismanen *et al*, 2000).

MSI contributes to the development of cancer when microsatellites within coding regions of genes are disrupted i.e. through DNA slippage or frameshift mutations. *TGFβRII* (transforming growth factor β receptor II), *TCF-4* (T-cell transcription factor), the apoptosis related genes *BAX*, *CASPASE-5* and *AXIN2* are all susceptible to MSI and implicated in the development of various carcinomas (Duval *et al*, 1999; Rampino *et al*, 1997; Liu *et al*, 2000; Markowitz *et al*, 1995; Myeroff *et al*, 1995).

1.4 Aims of this thesis

To address the potential role of rare inherited nonsynonymous variants of *APC* in inherited predisposition to CRAs.

To investigate the effect of the *APC* nonsynonymous variant E1317Q on mechanisms of somatic mutagenesis in colorectal tumourigenesis; specifically to examine itseffect on targeting of '2nd hit' somatic mutations towards the MCR of *APC*.

To determine the extent to which tumourigenesis in MAP is driven by deficient BER activity and the subsequent mutator phenotype.

Chapter Two: Materials and Methods

2.1 Suppliers

Consumables and equipment used throughout this study were purchased from the following companies:

Abgene (Surrey, UK),

Ambion (Cambridgeshire, UK),

GE Healthcare (Buckinghamshire, UK),

Anachem (Bedfordshire, UK),

Applied Biosystems (Cheshire, UK),

Beckman Coulter (Buckinghamshire, UK),

Bibby Streilin (Staffordshire, UK),

Bioquote (Yorkshire, UK),

Biorad (Hertfordshire, UK),

Cell Signalling Technologies (Danvers, MA, USA),

Corning CoStar (The Netherlands),

Difco Laboratories Ltd (Surrey, UK),

DuPont Instruments (Hertfordshire, UK),

Eppendorf (Cambrdgeshire, UK),

Eurogentec (Hampshire, UK),

European Collection of Cell Cultures (Salisbury, UK),

Fisher Scientific (Leceistershire, UK),

Fluka Biochemika (Dorset, UK),

IKA (Staufen, Germany),

Imgenex (San Diego, CA, USA)

Invitrogen Life Technologies (Strathclyde, UK),

Jencon-PLS (Bedfordshire, UK),

Kendro Laboratory Products (Hertfordshire, UK),

Labtech International (East Sussex, UK),

Leica (Wetzlar, Germany),

Millipore (Hertfordshire, UK),

MJ Research (Massachusetts, USA),

MWG Biotech (Buckinghamshire, UK),

New England Biolabs (Hertfordshire, UK),

Olympus Optical (London, UK),
PALM (Bernried, Germany),
Promega (Hampshire, UK),
Qiagen (West Sussex, UK),
Roche Biochemicals (East Sussex, UK),
Sartorius (Epsom, UK),
Santa Cruz Biotechnologies (Santa Cruz, CA, USA),
Sigma Ltd (Dorset, UK),
StarLab (Milton Keynes, UK),
Starstedt (Germany),
Stratagene (California, USA)
Thermo Electron Corporation (Hampshire, UK),
VWR International Ltd (Dorset, UK)

2.2 Materials

2.2.1 Chemicals

Analytical grade chemicals were purchased from either Sigma Ltd or Fisher Scientific unless otherwise stated.

2.2.2 Nucleic acid extraction and purification

QIAamp DNA Micro and QIAprep Mini kits were purchased from Qiagen. Trizol reagent was obtained from Invitrogen Life Technologies. DNA-*free* kit was obtained from Ambion Inc.

2.2.3 First strand synthesis

Superscript III First-Strand Synthesis System for RT-PCR was purchased from Invitrogen Life Technologies.

2.2.4 PCR and PCR purification

AmpliTaq Gold DNA polymerase and GeneAmp PCR buffer were obtained from Applied Biosystems. Deoxynucleotidetriphosphates (dNTPs) were purchased from GE Healthcare. HPSF purified oligonucleotide primers were supplied by either MWG-Biotech or Eurogentec. Exonuclease 1 was purchased from New England

Biolabs and Shrimp alkaline phosphatase as supplied by GE Healthcare respectively. QIAquick PCR purification kit was obtained from Qiagen.

2.2.5 Electrophoresis

Multipurpose agarose was purchased from Roche. Ethidium bromide was supplied by Fluka Biochemika whilst the 100bp and 1kb DNA ladders were supplied by New England Biolabs and Invitrogen Life Sciences, respectively.

2.2.6 Sequencing

BigDyeTerminator Cycle Sequencing Kit (Version 3.1), POP6 polymer, HiDi Formamide and Genescan 500-ROX size standard were all supplied by Applied Biosystems. Montage SEQ_{96} sequencing reaction clean up kits were obtained from Millipore and capillary electrophoresis running buffers were purchased from Sigma.

2.2.7 Antibodies

All antibodies were purchased from Cell Signalling Technology unless otherwise stated.

2.2.8 Protein purification and detection

Protein G-Sepharose beads were obtained from GE Health. Nupage Novex 3-8% Tris-Acetate Gels, Novex Sharp pre-stained protein ladders, SDS Running buffer and nitrocellulose transfer membranes were obtained from Invitrogen Life Technologies. Enhanced chemiluminescence (ECL) analysis system was purchased from GE Health.

2.2.9 Restriction enzymes

All restriction endonucleases were supplied, along with the appropriate buffer and BSA, by New England Biolabs.

2.2.10 Expression vector

The pCMV-APC vector contains a full length human *APC* cDNA under the control of a cytomegalovirus promoter and was a kind gift from B. Vogelstein at Johns Hopkins University, Baltimore, USA.

2.2.11 Site directed mutagenesis

Quickchange XL Site Directed - Mutagenesis kit (which included *Pfu*Turbo DNA polymerase and *Dpn*I restriction endonuclease) was purchased from Stratagene.

2.2.12 Cloning

pGEM-T Easy Vector System I was obtained from Promega and subcloning efficiency DH5α chemically competent *Escherichia coli* (*E. Coli*) cells were obtained from Invitrogen Life Technologies. Tryptone, yeast extract and agar were supplied by Difco Laboratories Ltd. Ampicillin, X-gal (5-bromo-4-chloro-3-indoyl-D-galactoside) and IPTG (isopropyl-β-D-thio-galactopyranoside) were obtained from Sigma Ltd.

2.2.13 Clinical Material

All tissue and blood samples were obtained with patient consent and ethical approval for research in accordance with guidelines of the Welsh Polyp study.

2.2.14 Cell lines

The SW480 colon cancer cell line was obtained from the European Collection of Cell Cultures (ECACC). Primary cell lines were established in-house from clinical biopsies.

2.3 Equipment

2.3.1. Plastics and glassware

Sterile Gilson pipette tips were supplied by StarLab. RNase- and DNAse-free sterile barrier tips were obtained from Promega. Distristrips and sterile tips for multi-channel pipettes were from Anachem. Sterile 5ml, 10ml and 25ml stripettes were from Corning CoStar. 0.65ml, 1.5ml and 2.0ml plastic eppendorfs were supplied by Bioquote and 1.5ml microcentrifuge tubes were supplied by Fisher scientific. 96 well Thermo-fast PCR reaction plates were obtained from Thermo Electron Corporation whilst adhesive PCR sealing sheets, 0.2ml plastic strip tubes and 96 well Thermo-fast skirted detection plates were purchased from ABgene. Sterile universals and petri dishes were obtained from Bibby Sterilin and Starstedt respectively. Glass flasks and beakers were provided by Jencon-PLS or Fisher Scientific. Optilux 96-well

luminometer plates were purchased from VWR International. Tissue culture flasks (T25, T75 and T125) were purchased from Nunc.

2.3.2 Laser microdissection

Laser capture microdissection was carried out using the PALM Microlaser system and visualised using the PALM Robo software.

2.3.3 Thermocycling

Thermocycling was carried out using either an MJ Research DNA Engine Tetrad PTC-225 or an Applied Biosystems GeneAmp 9700 for PCR and RT-PCR respectively.

2.3.4 Electrophoresis

DNA electrophoresis was carried out using a Horizon 11.14 gel tank from Invitrogen Life Technologies. Visualisation of ethidium bromide stained gels was achieved using a BioRad GelDoc XR transluminator. Protein gel electrophoresis was carried out using NuPAGE Novex Tris-Acetate Gels and the XCell SureLock Mini horizontal gel tank from Invitrogen Life Technologies. Power packs were supplied by BioRad.

2.3.5 Other equipment

DuPont Instruments supplied the Sorvall RT6000B refrigerated centrifuge and the Sorvall Legend RT centrifuge was provided by Kendro Laboratory Products. The minishaker MS2 was obtained from IKA. The Biofuge pico centrifuge and the vortex genie 2 were obtained from Jencon-PLS. Sartorius supplied the B1200 top pan balance. Quantitation of DNA and RNA was carried out using the NanoDrop ND-1000 spectrophotometer (Labtech International). The Olympus BX51 BF fluorescent microscope with an attached mercury lamp CCD (charge coupled device) camera was obtained from Olympus Optical.

2.3.6 Software

Fluorescent images were analysed using AxioVision software from Carl Zeiss Vision. Statistics and graphing was carried out using Minitab 15, Microsoft Excel and SPSS16.

2.4 Methods

2.4.1 General reagents

All solutions were made using MilliQ water and autoclaved at 15lb/sq.in at 121°C for 40 minutes where necessary.
TAE buffer: 40mM Tris-acetate, 1m MEDTA pH8

2.4.2 Freezing and sectioning clinical samples

Clinical samples were processed immediately after pathological investigation. Tissue was placed onto cork disks and covered with OCT embedding medium. Disks were snap frozen by being plunged into liquid nitrogen-cooled isopentane until frozen and stored in cryotubes at -70°C. Frozen sections were routinely sectioned at 10µm on a Bright cryostat and placed onto poly-L-lysine and UV treated (254nm) PALM membrane covered glass slides. Sections were air-dried at room temperature for 2 hours and stored at -20°C.

2.4.3 Laser capture microdissection (LCM)

Laser micromanipulation provides microscopic high-resolution control of sample composition by enabling the selection or rejection of user defined areas. For LCM, snap-frozen tissues were sectioned as detailed above and stained using toludene blue by immersion of a clean slide into solution for 2 seconds. Following a wash with water, sections were passed through 50%, 70% and 100% ethanol for 15 seconds and either air dried overnight or for 1 hour at 37°C in. A pulsed ultra-violet laser is interfaced into the microscope and focused through an objective. The laser cuts the tissue without the heating of adjacent material and results in a clear-cut gap between the desired sample area and the surrounding tissue. After microdissection, the isolated specimens are ejected out of the object plane and catapulted directly into the cap of a microfuge tube containing 1µl of mineral oil positioned above the slide. DNA was extracted from their tissue samples using the QIAamp DNA micro kit.

2.4.4 DNA extraction from laser microdissected tissue

DNA was extracted from laser captured tissue using the QIAamp DNA micro kit. Tissue was catapulted onto the lids of 0.6ml tubes. Forty micro litres of buffer ATL (contents trade secret, CTS) and 10µl of proteinase K were carefully placed into the lid of the tube and the tubes were left inverted at room temperature for ~3 hours. After the tissue was digested, 50µl of buffer AL (CTS) was added to the lysate and incubated at 70°C for 10 minutes. Fifty micro litres of 100% ethanol was added and the solution was applied to the QIAamp micro silica gel based spin column and centrifuged at 13,000 rpm for 1 minute. The column was transferred to a clean collection tube and 500µl of wash buffer AW1 (CTS) was added. The column was re-centrifuged at 13,000 rpm for 1 minute. The eluate was discarded before a second wash was carried out using 500µl of buffer AW2 (CTS). The column was re-centrifuged at 13,000 rpm for 3 minutes followed by an extra 1 minute spin in an empty collection tube to remove residual ethanol. DNA was eluted into ~40µl of DNAse free water by incubating for 1 minute at room temperature and centrifuging at 13000 rpm for 1 minute into a clean collection tube. Samples were stored at -20°C.

2.4.5 RNA extraction from adherent cell lines

Prior to RNA extraction adherent cell lines were washed 3 times using cold PBS. Samples were incubated for 5 minutes at room temperature in 1ml of Trizol reagent. Nought point two millilitres of 100% chloroform was added and gently agitated for 30 seconds before further incubation for 5 minutes at room temperature. Samples were transferred into 1.5ml eppendorfs and centrifuged for 15 minutes at 12,000 x g at 4°C. The aqueous phase of the solution was transferred to a fresh eppendorf (the remainder discarded) and RNA precipitated by addition of 500ml of isopropanol. The sample was then incubated at room temperature for 10 minutes and centrifuged for 10 minutes at 12000 x g at 4°C to provide a translucent RNA pellet. The supernatant was carefully discarded and the pellet washed with 1 ml of 75% ethanol. The sample was then centrifuged at 7,500 x g for 5 minutes at 4°C and the subsequent supernatant discarded. The remaining pellet was air dried for 10 – 20 minutes and re-suspended in diethylene pyrocarbonate (DEPC) treated RNase Free H2O before being quantified.

2.4.6 Quantification of nucleic acids

The concentration of eluted DNA and RNA samples was achieved using ultraviolet (UV) spectrophometry at wavelengths of 260nm and 280nm to determine the amount of DNA or RNA present and establish endogenous protein content. An absorbance ration of 1.8 at 260nm:280nm was used as an indicator of high sample purity (i.e. little contamination).

2.4.7 Polymerase chain reaction (PCR)

PCR allows the specific *in vitro* amplification of a defined DNA target sequence in an exponential manner. Double stranded DNA templates are heat denatured and oligonucleotides bind specifically to complementary target sites on each strand. Thermostable DNA polymerases extend the primers in the 5' to 3' direction by incorporating dNTPs to create a complementary DNA strand. This cycle is repeated 20 – 40 times enabling newly synthesised DNA molecules to be used as templates at each new round. (Mullis *et al*, 1986).

Complimentary oligonucleotide pairs were designed to have melting temperatures 2°C of each, to be between 15 and 25 nucleotides in length, lacked repetitive motifs and had little predicted dimerization or secondary structure formation.

For standard PCR, 25ng template DNA, 0.25mM dNTPs, 25pmol forward and reverse primers, 2µl 10X GeneAmp PCR buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, 15mM $MgCl_2$, 0.01% w/v gelatin), and 0.5U AmpliTaq Gold DNA polymerase were used in a total volume of 20µl. Cycling conditions were 95°C for 12 minutes, followed by 35 cycles of 95°C for 30 seconds, annealing temperature (52°C - 60°C) for 30 seconds, 72°C for 30 seconds and a final elongation step of 72°C for 10 minutes.

A 20µl RT-PCR reaction contained 1ng cDNA, 0.25mM dNTPs, 25pmmol forward and reverse primers, 2µl 10X GeneAmp PCR buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, 15mM $MgCl_2$, 0.01% w/v gelatine), and 0.5U AmpliTaq Gold DNA polymerase. Cycling conditions were 95°C for 12 minutes, followed by 35 cycles of

95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and a final elongation step of 72°C for 10 minutes.

2.4.8 Agarose gel electrophoresis

Agarose gels were prepared with 1X TAE buffer to a concentration of 2.0%. 0.05µg/ml ethidium bromide was added to the gel to allow for DNA visualisation since it is a DNA intercalating agent that fluoresces under UV light at a wavelength of 300nm. Three micro litres of loading dye (15%w/v ficol, 10mM Tris pH 8, 1mM EDTA, 0.2% orange G) was added to each sample before loading and electrophoresis was performed in 1X TAE buffer at 100V. 1kb or 100bp DNA ladders were used to allow fragment sizing. DNA was visualised on a UV transiluminator and photographed using the Bio-Rad XR system.

2.4.9 PCR purification

PCR products were purified using an ExoSap method. In a general 5µl reaction, 3µl of PCR product was purified by adding 10U exonuclease I and 0.5U shrimp alkaline phosphates. The sample was incubated at 37°C for 15 minutes to allow for digestion of excess primers and removal of phosphates groups from dNTPS before denaturation of the enzymes at 80°C for 15 minutes.

2.4.10 Cycle sequencing and purification

Sanger sequencing uses dideoxynucleotide triphosphates (ddNTPs) which lack the 3' hydroxyl group present in deoxyribose sugars. As a result of this, ddNTPs can be efficiently incorporated into a nascent strand by DNA polymerases but prevent further extension of the growing chain (Sanger *et al*, 1977). In automated sequencing the reaction can take place within a single tube because each ddNTP is labelled with a different fluorophore. The template DNA is denatured and bound by a single specific oligonucleotide. DNA polymerase extends this primer by incorporating either an unlabelled dNTP or chain terminating ddNTP at each position. The relative concentrations of dNTPs and ddNTPs are such that the labelled products formed differ in size by one nucleotide. Capillary electrophoresis is used to separate the single stranded DNAs, with smaller fragments migrating fastest through the polymer and passing through the laser beam first. The emitted wavelength of light is detected

and used to determine the ddNTP incorporated at a particular position. The order of the nucleotides provides a sequence read of up to 500bp.

Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing kit (Version 3.1) according to the manufacturer's instructions. A total reaction volume of 10µl contained 0.6µl – 1.5µl purified PCR product (~5ng), 1µl Terminator ready Reaction Mix (labelled ddNTPs and dNTPs, AmpliTaq DNA polymerase FS, $MgCl_2$ and Tris-HCl buffer, pH 8), 1.6pmol primer and 1.5µl BigDye terminator buffer (CTS). Cycle sequencing conditions were 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 3 minutes and 30 seconds.

The Montage SEQ_{96} Sequencing Reaction Cleanup Kit was used to purify sequencing reactions. Twenty micro litres of injection solution (CTS) was mixed with the reaction and transferred to a filter plate. A vacuum of was applied until the wells were empty, then a further 25µl injection solution was added and the vacuum applied again to ensure that all the contaminating salts and unincorporated dyes terminators were filtered out. Purified sequencing products were re-suspended in 25µl injection solution by shaking for 12 minutes. Samples were run on either an ABI 31000 or ABI 3730 Genetic Analyser.

2.4.11 Bacteriological methods

2.4.11.1 Bacteriological media and solutions

LB – Broth; 5g Bactotryptone, 2.5g yeast extract and 2.5g NaCl in 500ml dH_20. LB agar: 5g bactotryptone, 2.5g yeast extract, 2.5g NaCl and 8g Bactoagar in 500ml dH_20.

2.4.11.2 Site Directed Mutagenesis (SDM)

In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships and the effects of gene expression. Mutations were introduced into the *APC* open reading frame (ORF) of the pCMV-APC vector, using the Quickchange XL Site–Directed Mutagenesis Kit (Figure 2.1). Nonsynonymous or synonymous mutations involving one base substitution were created using mutagenic oligonucleotides that were between 30-34bp in length with the mismatch

near the centre, a melting temperature (T_m) ≥78°C, a minimum GC content of 40% and terminating in one or more C or G bases at the 3' end. The forward and reverse primers were complimentary.

2.4.11.3 Mutant Strand Synthesis Reaction

Reactions were carried out using 10ng of DNA, 125ng of each primer, 2µl of 10X reaction buffer, 0.5µl of proprietary dNTP mix, 1.5Ml of Quicksolution (CTS) and 1.25U *pfiUtra* DNA polymerase and H_2O to a volume of 25µl. This enzyme has proofreading activity and has to be added after the other components to prevent exonuclease digestion of the primers. Cycling parameters were 95°C for 1 minute, followed by 18 cycles of 95°C for 50 seconds, 60°C for 50 seconds and 68°C for 14 minutes (1 minute per kb of plasmid). Following thermal cycling, reaction tubes were placed on ice for approximately 2 minutes to cool the reactions to ≤37°C

The subsequent mixture was then incubated with 0.5µl *Dpn* I at 37°C for approximately 1 hour. *Dpn* I endonuclease is specific for methylated and hemimethylated DNA (target sequence: 5'Gm^6ATC-3') and is used to digest the parental DNA template and to select for mutation-containing DNA. The remaining nicked SDM products were transformed into XL10-Gold Ultracompetent Cells and recircularised vectors were purified using the QIAprep miniprep kit. Plasmids were sequenced to confirm the presence of the desired mutation and lack of any additional errors.

2.4.11.4 XL10-Gold Ultracompetent Cell Transformation

Transformation of XL10-Gold Ultracompetent cells is mediated via a heat shock process which leads to semi-permeabilisation of the cell membrane enabling the uptake of 'naked' DNA molecules from the surrounding environment into the cell.

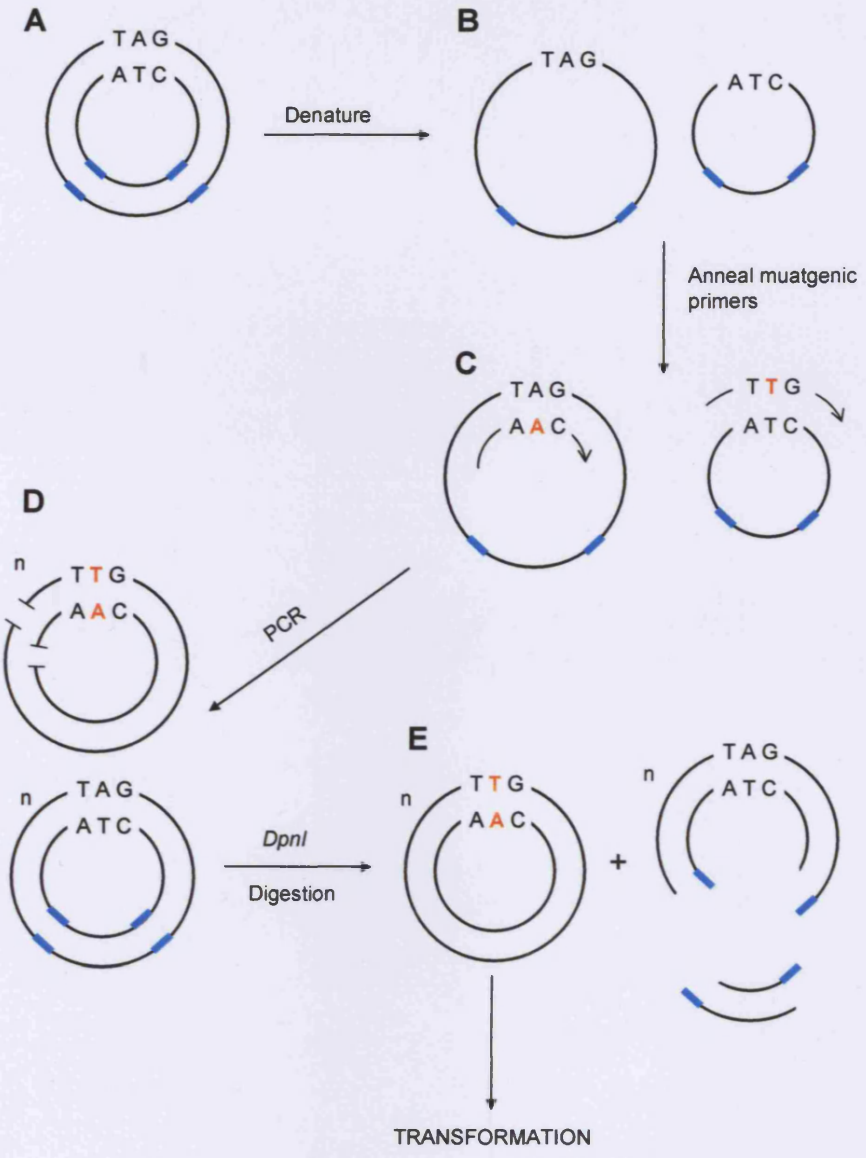


Figure 2.1 Overview of the QuikChange® XL site- directed mutagenesis method A) Methylated *p*CMV-*APC* vector containing the template codon sequence of interest (methylated sites indicated in blue); B) heat denaturation separates the double-stranded plasmid; C) mutagenic primers are then annealed to the region of interest (altered base in each primer is indicated in red); D) subsequent rounds of PCR exponentially amplify the plasmid containing the mutation using the non-strand displacing action of *Pfu* DNA polymerase. The mutated plasmids are 'nicked' circular strands; D) *Dpn I* digestion of non-mutated parental DNA (methylated wild-type) provides a population of 100% mutated DNA which can be then used to transform XL10-Gold ultracompetent cells.

One micro litre of β-mercaptoethanol (ME) mix was added to 22.5µl of cells, mixed gently and incubated on ice for 10 minutes ensuring that gentle agitation occurred approximately every 2 minutes. One micro litre of the *Dpn* I-treated DNA was added to the ultracompetent cells, gently mixed and incubated in ice for 30 minutes. Each sample was then heat shocked by placing in a 42°C water bath for exactly 30 seconds and incubated on ice for 2 minutes. The cells were then incubated at 37°C with agitation at 225rpm for 1 hour after adding 250µl of pre-warmed LB media. 200µl of the growing suspension was spread onto LB agar plates containing ampicillin, Xgal and IPTG. The transformation plates were incubated at 37°C overnight (>16 hours).The pCMV-APC vector carries the *LacZ* gene which encodes for the enzyme β-galactosidase and so it is possible to identify colonies that contain the insert according to their colour. Cells that contain the vector without the insert will produce β-galactosidase which will lead to the formation of blue colonies because of the utilisation of the enzymes' substrate Xgal. Cells that contain the vector with the successful insert will appear white since the presence of the insert will have disrupted the *LacZ* gene and therefore β-galactosidase will not be produced.

2.4.11.5 PCR confirmation for transformed colonies

Individual white colonies were picked, placed in 40µl of dH_20 and heated for 10min at 95°C to release the plasmid DNA. Cell debris was precipitated by centrifugation at 3000g for 1 minute. One micro litre of the supernatant was used as a template for a standard PCR reaction using primers that were suitable for the insert. Clones that were PCR positive were sequenced to confirm the presence of the desired point mutation.

2.4.11.6 Small scale plasmid preparation

High quantity plasmid DNA for transfection was prepared using QIAprep Spin Miniprep Kit according to the manufacturer instructions. Around 5-10µg of plasmid DNA is generated from a 1.5ml overnight LB culture. The process involves alkaline lysis of cells accompanied by gentle mixing to release intact plasmid DNA and to denature the proteins. This is followed by neutralisation and adjustment of conditions to a high salt medium to bind the DNA to the column. High salt conditions cause proteins to denature and chromosomal DNA and cellular debris to precipitate whilst

plasmid DNA stays in solution and binds to the silica-gel membrane. The membrane was washed to remove trace nucleases or carbohydrates and salts before the DNA was eluted.

Clones that successfully carried the correct insert were grown in 3ml of liquid LB containing ampicillin (50µg/ml) at 37°C with agitation (225rpm) for ~15 hours. Cells were centrifuged at 13000rpm for 1 minute and re-suspended in 250µl of Buffer P1 (100µg/ml RNase A, 50mM Tris/HCl and 10mM EDTA). Two hundred and fifty micro litres of Buffer P2 (200mM NaOH, 1% SDS) was added and the tubes were gently inverted 4-6 times to mix. Three hundred and fifty micro litres of Buffer N3 (3M potassium acetate) was then added, the tubes were gently inverted 4 – 6 times and centrifuged at 13,000rpm for 10 minute. The supernatant was added to a QIAamp column and centrifuged at 13,000rpm for 1 minute. The column was washed with 500µl of Buffer PB (CTS) and then centrifuged at 13,000rpm for 1 minute. The column was washed with 750µl of Buffer PE (containing ethanol and CTS) and centrifuged at 13,000rpm for 1 minute. Residual ethanol form Buffer PE was removed by centrifuging the column twice at 13,000rpm for 1 minute. DNA was eluted into 30µl of H_20 after placing the column in a fresh 1.5ml microfuge tube and centrifuging at 13,000rpm for 1 minute. Tubes were stored at 4°C for immediate use.

2.4.11.7 Storage of transformed XL10-told ultracompetent cells

For long term storage of transformed cells 900µl of bacteria culture was mixed with 300µl of 80% sterile glycerol. Labelled samples were stored in a -80°C freezer.

2.4.11.8 Transient transfection and the β-Catenin regulated transcription (CRT) assay

SW480 cells were seeded into 96 well plates at 10^4 cells per well and allowed to settle and adhere for 24 hours. Media was then aspirated and 25µl of transfection mastermix, containing 25ng of the luciferase reporter plasmids pTOPFLASH and pFOPFLASH, 25ng of pCMV-*APC* variant plasmids and 2ng of the transfection efficiency control plasmid, pRL-SV40, was added and placed in the 37°C incubator for 1 hour. Following this incubation a further 40µl of DMEM complete was added. Luciferase assays were performed using the Dual Luciferase assay kit.

2.4.11.9 Immunocytochemistry

SW480 cells were cultured on glass cover slips and transfected with mutant pCMV-*APC* and left 24 hours before staining. Slides were removed from culture and washed in warm PBS. Cells were fixed for 10minutes in 4% formalin before rinsing with TBS at room temperature. Cells were permeabilised with 100% methanol at -20°C for 30 minutes before rinsing. Slides were blocked with 1.5% goat serum in TBS for 1 hour before rinsing and incubated with primary antibody overnight at a dilution of 1:10,000. Slides were washed with TBS/Triton solution and then incubated with biotinylated secondary antibody (TBS/3% BSA; 1:250 Rabbit antibody) for 1 hour at room temperature.

2.4.11.10 qRT-PCR Analysis

Total RNA was extracted from transfected SW480 cells 24 hours post transfection with 25ng of mutant pCMV-APC vector. Quantitative real time PCR amplification and gene expression analysis were carried out in 20µl reaction volumes using 1ng cDNA, 10µl Taqman Universal PCR mix, 1µl Taq Gene Assay Mix and 8.5µl of DEPC treated RNase free water using an Applied Biosystems 7500 Real-Time PCR system. Four reactions were carried out using proprietary primers contained within the Taqman Gene Assay Mix for *cyclin D1*, *c-MYC* and TATA binding box protein (TBP).

2.4.11.11 Cell survival analysis

Cells were seeded in triplicate at $2x10^3$ cells per well into Optilux 96-well plates 24 hours before treatment with peroxide to allow sufficient adherence. ATP was quantified in wells containing medium alone, untreated control cells or treated cells treated immediately after treatment using the CellTiter-Glo Luminescent Cell Viability Assay with an Applied Biosystems Microplate Luminometer.

2.4.11.12 Detection of active Caspase-3

Active Caspase-3 quantification assays were performed using a Caspase-Glo™3/7 assay kit according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at a density of $2x10^3$ cells per well. After 24 hours, cells were exposed to H_2O_2 and t-butyl hydro-peroxide (TBH) solutions. Caspase-Glo 3/7 reagent (100µl)

was then added to each well including medium alone, untreated control cells or cells treated with peroxide 1, 24, 48 and 72 hours post exposure. The luminescence of each sample was measured with TR717 Microplate Luminometer.

2.4.11.13 Immunofluorescence

Cells were cultured on Lab Tek II Glass Chamber Slides to 75% confluence. Cells were fixed in 3.8% formalin for 10 minutes at room temperature and permeabilised with 100% methanol at -20°C for 5 minutes. Slides were blocked with 1.5% goat serum in TBS for 1 hour before rinsing and incubated with primary antibody overnight. Slides were washed with TBS/Triton solution and then incubated with fluorescent secondary antibody for 1 hour at room temperature

2.4.11.14 Immunoprecipitation and Western blotting

Treated cells were lysed on ice for 10 minutes in NP-40 lysis buffer (50mM Tris-HCl pH 8, 150mM NaCl, 1% NP-40, 50mM PMSF in isopropanol, 1ug/ml of Leupeptin, Aprotinin, and Pepstatin). Heat a sample to 95–100°C for 5 minutes; cool on ice. Half volume was used to determine phosphorylation of Chk1 whilst the remaining half was used to determine total Chk1 as a control. Briefly, lysates were incubated with 1µg of anti-phosphoChk1(Ser317) for 1 hour at 4°C (1:500 dilution). Twenty micro litres of Protein G-Sepharose bead solution was added and the mixture was incubated at 4°C for 2 hours. After three wash/centrifugation steps at 3000*g* for 1minute the pellet was resolved by SDS/PAGE and transferred to a nitrocellulose membrane. After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature. Wash three times for 5 minutes each with 15 ml of TBS/T. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C. Wash three times for 5 minutes each with 15 ml of TBS/T. The membranes were allowed to react with antibodies against CHK1 at dilutions stated above. Western blotting was detected by the enhanced chemiluminescence (ECL).

Chapter Three: Multiple Rare Nonsynonymous Variants in *APC* Predispose to Colorectal Adenomas

3. 1 Introduction

The 'rare variant hypothesis' proposes that genetic susceptibility to colorectal neoplasia within the general population is due to a number of low frequency variants in a number of different genes, where each variant confers a moderate, but detectable, increase in relative risk of developing the disease (Fearnhead *et al*, 2005). We reason that since germline mutations in *APC* that are predicted to result in a truncated protein product cause the CRC predisposition syndromes FAP and AFAP that rare nonsynonymous variants within important functional domains of *APC* may also contribute to colorectal tumourigenesis. The precedent has already been set in other settings such as coronary heart disease where it has recently been shown that rare nonsynonymous variants in the genes encoding apolipoprotein A1, the adenosine triphosphate binding cassette transporter A1 and lecithin cholesterol acyltransferase, are over-represented in individuals with low plasma levels of high-density lipoprotein cholesterol, a major risk factor for coronary atherosclerosis (Cohen *et al,* 2004).

Whether rare inherited nonsynonymous variants in *APC* might act as low penetrance alleles remains highly speculative; however, the variant I1307K has been shown to create a hypermutable tract that predisposes to somatic mutations (Laken *et al*, 1997) and E1317Q has been shown to be overrepresented in the germline of patients with multiple CRAs (Lamlum *et al*, 2000) and has also been identified as a somatic change in sporadic CRC (Frayling *et al*, 1998). To address the potential role of nonsynonymous variants of *APC* in inherited predisposition to CRAs, we undertook mutational analyses of *APC* in 691 unrelated North American patients with CRAs and 969 matched healthy controls. We also carried out functional and *in silico* analyses to compliment the genetic mutation analyses strategy.

3.2 Methods

3.2.1 Patients and control samples

Mutation analysis of *APC* and *MUTYH* genes was undertaken by Myriad Genetics Inc. in 691 unrelated North American patients that were referred by their physicians for genetic testing because of a clinical diagnosis of either FAP or "multiple" colorectal polyps but in whom conventional truncating mutations of *APC* and *MUTYH* had not been identified. All results were made completely anonymous in accordance with institutional approved guidelines. Myriad Genetics Inc. also sought rare non-synonymous variants in residual DNA samples from 969 unrelated North American healthy controls that were made available after routine carrier screening for cystic fibrosis. Samples were anonymised in accordance with institutional policies governing specimen use. No samples carried any of the 97 mutations that were tested for in the *CFTR* gene. The unrelated North American healthy controls were matched to the unrelated North American non-FAP non-MAP patients for age (mean, 41.6 years for controls and 47.8 years for patients), sex (~50% males/50% females in both groups), and self-reported ethnic backgrounds (Table 3.1).

	FAP/MAP patients	Non-FAP non-MAP patients	Healthy controls
African	2%	5%	5%
Ashkenazi	2%	4%	4%
Asian	2%	2%	2%
Caucasian	66%	61%	61%
Latin American/Caribbean	1%	4%	4%
Native American	3%	2%	2%
Near Eastern	0%	1%	1%
None Specified	14%	15%	15%
Other	9%	5%	5%

Table 3.1 Self reported ethnic backgrounds of the patients and healthy controls from North America. Data generated by Myriad Genetics.

3.2.2 Mutation analyses

Peripheral blood DNA samples from all patients were sequenced for the entire open reading frame (ORF) and splice sites of *APC* and exons 7 and 13 of *MUTYH* (that harbour the two common *MUTYH* mutations Y165C and G382D respecitively, which account for approximately 75-80%of *MUTYH associated polyposis incidences*; Sampson JR, *et al*, 2003) and screened for deletions at the *APC* locus by multiplex ligation-dependent probe amplification (MLPA) and by Southern blot analysis (mutational analysis performed by Myriad Genetics, Utah, USA). Samples with a single *MUTYH* mutation were then sequenced for the ORF and splice sites of *MUTYH* to identify biallelic mutations. Peripheral blood DNA samples from healthy controls were sequenced over a ~2.4-kb region of *APC* spanning the β-catenin down-regulating domain by Agencourt Biosciences using six overlapping PCR fragments (Table 3.2). Comparisons of numbers of patients harbouring variants were performed using either the Chi-squared test or Fisher's exact test.

Name	Primer Sequence (5' – 3')	Amplicon size (bp)
01F	GTAAAACGACGGCCAGTCAGTGAGAATACGTCCACACC	513
01R	CAGGAAACAGCTATGACCCTAAACATGAGTGGGGTCTCC	
02F	GTAAAACGACGGCCAGTGATCCTGTGAGCGAAGTTCC	564
02R	CAGGAAACAGCTATGACCGCTGGATGAACAAGAAAATCC	
03F	GTAAAACGACGGCCAGTTCAAACAGCTCAAACCAAGC	563
03R	CAGGAAACAGCTATGACCTAACATGCTTTTGGGGTTGC	
04F	GTAAAACGACGGCCAGTATGCCAACAAAGTCATCACG	491
04R	CAGGAAACAGCTATGACCATCTTTTTCACACGGAAAGG	
05F	GTAAAACGACGGCCAGTAGGCTCAAGGAGGAAAAACC	591
05R	CAGGAAACAGCTATGACCTGGTAACTTTAGCCTCTGATTCC	
06F	GTAAAACGACGGCCAGTTCATTACACGCCTATTGAAGG	507
06R	CAGGAAACAGCTATGACCTGAAATGATTTAGGAGCATAGCC	

Table 3.2 Primers used to sequence the β-catenin down-regulating domain of *APC*. Complementary primer pairs indicated by identical number prefix. F = forward, R = reverse.

3.2.3 Site directed mutagenesis

Mutant *APC* constructs were created by site directed mutagenesis of the pCMV-APC vector provided by B. Vogelstein. Complementary oligonucleotides containing the appropriate mismatches for single nucleotide substitutions were designed using the Quickchange Primer Design program (http://labtools.stratagene.com/QC/QCprimers). Primers sequences were annealed to the vector at 55°C and are shown in Table 3.3. In order to confirm the presence of the desired mutation and lack of any unwanted errors, the *APC* ORF was sequenced using the primers in Table 4.1.

3.2.4 Expression of stable *APC* constructs

The pCMV-APC vector is ~21kb in size. The large size of this vector means that efficient transfection may not be possible in all commercially available cell lines. Therefore, mutant and wild-type pCMV-*APC* constructs were initially transformed into a range of competent cells to establish the most efficient method of generating clones. ABLE K competent cells, SURE 2 Supercompetent cells and XL10-Gold Ultracompetent cells were chosen because of their efficacy in cloning of large DNA inserts. Transformations were carried out according to the manufacturer's instructions. Since all cells were amenable to blue-white colour screening for successfully recombinant plasmids, transformed bacteria were plated out and incubated on LB-ampicillin agar plates containing 80µg/ml X-gal and 20mM IPTG. White colonies were grown further and screened for the presence of mutation.

Name	WT Codon	Mutant Codon	Forward Primer Sequence (5' - 3')	Reverse Primer Sequence (5' - 3')
Truncating				
1309	AAA	TAA	CAAATAGCAGAAATAAAAGAATAGATTGGAACTAGGTCAGCTGAA	TTCAGCTGACCTAGTTCCAATCTATTCTTTTATTTCTGCTATTTC
1450	GAG	TAG	CCTCAAACAGCTCAAACCAAGTGAGAAGTACCTAAAAATAAAGCA	TGCTTTATTTTTAGGTACTTCTCACTTGGTTTGAGCTGTTTGAGG
1517	AGA	TGA	AGCCTCGATGAGCCATTTATATAGAAAGATGTGGAATTAAGAATA	TATTCTTAATTCCACATCTTTCTATATAAATGGCTCATCGAGGCT
1914	TGC	TGA	CACAGAACTAACCTCCAACTAACAATCAGCTAATAAGACACAAG	CTTGTGTCTTATTAGCTGATTGTTAGTTGGAGGTTAGTTCTGTG
Silent				
P1442P	CCT	CCG	AGAAGTAAAACACCTCCACCACCGCCTCAAACAGCTCAAACCAAG	CTTGGTTTGAGCTGTTTGAGGCGGTGGTGGAGGTGTTTTACTTCT
T1493T	ACG	ACT	GATACTTTATTACATTTTGCCACTGAAAGTACTCCAGATGGATTT	AAATCCATCTGGAGTACTTTCAGTGGCAAAATGTAATAAAGTATC
G1678G	GGG	GGT	GCTGGAGAAGGAGTTAGAGGAGGTGCACAGTCAGGTGAATTTGAA	TTCAAATTCACCTGACTGTGCACCTCCTCTAACTCCTTCTCCAGC
A1755A	GCG	GCT	GACCAGGTCCAGCAAGCATCTGCtTCTTCTTCTGCACCCAACAAA	TTTGTTGGGTGCAGAAGAAGAAGCAGATGCTTGCTGGACCTGGTC
S1756S	TCT	TCC	CAGGTCCAGCAAGCATCTGCGTCcTCTTCTGCACCCAACAAAAAT	ATTTTTGTTGGGTGCAGAAGAGGACGCAGATGCTTGCTGGACCTG
P1960P	CCg	CCT	AATTTTGCTATTGAAAATACTCCTGTTTGCTTTTCTCATAATTCC	GGAATTATGAGAAAAGCAAACAGGAGTATTTTCAATAGCAAAATT
Nonsynonymous				
I1307K	ATA	AAA	AATACCCTGCAAATAGCAGAAAAAAAAGAAAAGATTGGAACTAGG	CCTAGTTCCAATCTTTTCTTTTTTTTCTGCTATTTGCAGGGTATT
E1317Q	GAA	CAA	AAGATTGGAACTAGGTCAGCTCAAGATCCTGTGAGCGAAGTTCCA	TGGAACTTCGCTCACAGGATCTTGAGCTGACCTAGTTCCAATCTT
V1352A	GTT	GCT	GAATCAGCCAGGCACAAAGCTGCTGAATTTTCTTCAGGAGCGAAA	TTTCGCTCCTGAAGAAAATTCAGCAGCTTTGTGCCTGGCTGATTC
M1413V	ATG	GTG	TCAGAGTGAACCATGCAGTGGAGTGGTAAGTGGCATTATAAGCCC	GGGCTTATAATGCCACTTACCACTCCACTGCATGGTTCACTCTGA
T1445A	ACA	GCA	AACACCTCCACCACCTCCTCAAGCAGCTCAAACCAAGCGAGAAGT	ACTTCTCGCTTGGTTTGAGCTGCTTGAGGAGGTGGTGGAGGTGTT
A1446T	GCT	ACT	ACCTCCACCACCTCCTCAAACAACTCAAACCAAGCGAGAAGTACC	GGTACTTCTCGCTTGGTTTGAGTTGTTTGAGGAGGTGGTGGAGGT
K1454E	AAA	GAA	TCAAACCAAGCGAGAAGTACCTGAAAATAAAGCACCTACTGCTGA	TCAGCAGTAGGTGCTTTATTTTCAGGTACTTCTCGCTTGGTTTGA
P1467S	CCT	TCT	TGCTGAAAAGAGAGAGAGTGGATCTAAGCAAGCTGCAGTAAATGC	GCATTTACTGCAGCTTGCTTAGATCCACTCTCTCTCTTTTCAGCA
A1474T	GCT	ACT	ACCTAAGCAAGCTGCAGTAAATACTGCAGTTCAGAGGGTCCAGGT	ACCTGGACCCTCTGAACTGCAGTATTTACTGCAGCTTGCTTAGGT
I1572T	ATT	ACT	GATGATTCAGATGATGATGATACTGAAATACTAGAAGAATGTATT	AATACATTCTTCTAGTATTTCAGTATCATCATCATCTGAATCATC
C1578G	TGT	GGT	TGATATTGAAATACTAGAAGAAGGTATTATTTCTGCCATGCCAAC	GTTGGCATGGCAGAAATAATACCTTCTTCTAGTATTTCAATATCA
I1579V	ATT	GTT	TATTGAAATACTAGAAGAATGTGTTATTTCTGCCATGCCAACAAA	TTTGTTGGCATGGCAGAAATAACACATTCTTCTAGTATTTCAATA
R1676G	AGA	GGA	GTTAGCTGCTGGAGAAGGAGTTGGAGGAGGGGCACAGTCAGGTGA	TCACCTGACTGTGCCCCTCCTCCAACTCCTTCTCCAGCAGCTAAC
D1714N	GAT	AAT	ATCTGTAACCATACCTGAATTGAATGACAATAAAGCAGAGGAAGG	CCTTCCTCTGCTTTATTGTCATTCAATTCAGGTATGGTTACAGAT
G1921S	GGT	AGT	TGCAAAGCAGCCAATAAATCGAAGTCAGCCTAAACCCATACTTCA	TGAAGTATGGGTTTAGGCTGACTTCGATTTATTGGCTGCTTTGCA
P1934L	CCC	CTC	CTTCAGAAACAATCCACTTTTCTCCAGTCATCCAAAGACATACCA	TGGTATGTCTTTGGATGACTGGAGAAAAGTGGATTGTTTCTGAAG

Table 3.3 Primers used for site directed mutagenesis of the pCMV-APC vector. 'WT codon' refers to the codon sequence before mutagenesis and 'Mutant Codon' refers to codon sequence after successful mutagenesis.

3.2.5 β-Catenin–regulated transcription (CRT) assays.

The luciferase reporter plasmids pTOPFLASH and pFOPFLASH, which contain 3 copies of the optimal or mutant β-catenin/Tcf-binding motif, respectively, upstream of a firefly luciferase gene were used to assess the β-catenin–regulated transcription (CRT) mediated by transfected *APC* constructs. SW480 cells were transfected as described in section 2.4.11.4 with generated *APC* constructs.

Wildtype SW480 cells contain a large amount of cytoplasmic *β*-catenin due to truncated, non-functional APC, and would therefore result in the production of a relatively high level luciferase and subsequently produce high luminescent output. Transfection of functional *APC* into this system would result in degredation of the cytoplasmic β-catenin, resulting in a reduced luminescent signal. Functionally compromised *APC* would hypothetically produce a luminescent signal that was closer to that produced by wildtype SW480 cells alone and significantly different to that produced by functional *APC*. Results were standardized for transfection efficiency, and the average of at least 10 independent experiments was analysed using Students' t test.

3.2.6 qRT-PCR analysis

A total of 50ng RNA was used for first strand cDNA synthesis and subsequent PCR amplification and gene expression analysis were carried out as detailed in section 2.4.11.10.

3.2.7 Immunocytochemical staining for β-catenin

Immunostaining for β-catenin in cultured SW480 cells was performed 24h post-transfection with 25ng mutant *APC* using the rabbit VECTASTAIN ABC horseradish peroxidase kit as described in section 2.411.9

3.2.8 *In silico* analyses

Predictions using Polyphen (http://genetics.bwh.harvard.edu/pph/), were based on *Homo Sapiens* APC and predictions using Align-GVGD (http://agvgd.iarc.fr/), were based on a multiple sequence alignment (created using using t-coffee, http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/) of *APC* orthologues from *Homo sapiens* (ENSG00000134982), *Echinops telfairi* (ENSETEP00000013272), *Pan*

troglodytes (ENSPTRP00000029327), *Macaca mulatta* (ENSMMUP00000000446), *Oryctolagus cuniculus* (ENSOCUP00000013423), *Bos taurus* (ENSBTAP00000025099), *Xenopus tropicalis* (ENSXETP00000046491), *Monodelphis domestica* (NSMODP00000016609), *Rattus norvegicus* (ENSRNOP00000027691), *Mus musculus* (ENSMUSP00000078337), *Dasypus novemcinctus* (ENSDNOP00000006189) and *Loxodonta africans* (ESLAFP00000011382). All *in silico* analyses carried out by Mr. Edward Rawstorne.

3.3 Results

3.3.1 Contribution of germline mutations in *APC* and *MUTYH* to colorectal polyposis

Comprehensive mutation analysis of the *APC* and *MUTYH* genes in 691 unrelated North American patients presenting with a colorectal adenoma (CRA) phenotype was performed by Myriad Generics Inc. Truncating *APC* mutations were identified in 178 patients and biallelic *MUTYH* mutations in 33 patients, confirming the clinical diagnoses of FAP and MAP, respectively.

Approximately 98% of the germline truncating mutations in *APC* were nonsense point mutations and frame-shifts (due to small insertions and deletions) and were predominantly within the 5' region (codons 1 -1400; Figure 3.1) predicted to result in a C-terminal truncated protein. We also observed two common hotspots for deletion mutations at codons 1309 and 1062. Of the 33 MAP patients identified 36% of cases were biallelic G382D/Y165C, 24% were homozygous for G382D and 18% were homozygous for Y165C. The 7 remaining incidences were heterozygotes with Y165C and frameshift deletions.

Of the 211 FAP/MAP patients, 3.3% had ≤10 CRAs, 20.9% had 11 to 99 CRAs, 46% had ≥100 CRAs, and 29.9% had multiple CRA (number unknown), as recorded at colonoscopy or colectomy. Among the 480 non-FAP non-MAP patients, 15.4% had ≤10 CRAs, 33.5% had 11 to 99 CRAs, 9.2% had ≥100 CRAs, and 41.9% had multiple CRA.

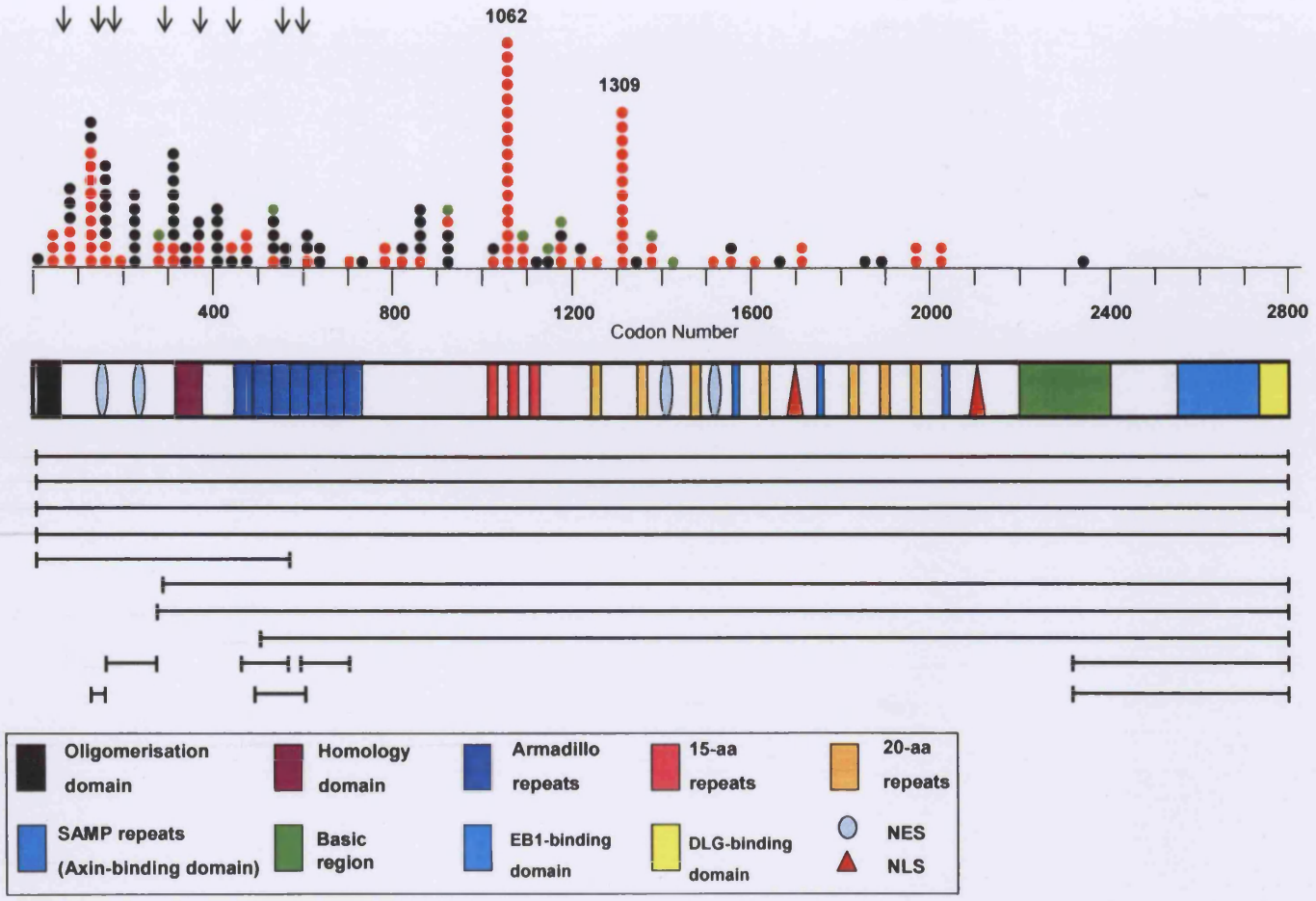


Figure 3.1 Distribution of germline *APC* mutations in 178 FAP patients. There is an over representation of mutations in the 5' region of the gene with the two corresponding hotspots at codons 1062 and 1309 annotated. Red dots indicate small deletion (≤2bp) mutations, green dots indicate small insertion (≤2bp) mutations, black dots indicate nonsense mutations, intronic mutations are shown as black arrows and large deletions are shown as black bars.

3.3.2 Nonsynonymous variants in patients with CRAs

From the sequencing performed by Myriad genetics Inc. forty-eight different rare (minor allele frequencies [MAFs] <2%) non-synonymous variants were identified spanning the *APC* ORF in 101 (14.6%) patients; 97 patients carried single heterozygous variants and 4 carried two heterozygous variants (three FAP patients carried A199T and L639S, R414C and G2502S, and, R1676G and P2467T, respectively, and one non-FAP non-MAP patient carried P870S and M949I). Only 20 of the 211 (9.5%) FAP/MAP patients carried rare nonsynonymous variants. In contrast, 81 of the 480 (16.9%) patients without FAP or MAP carried such variants (χ^2=6.42, *P*=0.0113), suggesting that some nonsynonymous variants in *APC* might predispose to CRAs (Table 3.4, Figure 3.2). When non-FAP non-MAP patients were classified according to the number of CRAs, the group with 11-99 CRAs had a higher frequency of rare nonsynonymous variants (18.6% of patients) as compared to the groups with ≤10 CRAs (13.5% of patients) or ≥100 CRAs (13.6% of patients; Table 3.4). Indeed, significantly more non-FAP non-MAP patients with 11-99 CRAs carried rare nonsynonymous variants as compared to the FAP/MAP patients (30/161 vs. 20/211, χ^2=6.579, *P*=0.0103).

I assessed whether the overrepresentation of the rare nonsynonymous variants in the non-FAP non-MAP patients was simply due to an overrepresentation of the previously studied variants I1307K and E1317Q. Similar proportions of non-FAP non-MAP and FAP/MAP patients were observed to carry I1307K (6 of 480 versus 2 of 211, *P* = 0.538). Although we did find that more non-FAP non-MAP patients carried E1317Q than FAP/MAP patients [13 of 480 (2.7%) versus 3 of 211 (1.4%)], this was not significant (*P* = 0.229). Importantly, when only alleles with MAFs of <0.5% were considered (thereby excluding G2502S (1.74%), E1317Q (1.16%), and I1307K (0.58%)), more non-FAP non-MAP patients were observed to carry rare nonsynonymous variants compared with FAP/MAP patients (43 of 480 versus 10 of 211, χ^2 = 3.68; *P* = 0.0549) and a significant overrepresentation in the non-FAP non-MAP cases with 11 to 99 CRAs (18 of 161 versus 10 of 211, χ^2 = 5.443; *P* = 0.0197) was observed. In contrast, similar proportions of non-FAP non-MAP and FAP/MAP patients carried rare (MAFs, <2%) synonymous variants, and the frequencies of seven common polymorphisms were almost identical between the two groups (Table

3.5). Both groups also had similar self-reported ethnic backgrounds (Table 3.1). It was therefore highly unlikely that these findings could be attributed to population stratification.

Category	Non-synonymous variants	Total (and frequency)
FAP/MAP patients (*APC* ORF)		
FAP patients	R106H, A199T+L639S, R414C+G2502S, S537C, L1129S, I1307K (2), E1317Q (2), R1676G+P2467T, H2149P, A2274V, G2502S (3), R2505Q, S2621C	17/178 (9.6%)
MAP patients	E1317Q, G2502S (2)	3/33 (9.1%)
	Total	**20/211 (9.5%)***
non-FAP non-MAP patients (*APC* ORF)		
≤10 CRAs	P981R, I1307K (2), E1317Q (2), T1445A, G2502S, R2505Q, N2593S, S2621C	10/74 (13.5%)
11-99 CRAs	R216Q, P981R, V1125A, L1129S, T1160K, I1307K (3), E1317Q (4), V1352A, M1413V, C1578G, I1579V, D1714N, G1921S, P2158R, H2232D, A2274V, G2502S (5), R2505Q, I2573V, S2621C, A2795T	30/161 (18.6%)
≥100 CRAs	K150R, S643P, R653K, G2502S (3)	6/44 (13.6%)
Multiple CRAs, number unknown	K150R, E538V, P870S, C947S, P870S+M949I, L1129S, T1160K, I1307K, E1317Q (7), A1446T, K1454E, P1467S, A1474T, I1572T, P1934L, R2066G, I2329V, G2502S (10), I2541V, I2756V	35/201 (17.4%)
	Total	**81/480 (16.9%)*** ***χ^2=6.42, *P*=0.0113** N.B. 32/480 (6.7%)** in the β-catenin downregulating domain
Healthy controls (β-catenin downregulating domain)		
	A1247T, I1307K (9), E1317Q (11), K1363I, M1413V (3), K1454E (2), P1458S, T1493M, P1584S, R1589G, R1589C, T1633K+T1655A+N1761T, R1676G, S1730F, Q1916K, I1975F	37/969 (3.8%)** **χ^2=5.74, *P*=0.0166

Table 3.4 Inherited nonsynonymous variants spanning the *APC* ORF in 691 North American patients with CRAs (classified according to FAP/MAP status and number of adenomas) and in the β-catenin down regulating domain in 969 North American healthy controls. In total, 61 different rare non-synonymous variants in *APC* were identified in this study. * Comparison between numbers of non-FAP non-MAP and FAP/MAP patients carrying rare nonsynonymous variants (MAFs, <2%) within the APC ORF (N.B. significantly more non-FAP non-MAP patients also carried rare nonsynonymous variants compared with FAP patients alone; χ^2 = 5.50, P = 0.0191). ** Comparison between numbers of non-FAP non-MAP patients and healthy controls carrying rare nonsynonymous variants (MAFs, <2%) within the β-catenin down-regulating domain.

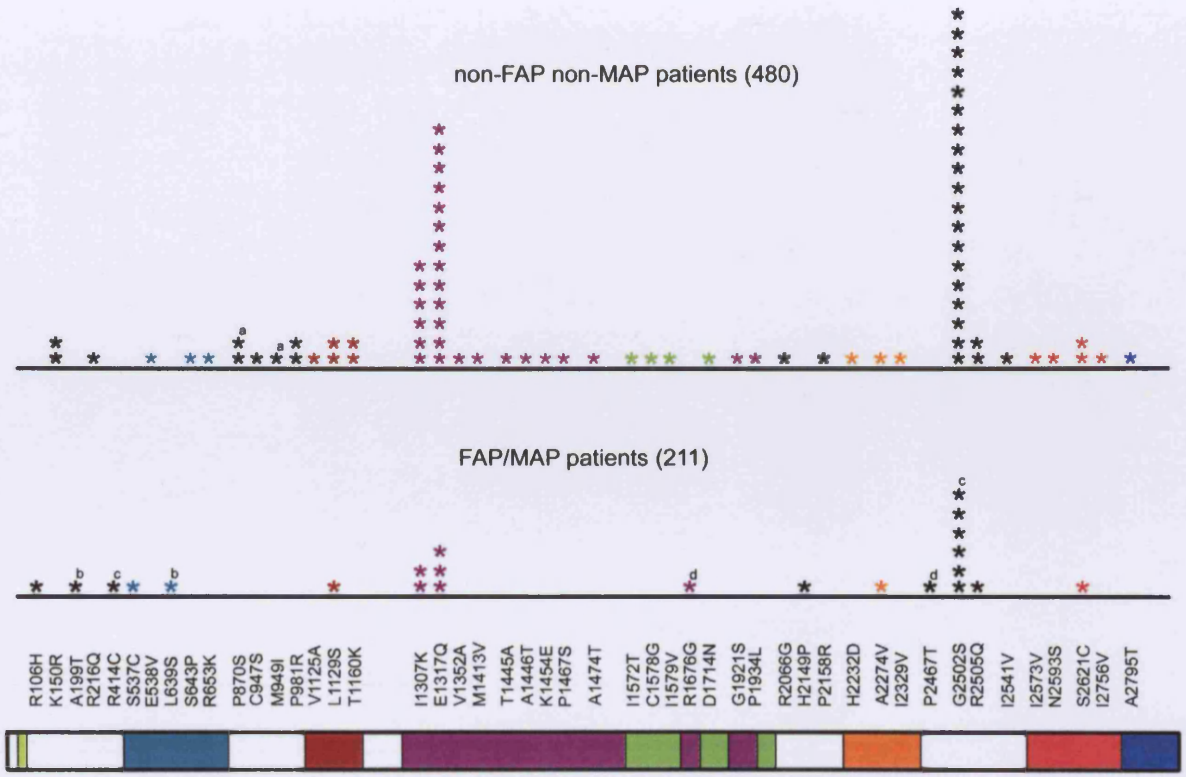


Figure 3.2 Distribution of inherited *APC* nonsynonymous variants in 480 non-FAP non-MAP patients and 211 FAP/MAP patients. Ninety-seven patients carried a single variant and four patients (*a–d*) carried two variants. Functional domains are coloured: ■ oligomerisation domain (codons 6-57), ■ armadillo region (codons 453-767), ■ 15 amino acid repeat β-catenin binding domain (codons 1020-1169), ■ 20 amino acid repeat β-catenin downregulating domain (codons 1262-2033), ■ SAMP repeats/axin binding domain (codons 1562-2056), ■ basic domain (codons 2200-2400), ■ EB1 binding domain (codons 2559-2771), and, ■ HDLG binding domain (codons 2771-2843) (domains not drawn to scale). Nonsynonymous variants are plotted as stars and coloured according to the domains in which they lie; *black stars,* nonsynonymous variants that do not lie within known functional domains.

	FAP/MAP patients (211)	Non-FAP non-MAP patients (480)	Healthy controls (969)
Rare Synonymous variants			
APC ORF	16.1%	14.8%	N/A
β-Catenin down-regulating domain	6.2%	6.0%	5.7%
Common polymorphisms			
D1822V	24.1%	22.8%	21.1%
A545 (1635A>G)	45.5%	44.4%	N/A
T1493 (4479A>G)	41.2%	39.7%	N/A
G1678 (5034A>G)	41.0%	39.8%	38.4%
S1756 (5268G>T)	40.5%	39.2%	38.1%
Y486 (1458C>T)	41.2%	39.2%	38.3%
P1960 (5880A>G)	41.0%	39.8%	38.0%

Table 3.5 Twenty nine different rare (MAFs, <2%) synonymous variants were found spanning the *APC* ORF in 105 patients (15.2%); 95 of these carried a single variant (93 in a heterozygous state), nine carried two heterozygous variants, and one carried three heterozygous variants. Eleven rare synonymous variants were found spanning the β-catenin down-regulating domain in 55 healthy controls (5.7%) N/A; not assessed.

3.3.3 Nonsynonymous variants in healthy controls

Significantly more non-FAP non-MAP patients had rare non-synonymous variants in the β-catenin down regulating domain (spanning codons 1262 to 2033) as compared to FAP/MAP patients (32/480 vs. 6/211, χ^2=4.12, *P*=0.0423). Using a re-sequencing approach, Myriad genetics Inc. were again commissioned to identify rare nonsynonymous variants in the ~2.4kb β-catenin down-regulating domain in 969 unrelated North American healthy controls that were matched to the unrelated North American non-FAP non-MAP patients for age, sex and race. In total, eighteen different rare non-synonymous variants were identified in the β-catenin down-regulating domain in 37 healthy controls; 36 controls carried single heterozygous variants and one carried three heterozygous variants (T1633K, T1655A and N1761T) (Table 3.4). Five out of eighteen of the nonsynonymous variants (I1307K, E1317Q, M1413V, K1454E and R1676G) were previously identified in the patient cohort, whereas the remaining thirteen variants were unique to the control group.

Significantly more non-FAP non-MAP patients carried rare non-synonymous variants in the β-catenin down-regulating domain as compared to the healthy controls (32/480 vs. 37/969, χ^2=5.74, *P*=0.0166) and this over-representation was highest in the non-FAP non-MAP patients with 11-99 CRAs (13/161 vs. 37/969, χ^2=5.914, *P*=0.0150; Table 3.4). In terms of individual variants, we did not observe an over-representation of I1307K in the non-FAP non-MAP patients versus controls (6/480 vs. 9/969, respectively). However, significantly more non-FAP non-MAP patients carried E1317Q were found, as compared to healthy controls (13/480 vs. 11/969, χ^2=4.88, *P*=0.0272), although even when this variant was excluded from the analyses, significantly more non-FAP non-MAP patients with 11-99 CRAs carried other rare non-synonymous variants in the β-catenin down-regulating domain as compared to controls (9/161 vs. 26/969, χ^2=3.887, *P*=0.0487). In contrast, the frequencies of rare synonymous variants in this region and common polymorphisms were almost identical between the non-FAP non-MAP patients and controls (Tables 3.5). These data suggest that a proportion of non-synonymous variants in the β-catenin down-regulating domain of *APC* are likely to alter β-catenin signalling to promote tumourigenesis.

3.3.4 Production of *APC* constructs for transfection.

We assessed the transformation efficiencies of three commercially available competent cells; ABLE K, XL-10 Gold Ultracompetent cells and SURE2 supercompetent cells. Transformations using the wildtype, I1307K nonsynonymous mutant and the T1493T synonymous mutant were carried out alongside an internal kit control. Only the XL10-Gold ultracompetent cells consistently yielded white colonies from each transformation reaction. Colonies from each transformation were screened for the presence of a mutation (where appropriate). Amplicons of the correct size were detected (Figure 3.3) and sequenced to ensure that site directed mutagenesis had resulted in synthesis of a mutant transcript; the Stratagene XL10-Gold ultracompetent cells were subsequently used for the synthesis of all DNA transfection constructs. We generated six synonymous, four truncating mutants and sixteen nonsynonymous pCMV-APC constructs to carry out functional assays (i.e. CRT assays, qRT-PCRs and immunocytochemistry). Examples of each construct are shown in figure 3.4.

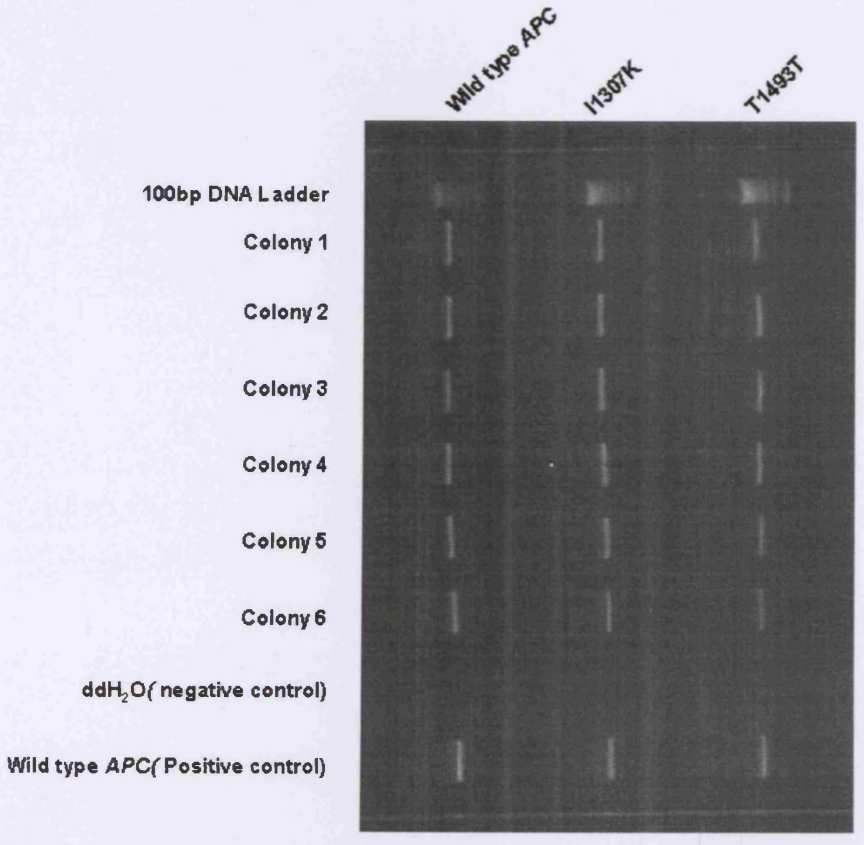


Figure 3.3 Agarose gel visualising PCR amplification of pCMV-*APC* vector in XL10-Gold ultracompetent cells using primers specific for exons human *APC*.

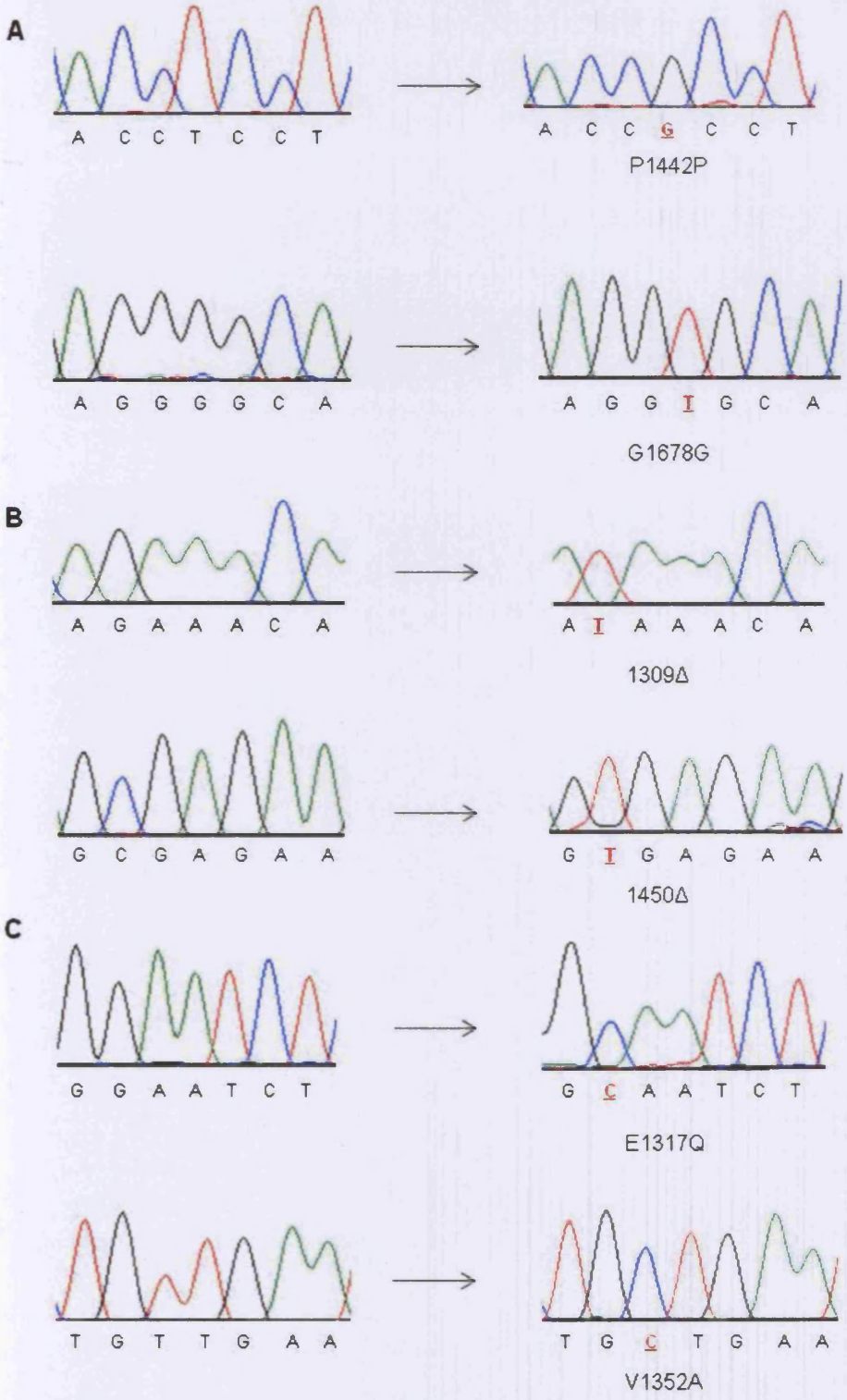


Figure 3.4 Examples of sequencing analyses of site-directed mutant pCMV-*APC* constructs. Wild type sequences are shown on the left and A) two nonsynonymous mutations, B) two truncating mutations and C) two nonsynonymous mutations are shown on the right. Mutated base is red, bold and underlined.

3.3.5 Functional analyses of nonsynonymous variants in the β-catenin down-regulating domain

We examined the functional consequences of 16 of the nonsynonymous variants identified within the β-catenin down-regulating domain using CRT assays as described in section 2.44.11.6. The four truncating mutations 1309Δ, 1450Δ, 1517Δ, and 1914 Δ were predicted to retain one, two, three, and five 20AARs, respectively. As expected, the six synonymous variants and the wild-type *APC* construct all suppressed CRT in an *APC*-deficient cell line (figure 3.5A). The 1309Δ construct, which is associated with a severe FAP phenotype, failed to suppress CRT ($P <$ 0.0001) whereas 1450Δ, 1517Δ, and 1914Δ suppressed CRT more effectively than 1309 Δ but not as effectively as the wild-type construct ($P < 0.05$; figure 3.5B). Next, we tested the 16 nonsynonymous variants, and in accordance with our hypothesis that some of these rare variants may compromise function, seven (43.8%) had a significantly reduced ability to suppress CRT (Figure 3.5C) Both I1307K and E1317Q were found to be functionally compromised.

3.3.6 Immunocytochemical analyses of β-catenin degradation

We aimed to explore whether the failure to suppress CRT in our *in vitro* model system would affect the subcellular distribution of endogenous β-catenin in transfected SW480 cells. No differences in β -catenin distribution could be detected after transfection with positive and negative control constructs (wild type *APC* and the three truncating mutations 1309Δ, 1450Δ and 1517Δ, Figure 3.6).

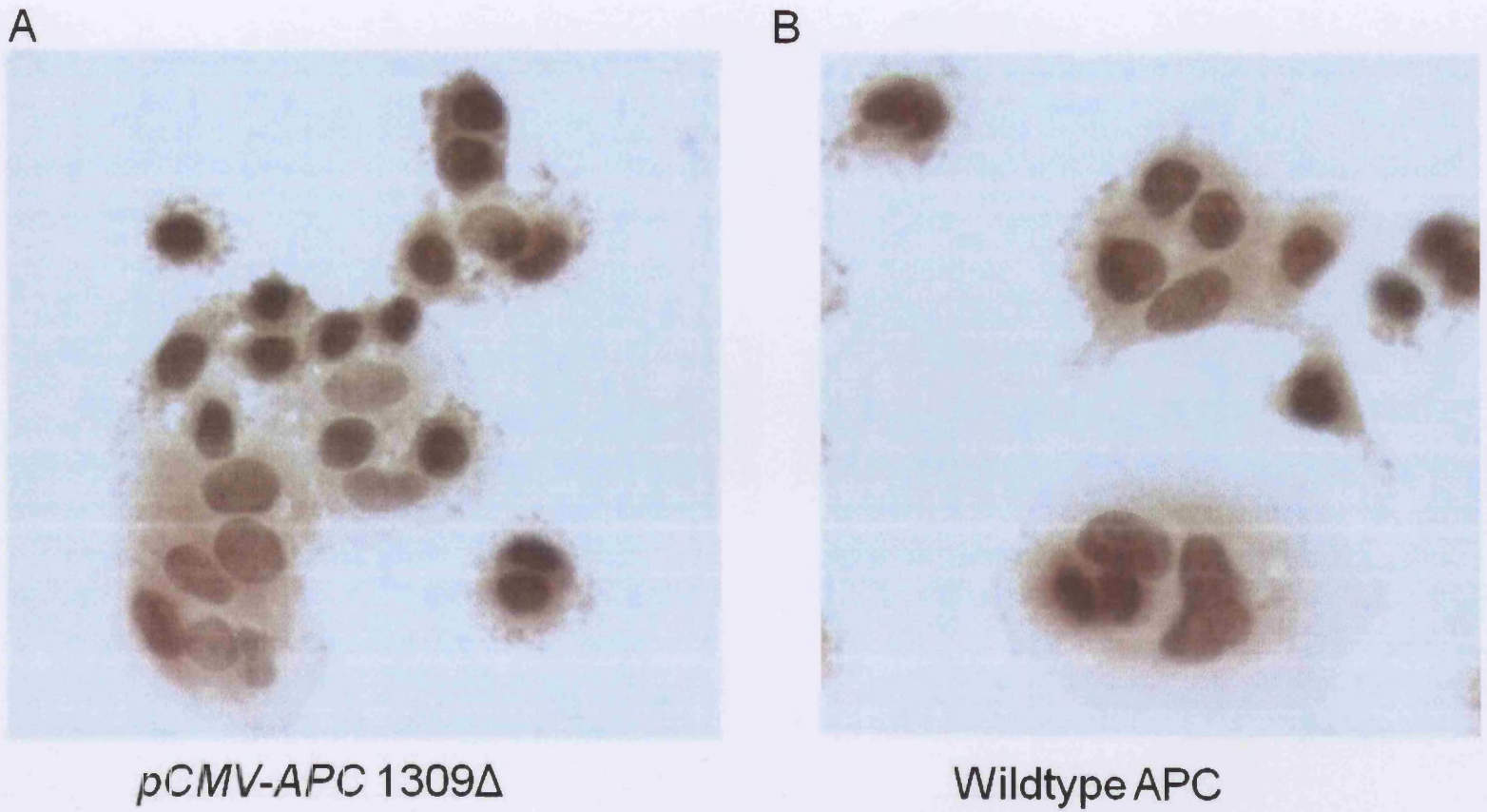


Figure 3.6 SW480 cell line transfected with A) 1309Δ mutant *APC* or B) wildtype *APC* and stained for β-catenin. No difference can be seen in the distribution of intracellular β-catenin. Cells were seeded in low numbers (~1000 per well) to prevent overgrowth and 'clumping' since SW480 is a transformed cell line that does not have a cell-cell contact mediated growth inhibition response and are presented as a selection of neighbouring cells and lone cells.

3.3.7 qRT-PCR analysis to examine gene expression of downstream targets of Wnt signalling

We attempted to analyse the expression of Wnt target genes following transfection with pCMV-APC constructs containing nonsynonymous variants. We carried out quantitative real time PCR analysis using total RNA isolated from SW480 cells transfected with either wildtype or the 1309Δ truncating mutant pCMV-*APC* construct to validate our *in vitro* model system. Detection of *c-myc* and CCND mRNA levels was multiplexed alongside TATA –box binding protein (*TBP*), the endogenous control for normalization of all assays. Relative quantification of gene expression levels was determined using the Comparative Ct method (Schmittgen D and Livak KJ, 2008). This method compares the relative amount of the target sequence to any of the reference values chosen and the result is given as relative to the reference value. The medians of the comparative Ct values ($2^{-\Delta\Delta Ct}$) values for *c-myc* and *cyclinD1* were compared using a Mann–Whitney U-test to determine if the relative fold change was significantly different between the two groups. No significant difference was found between the two groups (Figure 3.7).

3.3.8 *In silico* predictions of likely pathogenicity of nonsynonymous *APC* variants

An in silico approach was used to investigate whether nonsynonymous variants could interfere with APC's roles in mediation of intercellular adhesion, stabilisation of the cytoskeleton, chromosome stability or regulation of the cell cycle and apoptosis. I used the programs PolyPhen and Align-Grantham Variation/Grantham Deviation (Align-GVGD) to help predict the functional consequences of all 61 different non-synonymous variants that we identified in *APC*. PolyPhen uses empirically derived rules to predict the pathogenesis of SNPs. Results are scored as follows:

- 'probably damaging': it is with high confidence supposed to affect protein function or structure;
- 'possibly damaging': it is supposed to affect protein function or structure;
- 'benign':most likely lacking any phenotypic effect, and;
- 'unknown': when in some rare cases, the lack of data does not allow PolyPhen to make a prediction.

Variants are classified into 5 categories by Align-GVGD:

- deleterious 1': the position of interest is 100% conserved, so any mutation at the position is predicted as deleterious;
- 'deleterious 2': there is a small variation in amino acids at the given position (the residues encountered are biochemically similar) but the mutant amino acid does not fall within that range of variation;
- 'neutral 1': the composition, polarity and volume of the mutant amino acid fall within observed range of variation according to the alignment at that position so the mutant is predicted as neutral;
- 'neutral 2': the position tolerates more than 'conservative' substitution, and lastly;
- 'unclassified': the mutant does not fall in the previous categories.

Both *in silico* analysis tools predict the possible impact of an amino acid substitution on protein structure and function using proprietary algorithmic rules applied to the annotated sequence, the phylogenetic and structural information characterising the substitution i.e. changes in polarity, bulky structures, hrdrophilic changes within the presence of functional domains and distance to known active sites.

In silico analyses using PolyPhen predicted that 24 non-synonymous variants were likely to be damaging whereas Align-GVGD predicted that 28 variants, 17 of which were also called 'damaging' by PolyPhen, were likely to alter function (Table 3.6). Together, these analyses predicted that 35/61 (57.4%) non-synonymous variants were likely to alter function.

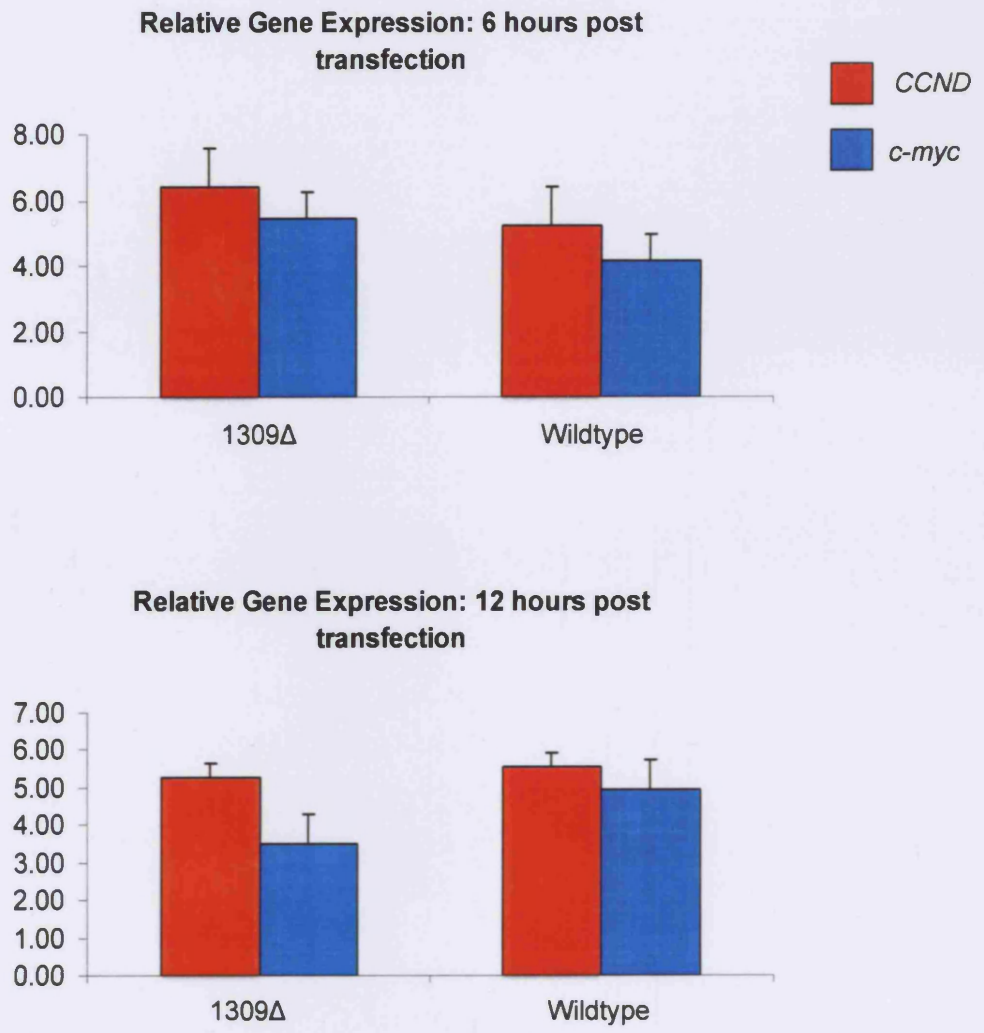


Figure 3.7 qRT-PCR analyses of *CCND* and *c-myc* gene expression 6 and 12 hours after transfection with pCMV-APC vector containing either the 1309Δ truncating or wildtype *APC*. Comparative Ct values of TATA binding box protein was used as endogenous control to normalise values

Non-synonymous	PolyPhen prediction	Align-GVGD prediction
R106H	benign	neutral 1
K150R	benign	deleterious 2
A199T	benign	deleterious 2
R216Q	benign	neutral 2
R414C	probably damaging	unclassified
S537C	possibly damaging	deleterious 1
E538V	possibly damaging	deleterious 1
L639S	possibly damaging	deleterious 1
S643P	possibly damaging	deleterious 1
R653K	benign	deleterious 2
P870S	benign	neutral 1
C947S	probably damaging	deleterious 1
M949I	benign	neutral 1
P981R	probably damaging	deleterious 1
V1125A	benign	neutral 1
L1129S	benign	unclassified
T1160K	benign	unclassified
A1247T	benign	neutral 1
I1307K	possibly damaging	neutral 2
E1317Q	benign	neutral 1
V1352A	benign	deleterious 1
K1363I	probably damaging	deleterious 1
M1413V	possibly damaging	deleterious 2
T1445A	benign	neutral 1
A1446T	benign	neutral 1
K1454E	benign	deleterious 2
P1458S	benign	neutral 2
P1467S	possibly damaging	deleterious 2
A1474T	benign	neutral 2
T1493M	possibly damaging	deleterious 1
I1572T	possibly damaging	deleterious 1
C1578G	probably damaging	deleterious 1
I1579V	benign	deleterious 2
P1584S	probably damaging	deleterious 1
R1589G	probably damaging	neutral 2
R1589C	benign	neutral 2
T1633K	possibly damaging	neutral 1
T1655A	benign	deleterious 2
R1676G	benign	neutral 1
D1714N	benign	deleterious 2
S1730F	possibly damaging	neutral 1
N1761T	possibly damaging	unclassified
Q1916K	benign	neutral 2
G1921S	benign	neutral 1
P1934L	probably damaging	unclassified
I1975F	benign	unclassified

R2066G	benign	neutral 2
H2149P	probably damaging	deleterious 1
P2158R	possibly damaging	deleterious 2
H2232D	probably damaging	deleterious 1
A2274V	benign	neutral 2
I2329V	benign	deleterious 2
P2467T	benign	neutral 2
G2502S	possibly damaging	deleterious 2
R2505Q	benign	deleterious 2
I2541V	benign	neutral 1
I2573V	benign	neutral 1
N2593S	benign	neutral 1
S2621C	benign	deleterious 2
I2756V	benign	neutral 1
A2795T	benign	neutral 1

Table 3.6 *In silico* predictions of the likely functional consequences of the non-synonymous variants identified in *APC*. See section 3.3.8 for results nomenclature

3.4 Discussion

3.4.1 Identification of pathogenic mutations in *APC* and *MUTYH* in a minority of cases within a cohort presenting with a CRA phenotype.

In FAP cases we found that, in agreement with published literature, the majority of germline truncating mutations in *APC* were either nonsense point mutations or frameshifts and resulted in a C-terminal truncated protein. In keeping with previous observations regarding the germline mutation spectrum of *APC* (Beroud and Soussi, 1996; Miyoshi *et al* 1992, Mandl *et al*, 1994) we identified two common mutation hotspots at codons 1309 and 1062. Thirty three were diagnosed with MAP, conferred by biallelic inactivation of *MUTYH*. We therefore concluded that 211 out of 691 individuals that presented with a CRA phenotype were associated with classical germline defects in known CRC predisposition genes.

3.4.2 An observed association between rare inherited nonsynonymous variants in *APC* and a CRA phenotype.

The potential role of rare non-synonymous *APC* variants in colorectal tumourigenesis is controversial. The *APC* nonsynonymous variant E1317Q was first described in 1996 in two siblings from an Ashkenazi Jewish family with late onset CRC (White S, *et al*; 1996). Since then the variant has been reported to confer an increased risk of CRAs and CRC in some, but not all, case control studies (Table 3.8): Frayling *et al,* (1998) found the E1317Q variant in 1.4% of patients with multiple CRAs (≥3 adenomas but without the 'classical' phenotype of FAP) and in 6.7% of CRC patients but in none of the 80 controls as well as also identifying the nonsynonymous mutation occurring as a somatic change in sporadic CRC. The combined data from the studies of White *et al,* (1996) and Frayling *et al,* (1998; n=80 and n=33 respectively) found that E1317Q conferred an increased risk in individuals for CRC or CRAs ($P = 0.035$). Lamlum *et al,* (2000) later concluded that that the E1317Q allele contributes to a predisposition to colorectal adenomas and carcinoma, but with low and variable penetrance since they identified E1317Q in 4.3% (7 of 164) of patients with multiple (3 – 100) CRAs compared to 0.4% (2 of 503) of a population based control sample (which also pooled together the control data of White *et al,* and Frayling *et al,*). Several other studies however (Table 3.7), have not supported an association between E1317Q and a significantly increased risk of CRC; Popat *et al,* (2000) identified the E1317Q variant a similar frequency amongst CRC patients and

the healthy spouse control group whilst Evertsson *et al,* (2001) failed to identify E1317Q in a Swedish CRC patient cohort. Hahnloser *et al,* (2003) screened 608 individuals with CRC and adenomas, and healthy controls 679 controls for the E1317Q variant and found that the frequency of heterozygotes for E1317Q among patients with CRC, with 4-100 adenomas and <3 adenomas did not differ from healthy spouse controls. Rozek *et al,* (2006) investigated the relationship between E1317Q and CRC in a large population-based case-control study of CRC in northern Israel using 1,834 matched cases and controls and concluded that it was unlikely that E1317Q was associated with a clinically meaningful risk of CRC.

Taken together, these reports infer that whilst E1317Q is a colorectal adenoma predisposition variant, that it does not confer any risk of CRC in the general populace. However, the concept that genetic predisposition to CRAs would not result in an increased risk for CRC is inconsistent with our current understanding of the adenoma –carcinoma progression sequence that results in the majority of CRCs (Muto *et al*, 1975; Fearon and Vogelstein 1990). Our data clearly shows that the E1317Q variant is indeed one of several colorectal adenoma predisposition variants and that by extension their presence within the general population would confer a slight risk of CRC. What is not clearly understood is the mechanism by which these nonsynonymous variants exert a pathogenic effect. I could speculate that E1317Q could result in compromised function of APC protein by altering the structure of the protein as a result of the charge change occurring because of the amino acid substitution. This could act to impede the the binding of APC to β-catenin by preventing accessibility to the 20AAR down regulating domain(s).

		Incidence of E1317Q in						
Study	Design	Multiple CRA patients	CRC patients	Controls	P-Value	OR	95% CI	Comments
White *et al.* 1996	Family Study		2/4 (siblings)	0/133				
Frayling *et al.* 1998	Case control, UK	2/134[a]	2/30[b]	0/80 0/213[c]	P>0.1 P=0.035 (a and b Vs c)			[c]Pooled controls from White and Frayling *et al.*
Popat *et al.* 2000	Case control, UK		2/364	2/290	P>0.01			
Lamlum *et al.* 2000	Case control, UK	7/184		2/503 (290 new) (213 from White/Frayling)	P<0.001	11.2	(2.3 - 54.3)	
Evertsson *et al.* 2001	Case series, Sweden		0/88 (hereditary) 0/106 (sporadic)					
Figer *et al.* 2001	Case series, Israel	1/21	4/85	2/148				
Heinimann *et al.* 2001	Case series, Germany	1/100 FAP						
Michils *et al.* 2002	Case control, Belgium	2/75 FAP or AFAP		1/180				E1317Q carrier identified within the control group was homozygous for the variant
Gismondi *et al.* 2002	Case control, Italian	2/182		2/235		1.29	(0.009 - 18.0)	
Hahnloser *et al.* 2003	Case control, USA	9/377 cases 2/145 (4-100 adenomas) 3/86 (1-3 adenomas) 9/377 cases 2/145 (4-100 adenomas) 3/86 (1-3 adenomas)		10/362 spouse controls 1/317 colonoscopy controls		0.84 0.52 1.16 9.01 3.69 16.59	(0.31 - 2.26) (0.10 - 2.68) (0.28 - 4.78) (1.1 - 73.6) (0.2 - 56.0) (1.5 - 178.8)	
Zhou *et al.* 2004	Case control, Sweden		1/97 (hereditary) 0/92 (sporadic)	0/188				
Guo *et al.* 2004	Case series, Singapore	0/147 Chinese 0/20 Malay 0/11 India						
Kapitanovic *et al.* 2004	Case control, Croatia		0/73	0/50				1 sporadic mutation identified in tumour DNA
Fearnhead *et al.* 2004	Case control, UK	3/124		6/480	P=0.4	2.0		
Fidder *et al.* 2005	Case control, Israel		6/538	5/440	P=0.975	0.98	(0.29 -3.24)	
Rozek *et al.* 2006	Case control, Israel		26/1,970	23/1,994 6/398 (polyp-free)		1.15 0.87	(0.65 - 2.02) (0.36 -2.14)	
Cleary *et al.* 2008	Case series, Canada Case contol, Canada	2/39	 17/971	 18/954		 1.08	 (0.59 - 2.74)	Pooled group of patients with CRC and multiple CRAs

Table 3.7 Studies of *APC* E1317Q in chronological order. Abbreviations: FAP, familial adenomatous polyposis; AFAP, attenuated adenomatous polyposis

The I1307K nonsynonymous variant, originally identified by Laken *et al* (1997) has been confirmed as a low penetrance pathogenic allele resulting in CRC predisposition amongst Ashkenazi Jews. The variant is a transversion mutation (T→A) in *APC* at codon 1307 that results in a hypermutable poly-adenine tract, causing mutations due to slippage during DNA replication. The frequency and epidemiology of I1307K and CRC within the Ashkenazi Jewish ethnic group has been extensively reviewed by Locker and Lynch (2004). In the initial Ashkenazi Jewish population studied by Laken *et al* (1997), the frequency of the variant was 6% in a sample set ascertained for Tay-Sachs testing,10% (P <0.033 by x2) in CRC patients, 16% (16%vs. 6.6%, P <0.033 by x2) in Ashkenazim diagnosedwith colorectal cancer under age 66, and 28% (P<0.001) in affected individuals with a family history of colorectal cancer. Multiple studies have assessed the frequency and epidemiology of I1307K and the incidence of I1307K amongst the Ashkenazi Jewish populace is estimated to be between 5.4 – 12.8% and most studies suggest significantly associated with multiple CRA and CRC incidence (table 3.8).

The role of I1307K outside of the Ashkenazim ethnic group has been examined and only one study has provided data supporting a significant association between I1307K and CRA or CRC development (Table 3.9). Frayling *et al,* (1998) identified I1307K in 3 of 134 individuals presenting with multiple CRAs in a UK based study. These individuals were later determined to be of Ashkenazim Jewish descent. A carrier rate of 8.1% in the British Ashkenazi Jewish control group was ascertained and 0% in the non-Jewish control group. Yuan *et al* (1998) identified I1307K in 4 members of a large French Canadian HNPCC kindred carrying a novel truncating mutation in hMLH1. Whilst the identification of the variant in this cohort is novel, no association between the CRC incidences was found. Lothe *et al,* (1999) determined that I1307K was rare in the Norwegian populace since it was present in 1 of 210 patients with CRC and they were later determined to be of Jewish descent. Prior *et al* (1999) assessed whether I1307K was unique to the Ashkenazi population and did not find the variant in 105 African-American controls, 38 Italian CRC patients, 148 Finnish CRC patients and 54 Hawaiian – Japanese CRC patients. Nathanson *et al,* (1999) did report a woman of Italian descent, affected with breast and ovarian cancer

Study	Design	Incidence of I1307K in		P-Value	Reported Frequency in AJ population (95% CI)	Comments
		Multiple CRA pts	CRC pts	Controls		
AJ Based Studies						
Laken *et al*. 1997	Case control, USA		22/211 AJs[c] 14/89 Pts<66 years old[d] 8/121 Pts ≥66 years old[e] 7/26 FH+[f] 0/13 FH-[g] 15/173 FH unkown[h]	0/243 Non-Jewish[a] 47/766 AJ[b]	*P*<0.0001 (a Vs. b) *P*<0.033 (b Vs. c) *P*<0.033 (d Vs. e) *P*<0.001 (b Vs. f) *P*<0.02 (f Vs. h)	6.1% (4.4 - 7.8)	
Woodage *et al*. 1998	Case series, USA	367/5,081 heterozygous* 2/5,081 homozygous*		4,712/5,081 wildtype*	*P*=0.06	7.0 % (6.3 - 7.7)	Samples obtained from an Ashkanizm DNA Biobank
Drucker *et al*. 2000	Case series, Israel		12/71 AJs[i] 0/34 Sepharidic Jews 3/4 Yemenite Jews[j]	17/298 AJs[k] 9/189 Yemenite Jews[l]	*P*=0.004 (i and j Vs k and l)	5.7% (0.018 - 0.045)	
Shtoyerman *et al*. 2001	Case series, assesing incidence amongst pts of Jewish descent		29/227 AJs 2/122 non-AJ	2/210 Moroccan Jews 2/160 Iraqi Jews		12.8% (4.0 - 7.4)	
Bahar *et al*. 2001	Australia			104/1200 Australian AJ		8.67% (7.07 - 10.26)	
Non-AJ Based Studies							
Frayling *et al*. 1998	Case control, UK	3/134 pts but 3/3 AJs		8/98 AJ			3 carriers were determined to be of Ashenazim descent
Yuan *et al*. 1998	Case study, Canada	4 individuals within a large French Canadian kindred with HNPCC carried the I1307K variant					2 individuals unaffected by HNPCC also carried I1307K
Lothe *et al*. 1998	Case series, Norway		1/210				Carrier was determined to be of Ashkenazim descent
Prior *et al*. 1999	Case control, misc.		0/38 Italian pts 0/148 Finnish pts 0/54 Hawaiian-Japanes pts	105 African Americans			
Nathanson *et al*. 1999	Case study, US	1 female out of 104 non-AJ pts with a family history of both breast and colon cancer.					
Evertsson *et al*. 2001	Case series, Sweden		0/194				
Michils *et al*. 2002	Case series/diagnostic validation, Belgium		1/75	0/180			Carrier was determined to be of Ashenazim descent
Guo *et al*. 2004	Case series, Singapore		0/147 Chinese 0/20 Malay 0/11 Indian				
Dundar *et al*. 2007	Case study, Turkey		26/56	0/18	*P*<0.05		

Table 3.8 Studies of *APC* nonsynonymous variant I1307K in Ashkenazi Jews and non-Ashkenazi Jewish groups. Pts= patients.

and with a family history of CRC who was heterozygous for the I1307K allele. A Swedish study (Evertsson *et al,* 2001) did not find I1307K in a 194 cases of familial CRC. However, Dundar *et al,* (2007) observed a significant over-representation of I1307K in 30 of 56 Turkish patients with CRC when compared to a small healthy control group (0/18; $P<0.05$).

In our study, we show that significantly more non-FAP non-MAP patients with CRAs carry E1317Q as compared to controls, clearly supporting a role for this variant in predisposing to CRAs. Although we did not find a similar over-representation of I1307K, this variant was less frequently observed since it is normally found in 6%-7% of the Ashkenazi Jewish population (Gryfe, *et al,* 1999; Woodage, *et al,* 1998) and <4% of our patients were from this ethnic background. Importantly, our study showed that even when I1307K and E1317Q were excluded, significantly more non-FAP non-MAP patients carried a variety of other rare non-synonymous variants, indicating that other rare variants in *APC* act as low penetrance disease alleles. However, apart from the variant I1307K, which has been shown to create a hypermutable tract that predisposes to somatic mutations (Laken, *et al* 1997), the mechanism of pathogenesis underlying almost all of these variants in *APC* remains unclear.

3.4.3 Functional analysis of nonsynonymous variants within the β-catenin down regulating domain.

We assessed the functional consequences of sixteen of the nonsynonymous variants identified within the β-catenin down-regulating domain using CRT assays. As expected, the six synonymous variants and the wild-type *APC* construct all suppressed CRT in an *APC*-deficient cell line. Consistent with the notion that mutations resulting in the retention of different numbers of 20AARs lead to different levels of β-catenin-associated signalling, 1309Δ, that is associated with a severe FAP phenotype, failed to suppress CRT ($P<0.0001$), whereas 1450Δ, 1517Δ and 1914Δ suppressed CRT more effectively than 1309Δ but not as effectively as the wild type construct ($P<0.05$). There was a clear relationship between the predicted number of 20AARs retained and CRT activity (7>5>3>2>>1). Next, we tested the sixteen non-synonymous variants and in accordance with our hypothesis that some of these rare variants may compromise function, seven (43.8%) had a significantly

reduced ability to suppress CRT. Amongst the seven, I1307K and E1317Q were found to be functionally compromised. This analysis implicates a reduced ability to degrade intracellular β-catenin levels as a mechanism of propagating aberrant Wnt signalling leading to unregulated cell growth.

We attempted to measure the effect of the nonsynonymous variants on the expression profiles of Wnt target genesin SW480 cell lines transfected with APC constructs by RT-PCR, but initial validation of our model system with the truncating mutant 1309Δ failed to show any significant difference when compared to wildtype *APC*. We also attempted to examine the effect of nonsynonymous variants on β-catenin distribution within the cell but immunostaining to visualise any partitioning between cytosolic and nuclear β-catenin following transfection with the 1309Δ truncating mutant and WT *APC* failed to demonstrate a difference. This is likely to reflect limitations of our *in vitro* model to assess the subtle changes required to elicit changes in gene expression for example, and highlights the difficulties faced when attempting to characterise the functional affects of rare nonsynonymous variants. A primary limitation may have been that the transient transfection of a colon cancer cell line with a pCMV-*APC* construct would have resulted in intracellular over expression of APC. Immunostaining for total β-catenin by Western blot (WB) could have been used to determine β-catenin turnover in our cell model. The use of hyper- and hypotonic lysis buffers followed by Western blotting for β-catenin could have been used to ascertain subcellular distribution of cellular β-catenin and may have been a better alternative to the immunocytochemical analyses performed.

3.4.4 *In silico* analyses

We used the programs PolyPhen and Align-Grantham Variation/Grantham Deviation (Align-GVGD) to help predict the functional consequences of 61 different nonsynonymous variants that we identified in *APC*. *In silico* analyses using PolyPhen predicted that 24 non-synonymous variants were likely to be damaging whereas Align-GVGD predicted that 28 variants, 17 of which were also identified as 'damaging' by PolyPhen. Taken together, these analyses predicted that (57.4%) of the nonsynonymous variants were likely to alter the function of APC. Functional studies' using the CRT assay show that six nonsynonymous variants (I1307K, E1317Q, V1352A, I1579V, R1676G, D1714N, G1921S) significantly failed to repress β -catenin regulated transcription; Polyphen results also indicated that I1307K was possibly deleterious to protein function and Align GVGD indicated that V1352A and I1599V were deleterious to protein function (Table 3.9). However, neither predicted that E1317Q, R1676G, D1714N or G1921S would result in aberrant protein function. Conversely M1413V, K1454E, P1467S, I1572T, C1578G and P1934L were highlighted by one, or both programs (Table 3.9) as possibly having a negative effect on protein function. In cases where the CRT assay show repressed β -catenin regulated transcription (i.e. non-pathogenic) but *in silico* predictions indicated deleterious effects, the latter might infer that a protein function other than β -catenin regulation is compromised by the variant. The accuracies of these programs have been assessed against known disease causing mutations; Ng and Heinkoff (2006) assessed program error for a number of i*n silico* programs summarising the rates of false negative and false positive predictions. False negatives were defined as amino acid changes that are known to be disease causing predicted as not damaging to the protein and false positives were defined as amino acid changes that are beliieved to have no disease causing association that were predicted as damaging to the protein. Polyphen has been shown to correctly identify pathogenicity 60% of the time and has a false positive rate of 9% and a false negative rate of 31%. Align has been shown to correctly identify pathogenicity 59% of the time and has a false positive rate of 12% and a false negative rate of 29%.

Variant	CRT Assay	Polyphen	Align-GVGD
I1307K	Pathogenic	Poss. deleterious	Neutral
E1317Q	Pathogenic	Benign	Neutral
V1352A	Pathogenic	Benign	Deleterious
M1413V	Non-pathogenic	Poss. deleterious	Deleterious
T1445A	Non-pathogenic	Benign	Neutral
A1446T	Non-pathogenic	Benign	Neutral
K1454E	Non-pathogenic	Benign	Deleterious
P1467S	Non-pathogenic	Poss. deleterious	Deleterious
A1474T	Non-pathogenic	Benign	Neutral
I1572T	Non-pathogenic	Poss. deleterious	Deleterious
C1578G	Non-pathogenic	Poss. deleterious	Deleterious
I1579V	Pathogenic	Benign	Deleterious
R1676G	Pathogenic	Benign	Neutral
D1714N	Pathogenic	Benign	Neutral
G1921S	Pathogenic	Benign	Neutral
P1934L	Non-pathogenic	Poss. deleterious	Unclassified

Table 3.9 Summary or results from the functional CRT assay and *in silico* predictions for the nonsynonymous variants within the β-catenin down regulating domain. Poss = possibly.

3.4.5 Multifactorial inherited susceptibility to CRAs

The rare variant hypothesis states that changes such as nonsynonymous variants in a number of genes may have a cumulative pathogenic effect resulting in a model of multifactorial inheritance for CRAs. Here, we found that individually rare, but collectively common inherited non-synonymous variants were significantly over-represented in patients who did not carry conventional pathogenic mutations in the *APC* or *MUTYH* genes (non-FAP/non-MAP patients; 81/480, 16.9%) as compared to patients with FAP or MAP (20/211, 9.5%; P=0.0113) and that this over-representation was highest in those non-FAP non-MAP patients with 11-99 CRAs (30/161, 18.6%, P=0.0103). Such variants are therefore likely to play a significant role in multifactorial inherited predisposition to CRAs and that a specific CRA phenotype of 11 to 99 CRAs is associated most significantly to the presence of rare nonsynonymous variants in *APC*. Only a minority of patients with this phenotype have been shown previously to have conventional mutations in *APC* of *MUTYH*. Further characterization of these and other low penetrance alleles promises to clarify the molecular basis of inherited predisposition to CRAS.

Chapter Four: The *APC* variant E1317Q predisposes to colorectal adenomas by a novel mechanism of relaxing the target for tumourigeneic somatic APC mutations

4.1 Introduction

Germline mutations that lead to AFAP cluster within exons 3 and 4 (5' of codon 169) of *APC*, in the alternatively spliced region of exon 9 (codons 312-412) and in the terminal region of exon 15 (3' of codon 1581) (Fearnhead *et al*, 2001; Knudson *et al* 2003). In agreement with Knudson's '2-hit' hypothesis, inactivation of both (Miyoshi *et al* 1992; Powell *et al* 1992) *APC* alleles is observed in most familial and sporadic CRAs and CRC (Miyoshi *et al* 1992; Powell *et al* 1992). However, it has recently been shown that type and location of the somatic '2nd-hit' in FAP tumours is determined by the type and location of the germline lesion (Lamlum *et al*, 1999), and that the position of the mutations in sporadic CRCs are also interdependent (Rowan, *et al*, 2000; Cheadle 2002). Careful examination of somatic mutations has revealed that one of the two mutant *APC* alleles within a tumour is generally selected for retention of one, or rarely, two of the 20-amino acid β-catenin binding and degradation repeats (20AARs). This has led to the suggestion that a 'just-right' level of β-catenin signalling within the Wnt pathway is required for colorectal tumour formation (Albuquerque *et al*, 2002).

In AFAP, a subset of tumours appear to harbour two somatic mutations, one on the wild type allele, the other on the attenuated *APC* (*AFAP*) allele carrying the germline mutation (Spirio *et al*, 1998; Su *et al* 2000; Sieber *et al*, 2006). The identification of this so-called '3rd hit' has suggested that *AFAP* alleles sometimes require further inactivation to confer the optimum signalling for tumourigenesis. Where the germline AFAP-associated mutation lies at the 5' end of the gene, the residual activity is most likely mediated via alternative splicing of exon 0.1 to an intra-exonic splice acceptor in exon 5 (Samowitz *et al*, 1995) or via translation initiation from the internal ribosome entry site (IRES) between codon 157 and the AUG at codon 184 (Heppner Goss *et al*, 2002). Likewise in AFAP patients carrying exon 9 mutations, direct splicing of exon 8 to exon 10 in a subset of *APC* mRNAs allows functional APC expression (Su *et al* 2000; Groden *et al*, 1991).

Mechanisms by which nonsynonymous variants act is unclear except for I1307K. This variant results in a hypermutable tract that predisposes to somatic mutations (Laken *et al,* 1997). We investigated the effect of E1317Q, which lies between the first and second 20AAR, by comparing the pattern of somatic *APC* mutations found in tumours from an AFAP family carrying an exon 4 truncating mutation as their only germline *APC* coding region alteration, to that found in tumours from members of a large AFAP family who carry similar exon 4 truncating mutation and the E1317Q variant on the same germline *APC* allele.

4.2 Methods

4.2.1 Patients and Samples

The study was approved by the Wales Multicentre Research Ethics Committee. Family S consisted of two generations affected with CRC, a mother (Patient L) and her daughter (Patient M). Patient L had no prior family history and presented with CRC at 44 years of age. Only one additional adenoma was noted at surgery. She re-presented with symptomatic polyposis at 58 years of age at which time several hundred macroscopic colorectal adenomas, ranging from 2-10mm, were noted. Patient M had 36 colorectal polyps removed following a total colectomy at 34 years of age. Following histopathological inspection 6 were CRAs and the remaining 17 were benign hyperplastic growths. Family B included at least four successive generations affected with CRAs or early onset CRC (Figure 4.1). Macroscopic adenomas ranged in number from less than ten to several hundred, but were generally small (<2mm), even in patients in their 4^{th} and 5^{th} decades, and affected predominantly the caecum and proximal colon. Apart from duodenal adenomas, no extracolonic features of FAP were observed in any patients.

DNA was prepared from venous blood samples taken from all available members of families S and B, and from 72 fresh frozen CRAs from Patients L and M of Family S and 105 fresh frozen CRAs from seven affected individuals from Family B (Patients A-G, Figure 4.1). RNA was extracted from fresh frozen normal colonic tissue. All tissues were examined histologically to verify their nature.

4.2.2 PCR and RT-PCR

Exons 1 to 14 of *APC* were amplified as 14 fragments and the first 2.8kb of exon 15 as 18 overlapping fragments, as previously described (Al-Tassan, N *et al*, 2002). We amplified the MCR as either a 1.1kb fragment using the primers MCR_F (5'-CCATCCAAGTTCTGCACAGAGTAGA) and MCR_R (5'-AGGTGGAGGTAATTTTGAAGCAGTC), or as two overlapping 0.8kb fragments using the primers 15.6F (5'-AATCAAAATGTAAGCCAGTCT) and 15.7R (5'-ATTCCACTGCATGGTTCAC), and, 15.8F (5'-CAAAAGTGGTGCTCAGACAC) and 15.9R (5'-CCCCGGTGTAAAACTAACA). The 3' region of exon 15 of *APC* was amplified as 22 overlapping fragments (Table 4.1), using genomic DNA as a template. For RT-PCR, we amplified a 158bp region of exons 11 and 12 using the primers APCRNA_11F (5'-CAGGCCATTGCAGAATTAT) and APCRNA_12R2 (5'-CAGCCTTTCATAGAGCATAGC), and a 1.2kb fragment from exon 3 to exon 12 using the primers APCRNA_3Fnew (5'-CTGTTCCTATGGGTTCATTTC) and APCRNA_12R2. RT-PCR products were cloned into pGEM-T Easy, propagated in *E. coli* JM109 and sequenced. In Family B, clones were assigned to the wild type or AFAP allele using the exon 11 polymorphism 1458 C→T.

4.2.3 Assays for the germline variants

Y159X was assayed using normal (N-ASO 5'-AAGTTGAGCGTAATACCAG) and mutant (M-ASO 5'-AAGTTGAGCCTAATACCAG) allele-specific oligonucleotide (ASO) probes. Five micro litres of denatured exon 4 PCR product was spotted onto duplicate membranes of Hybond N^+, dried, prehybridised at 42°C for 30mins, hybridised with either normal or mutant γ-^{32}P end-labelled ASOs at 42°C for 2 hrs, washed, and exposed to film. E1317Q was assayed using a *Pvu*II digest of a 506bp PCR product generated using the primers E1317QLF (5'-GCAGTAAAACCGAACATATG) and E1317QR (5'-TGGACTTTTGGGTGTCTG). 10μl of PCR product was digested according to manufacturers' recommendations and analysed on a 3% Nusieve 3:1 agarose gel. These were carried out by Dr. S Jones, Cardiff University, UK.

Exon/Primer	Primer sequence (5'-3')	PCR conditions (°C)	Sequencing	
			F	R
1	F-AGGTCCAAGGGTAGCCAAGG	55	Y	Y
	R-TAAAAATGGATAAACTACAATTAAAAG			
2	F-AAATACAGAATCATGTCTTGAAGT	55	N	Y
	R-ACACCTAAAGATGACAATTTGAG			
3	F-TGCTTAAAGCAATTGTTGTAT	55	Y	Y
	R-GTACACAAGGCAATGTTTACT			
4	F-TGCAGTCTTTATTAGCATTGTT	58	N	Y
	R-CAGGCCTAAAGTTGGGTAA			
5	F-CTTTTTTGCTTTTACTGATTAACG	55	Y	Y
	R-TGTAATTCATTTTATTCCTAATAGCTC			
6	F-GGTAGCCATAGTATGATTATTTCT	55	Y	Y
	R-CTACCTATTTTTATACCCACAAAC			
7	F-AAGAAAGCCTACACCATTTTTGC	57	Y	Y
	R-GATCATTCTTAGAACCATCTTGC			
8	F-ACCTATAGTCTAAATTATACCATC	55	Y	Y
	R-GTCATGGCATTAGTGACCAG			
9	F-AGTCGTAATTTTGTTTCTAAACTC	55	Y	Y
	R-CTTTGAAACATGCACTACGA			
10	F-AAACATCATTGCTCTTCAAATAAC	55	Y	Y
	R-TACCATGATTTAAAAATCCACCAG			
10a	F-CCTGTATTCCAATGGATTGTAG	55	Y	Y
	R-CTATGTCCCCAGCAGTCAC			
11	F-GATGATTGTCTTTTTCCTCTTGC	58	Y	Y
	R-CTGAGCTATCTTAAGAAATACATG			
12	F-AAGCTTGGCTTCAAGTTGTC	55	Y	Y
	R-CAGAGTGAGACCCTGCCTC			
13	F-TCCCAAAGTGATAGGATTACA	58	Y	Y
	R-AGGGAATCTCATGGCTAAA			

14	F-TAGATGACCCATATTCTGTTTC R-CAATTAGGTCTTTTTGAGAGTA	55	Y	Y
15.1	F-CAAAAGGAGATGTGGAATACT R-AGCCAGGAGACATAATATTG	55	Y	Y
15.2	F-CAGGAAGCATTATGGGACAT R-GAGGAGCTGGGTAACACTGTAG	55	Y	Y
15.3	F-AAGGCATCTCATCGTAGTAA R-TTCCGACTTAGTGAAATTGTA	55	Y	Y
15.4	F-CTGGGTCTACCACTGAATTAC R-AGTTGTACTTTGATTCCTTGAT	55	Y	Y
15.5	F-CCTAGCCCATAAAATACATAGT ATAGGCTGATCCACATGAC	52	Y	Y
15.6	F-AATCAAAATGTAAGCCAGTCT R-AGCTGATGACAAAGATGATAA	55	Y	Y
15.7	F-CTGCCACTTGCAAAGTTTC R-ATTCCACTGCATGGTTCAC	52	Y	Y
15.8	F-CAAAAGTGGTGCTCAGACAC R-TTCCTGAACTGGAGGCATTA	58	Y	Y
15.9	F-GACCTAAGCAAGCTGCAGTA R-CCCCGGTGTAAAACTAACA	55	Y	Y
15.10	RF-CCCAGACTGCTTCAAAATTAC R-CACGGAAAGGCTTGTGACT	55	Y	Y
15.11	F-AAAACCTCATCTGTAACCATAC R-TTCCTTCAATAGGCGTGTA	55	Y	Y
15.12	F-AATTTAAATGCTGAGAGAGT R-CCCTCTGTCTGGTATGTCT	55	Y	Y
15.13	F-TACCAGCCACACAGAACTAAC R-CACCCATATTTCTGGGACTAT	58	Y	Y
15.14	F-CACAGGGAGAACCAAGTAAAC R-AATGGTGATCCCAGAGAGAT	58	Y	Y
15.15	F-GAAGGTGCAAATTCCATAGTA R-GGCTGTTTGACCTTCACTAG	55	Y	Y

15.16	F-AGGCAGGACAATGATTCATAT R-GGCATTCTTGGATAAACCTG	55	Y	Y
15.17	F-CCCTAGTACTGCTTCAACTAAG R-TGGTCTTCCATCATTATACTCT	55	Y	Y
15.18	F-AGACCAGCTTCTCCCACTAG R-ACCTGAGGAAACGGTCTGA	55	Y	Y
15.19	F-ACAAAGTAAAGAAAACCAAGTA R-GGGGTACGTTCCACTATAG	55	Y	Y
15.20	F-TCGCCTGAACTCCTTTATTC R-TTCCAGAACAAAAACCCTCTA	55	Y	Y

Table 4.1 Oligonucleotide primers used to amplify exon 15 of *APC*.

4.2.4 Assays for LOH at the APC locus

FAM-labelled PCR products encompassing E1317Q were digested with *Pvu*II, mixed with highly deionised formamide and heat denatured before running on an ABI PRISM 3100 Genetic Analyser. Results were analysed using Genescan software and peak heights compared to a series of five control DNAs extracted from adjacent normal colon. Ratios exceeding 1.8:1, after adjusting for normal variation, were taken as indicative of LOH. The nonsense mutation Q163X in exon 4, the variant in intron 5 (647+32 C→T), and the silent polymorphisms in exon 11 (1458 C→T) and 15 (1493 G→A and 5880 A→G) were similarly assayed in informative individuals using *Hpy*188I, *Tsp*RI, *Rsa*I, *Btg*I and *Bsa*WI restriction digests, respectively. All assays were carried out in duplicate. These were carried out by Dr. S Jones, Cardiff University, UK.

4.2.5 dHPLC analysis

dHPLC was carried out using the 3500HT WAVE nucleic acid fragment analysis system. To enhance the formation of heteroduplexes prior to analysis, the PCR products were denatured at 94°C and re-annealed by cooling to 50°C at a rate of 1°C per min; dHPLC was carried out at the melting temperatures predicted by Wavemaker (version 4.1) software with a 12% acetonitrile gradient over 2.5 min.

Samples displaying aberrant dHPLC elution profiles were sequenced directly. These were carried out by Dr. S Jones, Cardiff University, UK.

4.2.6 Direct sequencing

Amplification products were purified by incubation with exonuclease I and shrimp alkaline phosphatase. Automated sequencing was carried out using the Big Dye Terminator Cycle Sequencing kit version 3.1. Sequencing reactions were purified using the Montage SEQ96 Sequencing Reaction Cleanup kit, and analysed on an ABI PRISM 3100 Genetic Analyser.

4.2.7 Clone based re-sequencing

Fragments spanning the MCR (primers MCR_F and MCR_R) were purified using the PCR purification kit, cloned into pGEM-T Easy, and propagated in *E. coli* JM109; at least twelve recombinant clones from the wild type and AFAP alleles were sequenced (Oswel Services). Sequence data was aligned and variants in two or more clones from the same allele were analysed by an independent assay to confirm that they were genuine sequence variants and not PCR or cloning induced errors.

Somatic mutations identified in Family B were assigned to an *APC* allele by cloning and sequencing PCR products that included both the somatic mutation and a known heterozygous germline variant. These were carried out by Dr. A Dallosso, Cardiff University, UK.

4.2.8 Statistical analyses

The pattern of somatic mutations in Family S and Family B were compared using a likelihood approach, similar to the one previously described by Cheadle, JP *et al* (2002). Only tumours with single somatic mutations were used for these analyses. Tumours with 2 or more somatic mutations were excluded since those mutations may not represent independent events. To estimate the Poisson distribution parameter (λ), we considered a number (n= 2, 3 and 4) of *APC* coding sequence sub-regions where the value of the parameter was constant, allowing different parameters of the Poisson distribution for each dataset and each region:

$$L_1(\lambda^{(S)},\lambda^{(B)},b)=\underbrace{\prod_{i=1}^{n}\prod_{j=b_{i-1}+1}^{b_i} e^{-\lambda_i^{(S)}}\frac{(\lambda_i^{(S)})^{k_j^{(S)}}}{k_j^{(S)}!}}_{\text{Likelihood for family}(S)}\cdot\underbrace{\prod_{i=1}^{n}\prod_{j=b_{i-1}+1}^{b_i} e^{-\lambda_i^{(B)}}\frac{(\lambda_i^{(B)})^{k_j^{(B)}}}{k_j^{(B)}!}}_{\text{Likelihood for family}(B)},$$

where b is the vector of the borderlines $b=(b_1,...,b_{n-1})$, b_0+1 - is the beginning and b_n is the end of the region; $\lambda^{(S)}=(\lambda_1^{(S)},...,\lambda_n^{(S)})$ and $\lambda^{(B)}=(\lambda_1^{(B)},...,\lambda_n^{(B)})$ are parameters of the Poisson distribution and $k_j^{(S)}$, $k_j^{(B)}$ are the number of mutations at the position *j* for S and B mutations, respectively. This likelihood was maximised with respect to the borderlines b and parameters $\lambda^{(S)}$ and $\lambda^{(B)}$. When these parameters were identified, the maximum likelihood L_0 which assumes the same parameters $\lambda=(\lambda_1,...,\lambda_n)$ in both families (Null hypothesis) for each of these sub-regions was calculated,

$$L_0(\lambda,b)=\underbrace{\prod_{i=1}^{n}\prod_{j=b_{i-1}+1}^{b_i} e^{-\lambda_i}\frac{(\lambda_i)^{k_j^{(S)}}}{k_j^{(S)}!}}_{\text{Likelihood for family}(S)}\cdot\underbrace{\prod_{i=1}^{n}\prod_{j=b_{i-1}+1}^{b_i} e^{-\lambda_i}\frac{(\lambda_i)^{k_j^{(B)}}}{k_j^{(B)}!}}_{\text{Likelihood for family}(B)}$$

The two likelihoods $L_1(\lambda^{(S)},\lambda^{(B)},b)$ and $L_0(\lambda,b)$ were then compared using the Likelihood Ratio test with *n* degrees of freedom. Likelihood comparison approach carried out by Dr V. Moskvina from the Bioinformatics unit, Cardiff University.

4.2.9 β-catenin/TCF regulated transcription (CRT) assays

Variants were introduced into pCMV-APC constructs using the Quikchange XL site-directed mutagenesis kit. All clones were sequenced for the entire *APC* ORF to confirm that the correct variants were incorporated and to ensure that no unwanted errors had been introduced. The luciferase reporter plasmids pTOPFLASH and pFOPFLASH which contained wild type or mutated TCF-response elements were used to assess CRT as previously described (section 2.4.11.8).

4.3 Results

4.3.1 Germline APC defects in Family S and Family B

We sequenced the entire APC open reading frame (ORF) in a peripheral blood DNA sample from an AFAP patient (Patient L) from Family S and identified the exon 4 nonsense mutation Q163X (487 C→T) as the only coding region variant. We confirmed the presence of this mutation in the probands affected daughter (Patient M).

We also sequenced the entire *APC* ORF in a peripheral blood DNA sample from an affected member (III.3, Fig. 1) of a second, larger, AFAP family (Family B) and identified the nonsense mutation Y159X (477 C→G) in exon 4 and the non-synonymous variant E1317Q (3949 G→C) in exon 15. Screening of 26 living members of Family B for these two variants, using either ASO or restriction digestion based assays, showed that both variants co-segregated with the AFAP phenotype (Figure 4.1). Y159X and E1317Q therefore lie on the same *APC* allele (hereafter termed the 'AFAP allele'). We carried out RT-PCR from exons 3-12 and/or exons 11-12 of *APC* using RNA extracted from normal colonic mucosa of one member of Family S and two members of Family B. Cloning and sequencing of these products showed that the mutant AFAP allele was expressed at the mRNA level in both families, albeit at a ~2.4 - 3 fold lower level compared to the wild type allele (46 AFAP vs. 138 wild type clones from Family S and 96 AFAP vs. 233 wild type clones from Family B).

4.3.2 Somatic APC mutations in Families S and B

We sought somatic *APC* mutations in 72 CRAs from 2 affected individuals from Family S by assaying for LOH using 2 intragenic markers and by direct sequence analysis of the entire *APC* ORF. We identified somatic mutations in 58 (80.6%) tumours (Table 4.2, Figure 4.2); 55 (94.8%) of these carried single somatic mutations and 3 (5.2%) carried 2 somatic mutations.

We also sought somatic *APC* mutations in 105 CRAs from 7 affected individuals from Family B by assaying for LOH at 4 intragenic loci and by using a combination of dHPLC, direct and clone based re-sequencing of the entire *APC* ORF. We identified

somatic mutations in 46 (43.8%) tumours; 36 (78.3%) of these carried single somatic mutations, 9 (19.6%) carried 2 somatic mutations, and 1 carried 3 somatic mutations (Table 4.2, Figure 4.2). Of the 36 tumours with single somatic mutations, 17 were proven to have a mutation on the wild type *APC* allele and 1 was proven to have a mutation on the AFAP allele. In 2 tumours, two somatic mutations were observed independently on the WT allele, likely reflecting a mixed population of tumour cells. In 6 of the remaining 7 tumours with two somatic mutations, one of the somatic mutations was proven to affect the AFAP allele and therefore these are likely to represent genuine 3rd hit tumours. In the single tumour with 3 somatic mutations (A5S), one mutation was proven to lie on the AFAP allele and the other 2 somatic mutations were proven to lie on different WT alleles. This tumour likely contained a mixture of "2-hit" and "3-hit" cells. In total therefore, mutations were identified on the AFAP allele in 8/49 (16.3%) tumours from Family B. In terms of pathology, we found no obvious differences between tumours with 2 or 3-hits: adenomas with somatic mutations on the AFAP allele were 1.5mm to 3.5mm in size with mild dysplasia and tubular morphology and adenomas with somatic mutations on only the wild type allele were 0.5mm to 10mm in size with varying degrees of dysplasia (77.8% mild, 11.1% moderate, 5.6% severe, 5.6% unknown) and morphology (50% tubular, 11.1% tubulovillous, 38.9% unknown).

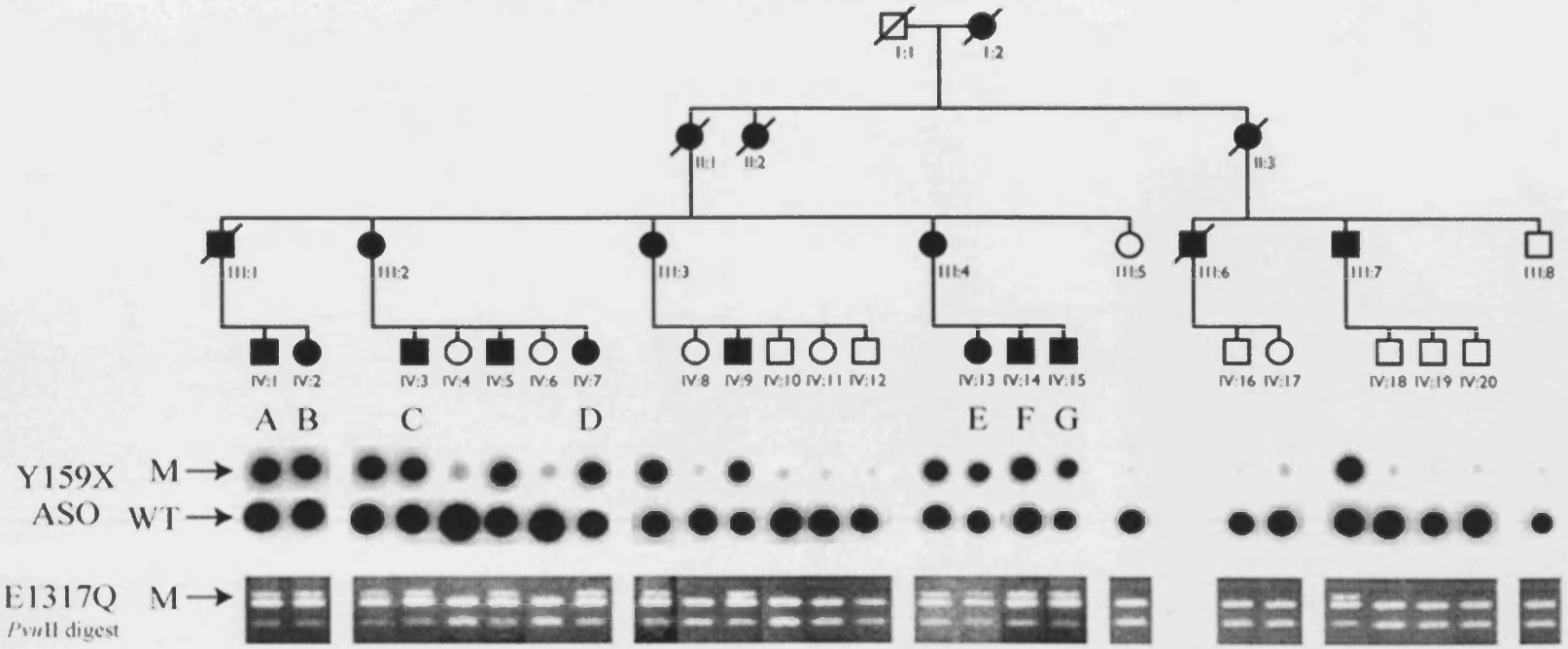


Figure 4.1 Pedigree of Family B and cosegregation of the *APC* variants Y159X and E1317Q, with AFAP. ASO and restriction digest assays were used to screen for the germline mutation Y159X (477 C→G) and the non-synonymous variant E1317Q (3949 G→C), respectively. E1317Q was assayed using a *Pvu*II digest of a 506bp PCR product, the wild type allele was cut at two sites to generate 234bp, 173bp, and 99bp products, while the E1317Q allele was cut at only one site generating 272bp (lower arrow marked M) and 234bp products. Both variants cosegregated with the multiple CRA phenotype indicating that they were present on the same *APC* allele. Upper arrow marked M – mutant ASO, middle arrow marked WT – wild type ASO.

Tumours with 1 somatic mutation						
Family S (Germline – Q163X)				**Family B (Germline - Y159X and E1317Q)**		
		No. of 20AARs retained				No. of 20AARs retained
L1	3967 del13bp	1		A1S	R213X^{WT}	0
L3	Q1367X	1		A3	3476 delC^{WT}	0
L6	Q1367X	1		A3S	R232X^{AFAP}	0
L7	Q1291X	1		A6	LOH^{WT}	0
L9	E1317X	1		B3	Q1378X^{WT}	1
L10	E1322X	1		B4	LOH^{WT}	0
L13	3925 delG	1		C1S	R1114X	0
L22	E1322X	1		C6S	S1281X	0
L23	4188 delT	2		C11S	Q1242X	0
L24	R216X	0		C16S	3921 del5bp	1
L26	E1285X	1		C17S	R1450X	2
L27	3840 delGT	0		C49S	R216X	0
L29	E1379X	1		C51S	R232X	0
L30	LOH^{WT}	0		C56S	R216X	0
L35	3511 insA	0		C57S	R213X	0
L40	R876X	0		C65S	R302X	0
L41	3927 del5bp	1		C73S	4390 delAG	2
L43	E1285X	1		C76S	E1209X	0
L45	Q1338X	1		C78S	4463 delT	2
L47	Q1309X	1		C80S	R216X	0
L49	3921 del5bp	1		C84S	R232X	0
L53	Q423X	0		C96S	S1282X	0
L54	Q1367X	1		C99S	Q1228X	0
L55	Q1378X	1		C106S	R232X	0
L56	R1090X	0		D7	4661 insA^{WT}	3
L57	3917 delA	1		E10S	4575 delG^{WT}	3
L59	R216X	0		F1S	4470 insT^{WT}	2
L60	E1283X	0		F4S	4641	3

					insLINE1^{WT}	
L61	3921 del10bp	1		F6S	R232X^{WT}	0
L64	4080 delTC	1		F11S	3631-2 delAT^{WT}	0
L65	G1312X	1		G1S	4470 delT^{WT}	2
L65	Q1345X	1		G3S	4461-4468 del8bp^{WT}	2
L67	E1309X	1		G4S	4370-4371 insCA^{WT}	2
L68	Q1156X	0		G6S	Q1447X^{WT}	2
L69	Q1291X	1		G7	R1450X^{WT}	2
L70	3921 del5bp	1		G8S	R1450X^{WT}	2
L73	3634 del8bp	0				
L75	Q1406X	2				
L76	3873 ins8bp	1				
L78	S1392X	1				
L79	3941 delG	1				
L83	3473 FS	0				
L85	S1281X	0				
L89	3471 delA	0				
L94	Q1294X	1				
L95	E1306X	1				
L101	R1450X	2				
L102	4023 delT	1				
L108	Q1193X	0				
MA2	R1450X	2				
MA3	E1309X	1				
MA4	E1309X	1				
MA5	C1289X	1				
MA9	Q1406X	2				
MA12	Q1317X	1				

Tumours with 2 somatic mutations						
L25	Y1166X + 4373 insAC	0 ; 2		A2S	Q1517X^{AFAP} + 2814-2817 insTATA^{WT}	3 ; 0
L36	Q1294X + 4373 insAC	1 ; 2		[a]B2	S1346X^{WT}; LOH^{WT}	1 ; 0
L84	R213X + 4210 delT	0 ; 2		C2S	R213X+ LOH^{AFAP}	0 ; 0
				C4S	S1282X +LOH^{AFAP}	0 ; 0
				C63S	4390 insA + W2504X	2 ; 7
				C68S	4316 delC + LOH^{AFAP}	2; 0
				E11S	R1450X^{AFAP} + 4463 insA^{WT}	2 ; 2
				F12S	R232X^{AFAP} + 4479 delG^{WT}	0 ; 2
				[a]G5S	R1450X^{WT}; LOH^{WT}	2 ; 0
Tumours with 3 somatic mutations						
				[a]A5S	LOH^{AFAP}, 4461-2 delTT^{WT}, E1286X^{WT}	0 ; 1; 2

Table 4.2 Somatic *APC* mutations identified in Family S and Family B

^{WT}, inherited wild type *APC* allele; ^{AFAP}, AFAP allele; [a]Two somatic mutations were detected on different wild type alleles in samples A5S, B2 and G5S and are likely to reflect mixed populations of tumour cells. Therefore, in total, 49 tumours from Family B harboured somatic mutations.

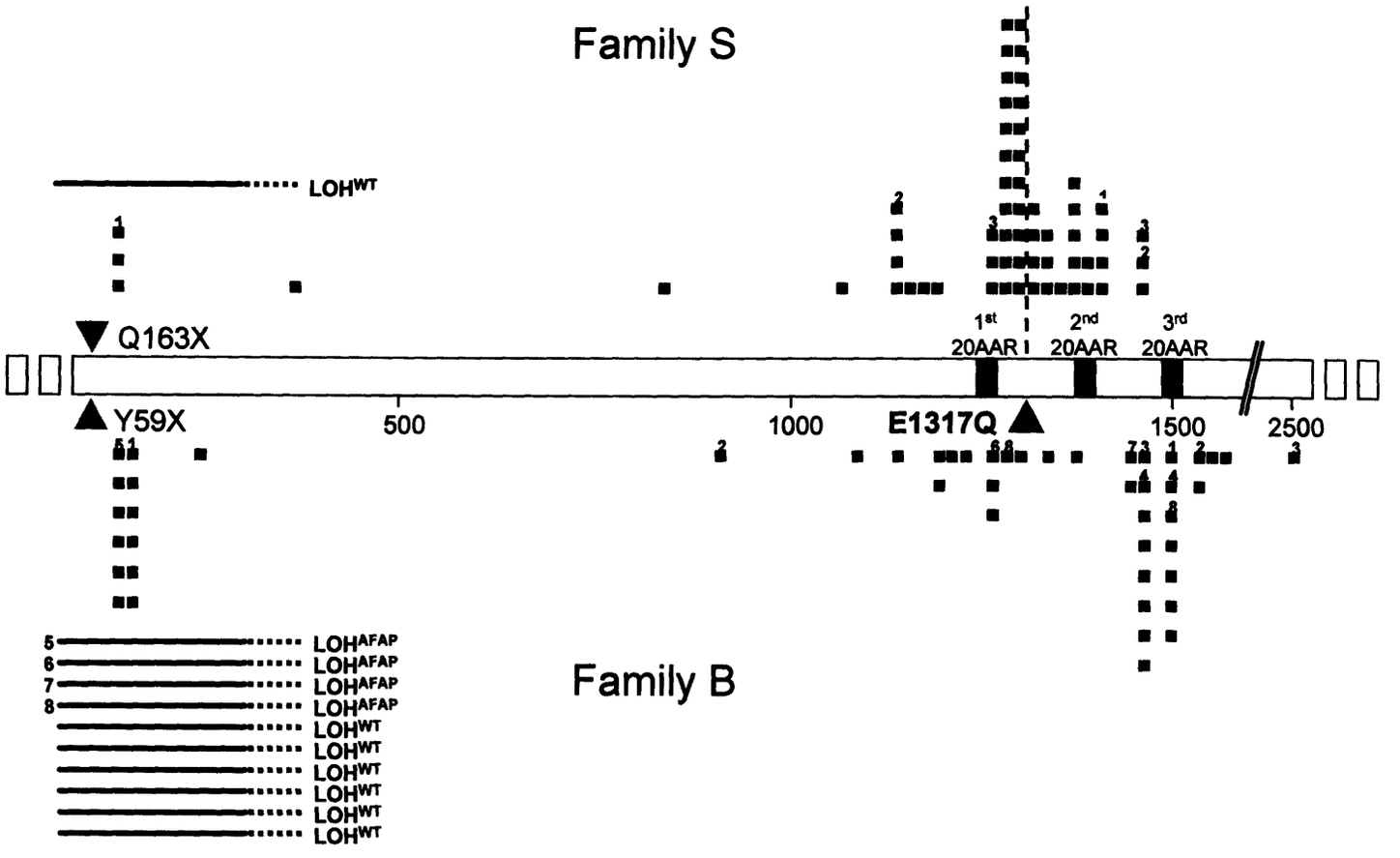


Figure 4.2 Distribution of somatic *APC* mutations in Family S and Family B. Somatic mutations (black squares) from Family S are plotted above, and those from Family B are plotted below, the schematic *APC* locus. The positions of the 1st, 2nd and 3rd 20AARs are shown as black vertical lines, the germline exon 4 truncating mutations are shown as grey triangles and the position of the germline E1317Q variant is shown as a black triangle and dashed line. LOH^{WT} and LOH^{AFAP} indicate tumours exhibiting loss of the wild type and attenuated alleles respectively, and pairs of numbers indicate somatic mutations that occur in the same tumour.

4.3.3 Comparing the somatic mutation spectra in Families S and B

We compared the somatic mutational spectra in Families S and B and found that the greatest difference was observed by considering four regions: nucleotides 637-694, 695-3840, 3841-3967 and 3968-4661 and the results of maximum likelihood Poisson parameters (λ_1-λ_4) are shown in Table 4.2. The patterns of somatic mutation in Families S and B are clearly different (P= 2.44 x 10^{-5}) and this difference is most marked in the sub-region λ_3 (nucleotides 3841-3967). This region encompasses the MCR (Miyaki *et al,* 1994) and first three 20AARs, as well as the variant E1317Q at nucleotide 3949.

4.3.4 Retention of 20AARs in tumours from Family S and Family B

In Family S, there was a distinct clustering of somatic mutations around codon 1300 and 62.1% (36/58) of the tumours had somatic mutations that were predicted to generate mutant APC polypeptides retaining one 20AAR (Figure 4.3). This pattern of mutant alleles is very similar to that found by two other studies looking at somatic *APC* mutations in tumours from families with inherited exon 4 truncating mutations (4/7, 57%, Sieber *et al*, 2006; and 6/8, 75%, Albuquerque *et al*, 2002) and is in close agreement with the just-right signalling hypothesis. In contrast, only 8.2% (4/49) of tumours collected from Family B (that carry E1317Q between the 1st and 2nd 20AARs) had somatic mutations that were predicted to result in mutant polypeptides that retained a single 20AAR (Figure 4.3), which is significantly less than found in Family S (P=5.64x10^{-9}) and the two other aforementioned studies (P=0.005, Sieber *et al*, 2006; P=0.0001, Albuquerque *et al*, 2002).

Consistent with the just-right signalling hypothesis, a low level (8/58, 13.8%) of tumours from Family S had somatic mutations that were predicted to generate mutant APC polypeptides retaining two 20AARs and no tumours contained somatic mutations predicted to generate mutant polypeptides retaining three or more 20AARs (Figure 4.3). In contrast, 40.8% of tumours from Family B had somatic mutations predicted to generate mutant polypeptides retaining two (16 tumours) or three (4 tumours) 20AARs (P=0. 002) (Figure 4.3). Furthermore, only 5/58 tumours from Family S had LOH or a somatic mutation early within the *APC* ORF (upstream of codon 500), whereas 20/49 (40.8%) of tumours from Family B carried these types

of mutation (Figure 4.3). Therefore, complete inactivation of an *APC* allele was considerably more frequent in tumours from Family B as compared to Family S (*P*=0.00009).

Region Nucleotide N°.	λ_1 (637-694)	λ_2 (695-3840)	λ_3 (3841-3967)	λ_4 (3968-4661)
Family S	0.035	0.0032	0.213	0.022
Family B	0.193	0.0022	0.032	0.023

Table 4.3 Comparing the patterns of somatic *APC* mutations in Family S and Family B using a maximum likelihood approach. The greatest difference between the somatic mutation spectra was observed when the region was split into four sub-regions: nucleotides 637-694, 695-3840, 3841-3967 and 3968-4661 and the results of maximum likelihood Poisson parameters (λ_1-λ_4) are shown. The two distributions are significantly different overall ($P=2.44\times10^{-5}$), and sub-region λ_3 demonstrates the maximum difference.

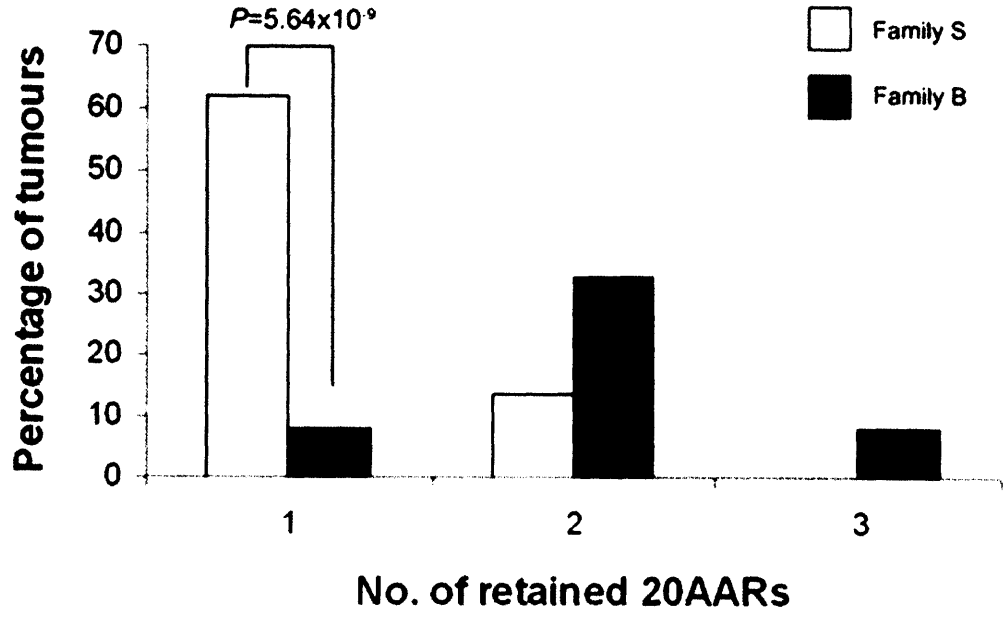


Figure 4.3 Graphical illustration of the numbers of retained 20AARs in tumours from Family S and Family B. In agreement with the just right signalling hypothesis, 62.1% of tumours from Family S were predicted to generate mutant APC polypeptides retaining one 20AAR, which contrasts with only 8.2% of tumours from Family B (P=5.64x10^{-9}). Furthermore, 40.8% of tumours from Family B had somatic mutations predicted to generate mutant polypeptides retaining two or three 20AARs and such mutations were rare in Family S (P=0.002). These data suggest that E1317Q relaxes the target of somatic mutagenesis.

4.3.5 E1317Q alters β-catenin-regulated transcription (CRT)

Previously work investigating the ability of rare *APC* nonsynonymous variants to repress β-catenin-regulated transcription (CRT) has shown that E1317Q on an otherwise wild-type *APC* background has a reduced ability to repress CRT relative to the normal wild type construct ($P<0.005$) (Chapter 3). Here, we studied the effect of E1317Q on CRT in the context of *APC* alleles predicted to retain one, two, three and five 20AARs, respectively, by introducing E1317Q onto four different truncating mutant backgrounds - *APC*1309Δ, *APC*1450Δ, *APC*1517Δ and *APC*1914Δ. When E1317Q was introduced into the 'weak mutant' constructs *APC*1914Δ and *APC*1517Δ (predicted to retain five and three 20AARs, respectively), the E1317Q containing mutants showed on average a 1.20 – 1.21 fold reduced ability to repress CRT relative to the normal truncating mutants ($P<0.05$). However, when E1317Q was introduced into the 'strong mutant' constructs *APC*1450Δ and *APC*1309Δ (predicted to retain two and one 20AARs, respectively), the E1317Q containing mutants showed no difference in activity relative to the normal truncating mutants (Figure 4.4).

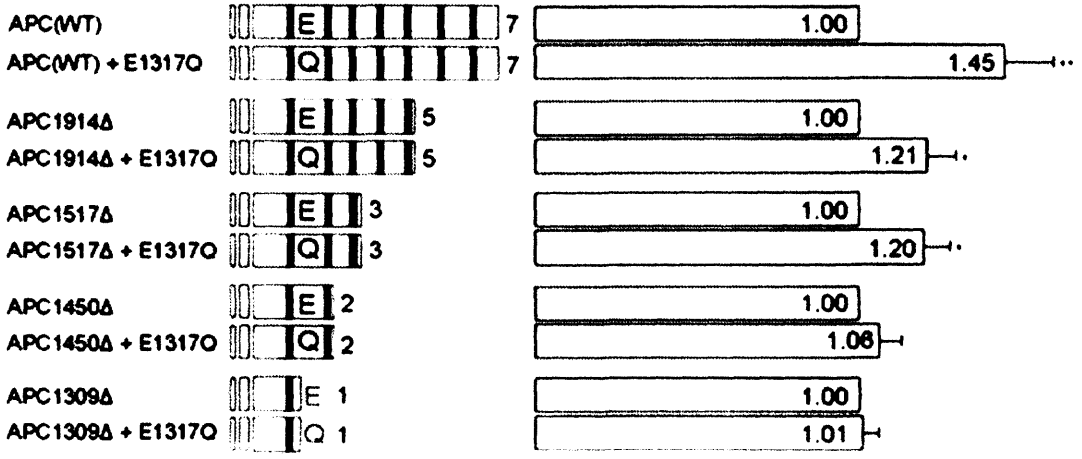


Figure 4.4 Functional analysis of E1317Q in both wild type and truncating mutant contexts.

Relative CRT levels following transfection of wild type *APC* and the four truncating mutants, together with their corresponding glutamine (Q) containing clones at residue 1317. Corresponding schematics indicate the position of the truncating mutation relative to the number of retained 20AARs (black lines and associated numbers) and showing the presence or absence of E1317Q ('Q' and 'E', respectively) (N.B. Grey font E and Q indicate that the truncating mutation occurs upstream of codon 1317). E1317Q significantly impaired β-catenin regulated transcription in both the wild type ($P<0.005$) and 'weak' truncating mutant ($P<0.05$) contexts (data is presented as fold values relative to the corresponding E1317-containing clones).

4.4 Discussion

Our recent work has shown that multiple rare non-synonymous variants in *APC*, including E1317Q, predispose to CRAs (Chapter 3) and whilst we have already demonstrated that a proportion of these variants have a direct effect on CRT, the exact mechanism of the tumour predisposition still remains unclear. Since the nature of the germline *APC* mutation in patients with FAP dictates the position and type of somatic *APC* mutations that are selected in colorectal tumours (Lamlum *et al,* 1999), we investigated whether the germline non-synonymous variant E1317Q also has an effect on the pattern of somatic *APC* mutations. Using a likelihood approach, we showed that there was a significant difference in the distributions of somatic mutations between Family S, who carried only a germline exon 4 truncating mutation, and Family B, affected members of which carried an exon 4 truncating mutation and E1317Q on the same germline allele. We examined these differences in relation to the numbers of retained 20AARs in the predicted mutant polypeptides. We found that in Family S, 62.1% of tumours had somatic mutations that were predicted to generate mutant APC polypeptides retaining one 20AAR. This was very similar to the pattern of somatic *APC* mutation found in two previously reported families with inherited exon 4 truncating mutations (Albuquerque *et al,* 2002, Sieber *et al*, 2006) and in close agreement with the pattern predicted by the "just-right" signalling hypothesis (Albuquerque *et al,* 2002). In contrast, only 8.2% of tumours from Family B had somatic mutations that were predicted to result in mutant polypeptides that retained a single 20AAR, suggesting that E1317Q has an effect of relaxing the requirement for this type of somatic mutation. Indeed, 40.8% of tumours from Family B had somatic mutations predicted to generate mutant polypeptides retaining two or more 20AARs and such mutations were rare in Family S. These data suggest that mutant polypeptides retaining two or three 20AARs offer the necessary growth advantage when found in combination with a mutant allele expressing E1317Q.

Complete inactivation of the entire wild type *APC* allele is generally only found in tumours from classical FAP patients with 'strong' germline mutations that are predicted to generate mutant polypeptides retaining a single 20AAR, such as 1309del5bp (Lamlum *et al,* 1999). Interestingly, 40.8% of tumours from Family B had LOH or a somatic mutation early within the *APC* ORF which was significantly more

frequent than in Family S. These data suggest that an AFAP allele carrying an exon 4 truncating mutation in combination with E1317Q can sometimes mimic a more highly selected mutation, further illustrating how E1317Q may play a role in colorectal tumourigenesis. Although all individual members of Family B showed a paucity of somatic mutations that were predicted to generate polypeptides retaining one 20AAR, it is interesting to note that tumours from family member G showed a preference for mutant alleles that were predicted to encode polypeptides retaining two 20AARs (7/8 tumours) whereas those from family member C showed a preference for retaining no 20AARs (16/22). No additional inherited *APC* coding region variants were identified in these individuals after screening their entire *APC* ORFs, suggesting that factors independent of *APC* may also play a role in the targeting of somatic mutations.

We showed that the mutant AFAP allele was expressed at the mRNA level in normal colonic tissue from both Patient S and two members of Family B demonstrating a potential for APC protein expression from these AFAP alleles either through internal translation initiation or translation of alternatively spliced transcripts (Samowitz *et al,* 1995, Heppner *et al,* 2002). Despite the potential for this residual activity, we found a low level of somatic (3rd hit) mutations on the AFAP alleles. In Family S, we identified only three (5.2%) tumours with two somatic mutations that may represent genuine 3-hit lesions (although some of these may simply represent tumours that have grown together after arising from neighbouring crypts). In Family B, ~16% of tumours potentially carried 3-hits. It remains unclear why only a minor proportion of tumours require these 3rd hits; however, the most likely explanation comes from careful examination of the pattern of somatic mutations in the three potential 3-hit tumours from Family S. Of the six somatic mutations in these tumours, only one is predicted to generate a polypeptide that retains a single 20AAR, which is significantly less than in tumours from Family S with single somatic mutations (P=0.038). Therefore, we hypothesize that a 3rd hit may be required in tumours where the 2nd hit is a weak mutation (i.e. those that are predicted to generate mutant polypeptides that do not retain a single 20AAR). An alternate hypothesis is that the requirement for a 3rd hit could be reduced by variants in other Wnt signalling genes, such as β-catenin (activating mutations of β-catenin are thought to be functionally equivalent to inactivating *APC* mutations in their effect on Wnt signalling, Sparks *et al,* 1998).

We previously demonstrated that E1317Q has a direct effect on CRT using an *in vitro* assay and show in the current study that the magnitude of this effect depends upon the number of 20AARs in the expressed polypeptide. On a wild type background, or when expressed with 'weak' truncating mutants that would generate polypeptides with three or five 20AARs, the effect is significant; however, when expressed with 'strong' truncating mutants that would generate polypeptides with one or two 20AARs, there is no measurable effect. These results are consistent with our hypothesis that E1317Q itself acts as a weak mutant allele that has a subtle yet significant effect on the level of β-catenin within the cell thereby relaxing the target for somatic mutagenesis on the wild type allele.

Chapter Five: MUTYH-deficient cells fail to engage apoptosis upon exposure to oxidative stress

5.1 Introduction

Cell cycle checkpoints are surveillance mechanisms that monitor cellular physiology, maintain telomere stability and preserve genomic integrity (Zhou and Elledge 2000). DNA damage triggers signal transduction pathways involving sensor, transducer and effector proteins. Human ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related protein) are phosphoinositol phosphate 3-kinase-related kinases. ATM or ATR activation occurs in response to stress and transduces the DNA damage signal by phosphorylating many downstream proteins in a Rad9, Rad1, Hus1 and Rad17-dependent manner. Rad9, Rad1 and Hus 1 form the so-called '9-1-1' complex; a heterotrimeric complex that has predicted structural homology to proliferating-cell nuclear antigen (PCNA) sliding clamp (Shiomi *et al,* 2002; Venclovas and Thelen, 2000). Rad17 is a paralogue of the largest subunit of replication factor C (RFC), and it forms the alternative clamp loader with RFC2-5. The 9-1-1 complex is loaded on to DNA lesion sites by RAD17-RFC (Bermudez *et al,* 2003). ATM/ATR, the 9-1-1 complex and Rad17 are proposed to act as an early step of DNA damage response to sense the DNA damage and to lead to cell cycle arrest or apoptosis (Zhou and Elledge, 2000). These checkpoint proteins may detect a common intermediate, such as a single stranded DNA coated with replication protein A (RPA), which is processed by various DNA repair pathways (Bermudez, *et al,* 2003). Several reports support a hypothesis that checkpoint proteins may require a series of 'adaptors' to recognise DNA damage (Zou and Elledge, 2003; Giannattasio *et al,* 2004; Lavin 2004). These adaptor proteins may be DNA damage recognition proteins involved in mismatch repair (MMR), nucleotide excision repair (NER) and double-strand break (DSB) repair.

MUTYH has been directly associated with PCNA in *Schizosaccharomyces pombe* (*S. pombe*) and human cells (Chang and Lu, 2002; Parker *et al,* 2001). The coupling between the MUTYH BER pathway and DNA replication may provide a signal to target MUTYH repair to daughter DNA strands (Parker *et al,* 2001). DNA damage induced SpHus1 phosphorylation is dependent on SpMYH expression (Chang and Lu, 2002). Shi *et al,* (2006) have shown that human MUTYH physically interacts with

Hus1 and Rad1, and co-localises to nuclear foci with Rad9 following oxidative damage and recently the 9-1-1 complex has been shown to interact with and stimulate the enzymes involved in BER, which include polymerase β (Toueille *et al,* 2004), FEN1 (flap endonuclease 1) (Friedrich-Heineken *et al,* 2005) and DNA ligase 1(Wang *et al,* 2006). Thus the 9-1-1 complex serves both as a damage sensor and as a component of BER.

We assessed the response of primary *MUTYH* defcient cell lines to oxidative damage to examine the mechanisms of MAP-associated cell survival/death.

5.2 Methods

5.2.1 Cell Lines

MUTYH-deficient primary cell lines MAP1, MAP2, and MAP3 are human epithelial fibroblast cell lines derived from clinical biopsies obtained from colectomy specimens from unrelated MAP patients. 161BR is a *MUTYH*-proficient human epithelial fibroblast cell line obtained from the ECACC. All cells were maintained in 5% CO_2 atmosphere in DMEM medium with 15% (v/v) foetal bovine serum, 1% non essential amino acids, 1% L-glutamine and 1% penicillin/streptomycin at 37°C.

5.2.2 Cell Survival Assays

Cells were seeded in triplicate at $2x10^3$ cells per well into Optilux 96-well plates 24 hours before treatment to allow sufficient adherence. Hydrogen peroxide (H_2O_2) and *t*-butyl hydroperoxide (TBH) treatments (0, 12.5, 25, 50, 100 and 200μM) were given for 1 hour at room temperature. Cells were rinsed twice with cold PBS before replenishing with media. ATP measurement medium alone, untreated control cells and treated cells (with peroxide) were assessed immediately after peroxide treatment and at 24 hour intervals for 7 days using the CellTiter-Glo Luminescent Cell Viability Assay that quantifies the amount of ATP present in a homogenous lysis solution, according to the manufacturer's instructions. The luminescence of each sample was measured with TR717 microplate luminometer.

5.2.3 Caspase-3 Activation Assay

Caspase-3 activation assays were performed using a Caspase-Glo™3/7 assay kit according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at a density of $2x10^3$ cells/well. After 24 hours, cells were exposed to H_2O_2 and TBH solutions (0, 12.5 and 25 μM) as described in section 5.2.2. Caspase-Glo 3/7 reagent (100μl) was then added to each well including medium alone, untreated control cells or cells treated with peroxide 1, 24, 48 and 72h post exposure. The luminescence of each sample was measured with TR717 Microplate Luminometer.

5.2.4 Immunoprecipitation and Western blotting

Following exposure to peroxide at 1, 3, 6 and 9 hours, cells were lysed on ice for 10 minutes in NP-40 lysis buffer (50mM Tris-HCl pH 8, 150mM NaCl, 1% NP-40, 50mM PMSF in isopropanol, 1ug/ml of Leupeptin, Aprotinin, and Pepstatin). Half volume was used to determine phosphorylation of Chk1 whilst the remaining half was used to determine total Chk1 as a control. Briefly, lysates were incubated with 1μg of anti-phosphoChk1(Ser317) for 1 hour at 4°C (1:500 dilution). Twenty microlitres of Protein G-Sepharose bead solution was added and the mixture was incubated at 4°C for 2 hours. After three wash/centrifugation steps at 3000*g* for 1minute the pellet was resolved by SDS/PAGE and transferred to a nitrocellulose membrane. The membranes were allowed to react with antibodies against phCHK1. Western blotting was detected by the enhanced chemiluminescence (ECL) analysis system according to the manufacturer's protocol. Each Western blot experiment was repeated.

5.2.5 Immunofluorescence

After exposure to H_2O_2 for 3 hours, cells were fixed with 0.4% formal saline for 15 minutes at room temperature. Cells were permeabilised with 100% methanol for 5 minutes at -20°C. After being blocked in PBS containing 15% foetal bovine serum (FBS) for 1 hour at room temperature, the cells were reacted with rabbit polyclonal antibodies for MUTYH (1:250 dilution) and mouse monoclonal antibody for RAD9 (1:20 dilution) at 37°C for 30 minutes. Next the cells were washed three times for 15 minutes each in TBS-Triton and incubated with Alexa Fluor® 594 goat anti-rabbit and Alexa Fluor® goat anti mouse IgG antibodies at a 1:250 dilution in PBS for 30 minutes at 37°C. The cells were then washed three times in PBS. Nuclear DNA was counterstained with DAPI (4'6'-diaminido-2-phenylindole). Images were captured

with an Olympus BX51 BF fluorescent microscope with an attached mercury lamp charge coupled device (CCD) camera.

5.2.6 Statistical analysis

The students' *t*-test was used to assess statistical significance.

5.3 Results

5.3.1 MUTYH-deficient cells are resistant to the cytotoxic effects of peroxides

We established primary fibroblast lines from three unrelated patients with MAP (MAP1, MAP2 and MAP3), all of whom shared the same common MUTYH genotype Y165C/G382D (Sieber *et al,* 2003), and a wild type control. We determined the growth rate of each cell line under normal conditions to ensure that cell survival assay results were not skewed due to differences in population size and variable growth rates. We used standard luminescent cell viability assays to quantify the number of metabolically active cells based on the detection of cellular ATP. Each cell line grew at a similar rate at every time point measured (0 – 7 days) validating their inclusion in this study (figure 5.1).

To determine the role of *MUTYH* in oxidative stress-mediated cytotoxicity, we compared the relative sensitivities of the cells to H_2O_2 and TBH. *MUTYH*-deficient cells were significantly more resistant to H_2O_2 induced cytotoxicity as compared to wild type cells (Figure 5.2). At 48 hours post exposure to 12.5μM or 25μM H_2O_2, the *MUTYH* deficient cells had a mean viability of 45-64% and 43-49%, respectively, whereas the wild type cells had a viability of 20 and 9%, respectively ($P<0.001$). Similarly, after 7 days post exposure to 12.5-25μM, MUTYH deficient cells had an average cell viability of 70-83% and 60-77%; significantly higher than that of the wild-type (25 and 15%), At a higher dose of 50μM H_2O_2, cell lines MAP1 and MAP2 had a mean viability of 52-77% whereas the mean viability of MAP3 had fallen to 23%; these levels were still significantly higher than the wild-type ($P<0.001$). *MUTYH*-deficient cells were also significantly more resistant to TBH induced cytotoxicity as compared to wild type cells. At 4 days post exposure to 12.5μM or 25μM TBH, the *MUTYH* deficient cells had mean viabilities of 30-36% and 29-33% compared to the wild-type cell lines that showed 17% mean viability to both concentrations($P>0.001$).

At 100-200μM H_2O_2 or TBH, both MUTYH-deficient and wild type cells showed similar sensitivities.

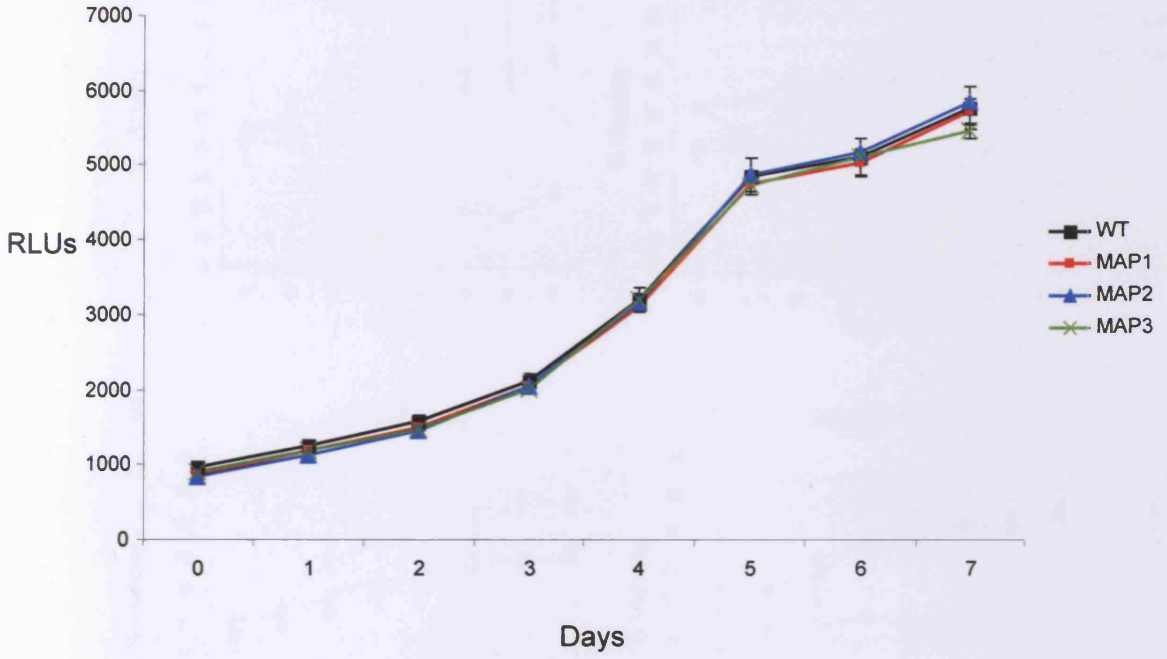


Figure 5.1 MUTYH proficient (WT) and MUTYH deficient (MAP1, MAP2 and MAP3) growth rates. Cell survival assays of untreated cell lines over a seven day period indicate that all cell lines grow at a similar rate after initial seeding into Optilux plates.

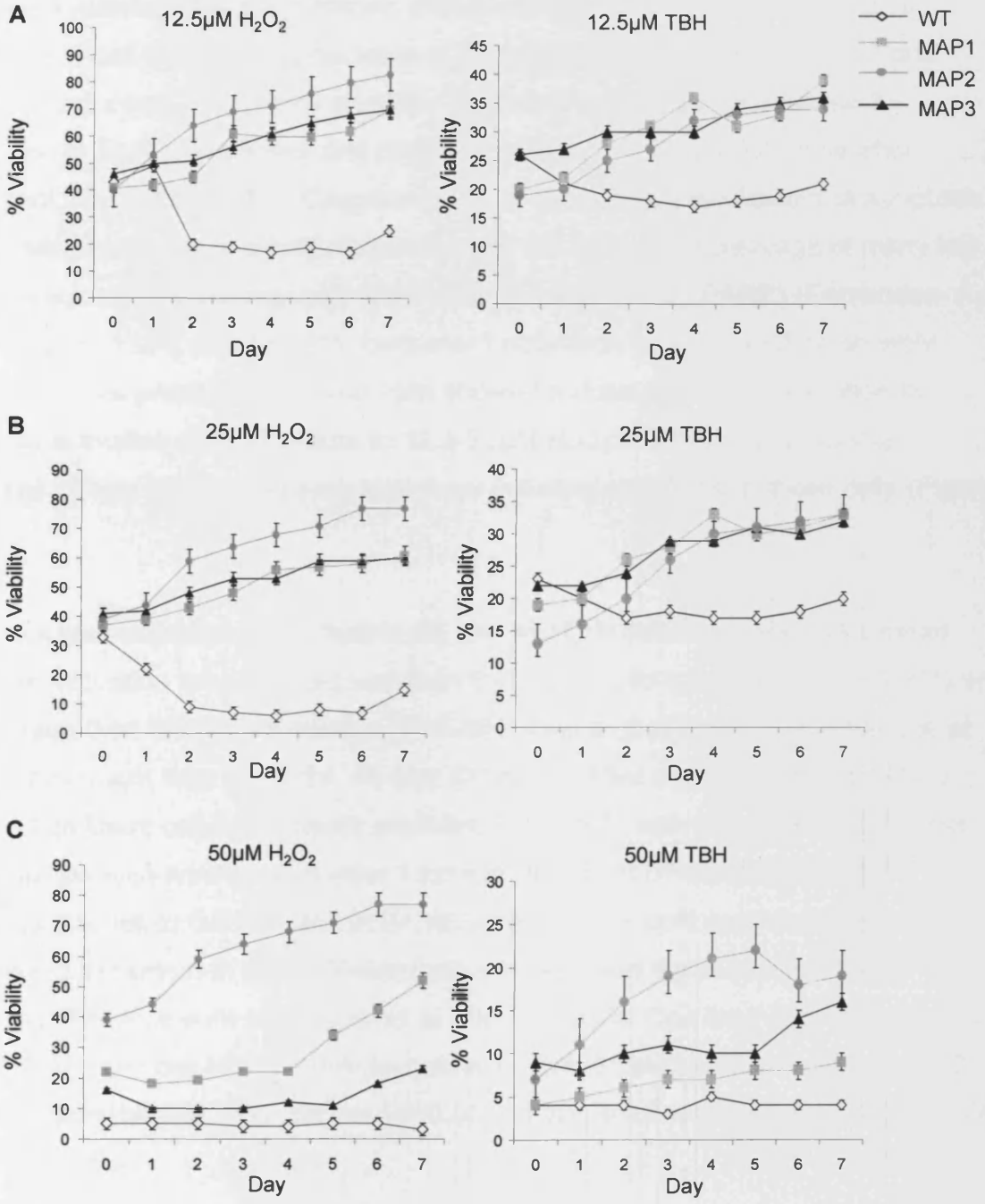


Figure 5.2 Cell viability in *MUTYH* proficient (WT) and *MUTYH* deficient (MAP1, MAP2, MAP3) cell lines after treatment with peroxides. (A-C) show percentage viability of cell lines over a period of 7 days following exposure to H_2O_2 and TBH. Results are shown as the percentage relative to do mock treated control and represent the mean ±SD of experiment carried out in triplicate.

5.3.2 MUTYH-deficient cells fail to undergo apoptosis

Increased resistance to the cytotoxic effects of H_2O_2 or TBH may result from a decrease in cell death due to necrosis or apoptosis. To determine the route and extent of cell death induced by peroxide treatment we measured caspase 3 activation in *MUTYH*-deficient and proficient cells 1, 24, 48 and 72 hours after treatment with H_2O_2 or TBH. Caspase-3 is one of the key executioners of apoptosis as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear poly (ADP-ribose) polymerase (PARP) (Fernandes-Alnemri *et al,* 1994) and as such, caspase-3 activation is often used as an early indicator of this process. Wild type cells showed a dose dependent increase in caspase activation after exposure to 12.5-25μM H_2O_2 or TBH, indicating that cytotoxic effects of peroxide exposure were initiating apoptosis in these cells (Figure 5.3).

At 1 hour post-exposure to 12.5μM H_2O_2, the *MUTYH* deficient cells had a mean caspase activation level 0.7 fold less than that of the wild-type cell line ($P<0.001$) and on average 0.35 fold less activation ($P<0.001$) than that of the wild-type cell line at each subsequent time point (24, 48 and 72 hours). After exposure to 25μM H_2O_2, *MUTYH*-deficient cell lines initially exhibited caspase-3 activation at levels 0.6 fold less than the wild-type cell line after 1 hour of treatment ($P<0.0001$) which subsequently fell to 0.2 fold less at 24, 48 and 72 hours post exposure ($P<0.001$). Caspase-3 activation in *MUTYH*-deficient cells was also significantly reduced following TBH exposure as compared to wild type cells. One hour after exposure to 12.5-25μM TBH, the *MUTYH* deficient cells exhibited caspase-3 activation 0.5-0.6 fold less than the wild type cell line ($P<0.001$) and 0.4-0.6 fold less ($P<0.0001$) at 24, 48 and 72 hours.

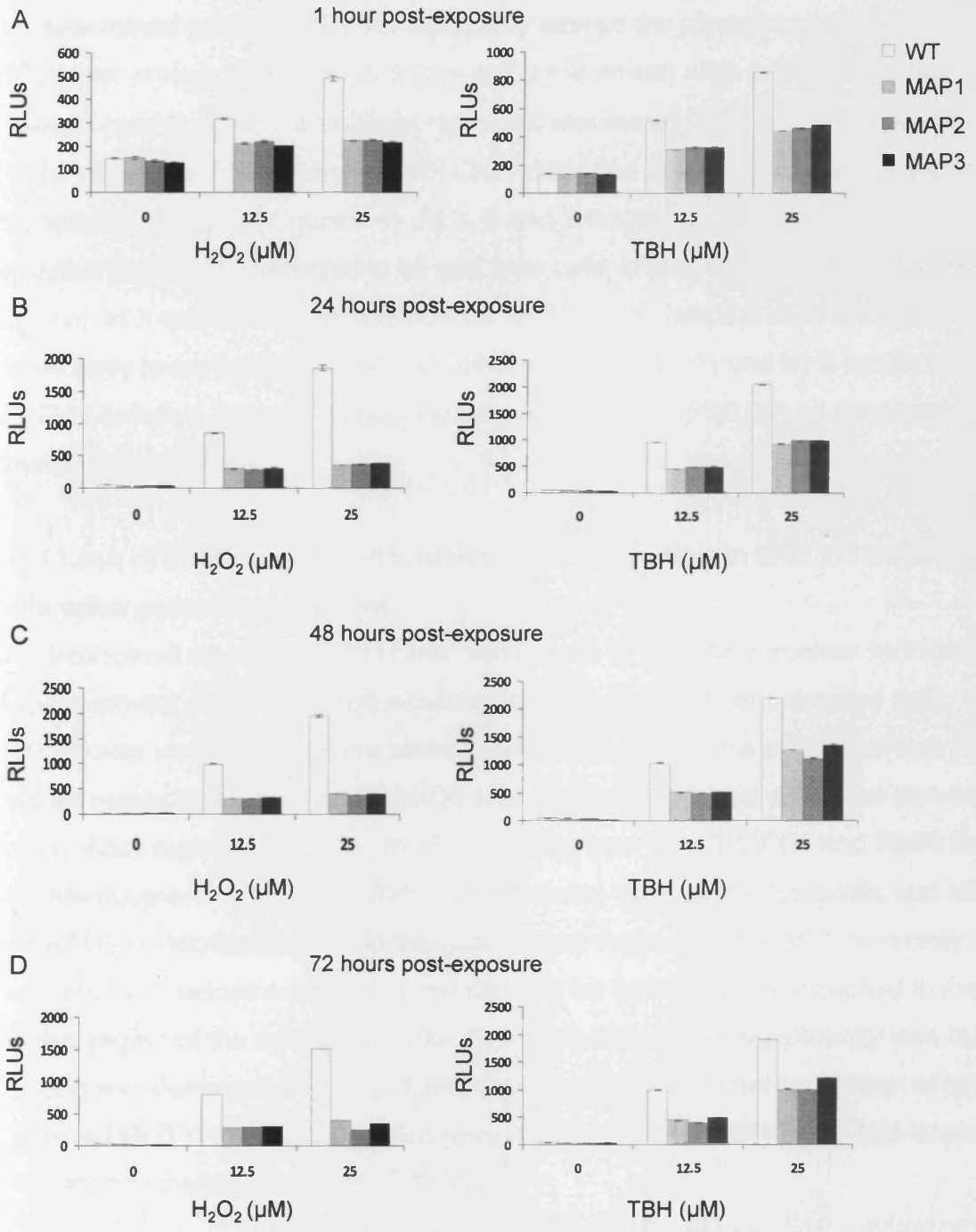


Figure 5.3 Activation of caspase-3 in *MUTYH* (WT) proficient and *MUTYH* (MAP1, MAP2 and MAP3) deficient cell lines after treatment with peroxides. Normalised luminescence values (RLUs) indicate level of active caspase-3 detected following exposure to stated concentrations of H_2O_2 (left) and TBH (right). Error bars indicate the ± SD of experiments carried out in triplicate.

5.3.3 Lack of Chk1 phosphorylation/activation post damage permits cell survival

We determined whether *MUTYH*-deficiency altered the phosphorylation status of the checkpoint protein Chk1 (which drives cell cycle arrest) after oxidative stress. We exposed cells to 0, 12.5 and 25μM H_2O_2 and measured Chk1 phosphorylation over a 9 hour period. No phosphorylation of Chk1 occurred in any lines after exposure to the control (no H_2O_2) (Figure 5.4). At 3, 6 and 9 hours post exposure to H_2O_2, Chk1 phosphorylation was detected in all wild type cells in a dose-dependent manner. In contrast, at 3 and 6 hours post exposure, all *MUTYH*-deficient cells showed significantly lower levels of Chk1 phosphorylation ($P \leq 0.01$) and by 9 hours, no *MUTYH*-deficient lines exhibited Chk1 phosphorylation ($P \leq 0.05$) as assessed by Western blot analysis.

5.3.4 Lack of MUTYH and Rad9 nuclear co-localisation in MUTYH deficient cells upon peroxide treatment

We determined whether MUTYH and Rad9 move to the same nuclear foci following H_2O_2 treatment using immunofluorescent staining analysis. In untreated cells MUTYH was visible as granular staining distributed within the cytoplasm and peri-nuclear region of the cell, whilst RAD9 was consistently found within the peri-nuclear region. After exposure to H_2O_2, in ~82% of wild-type cells MUTYH and Rad9 formed discrete nuclear foci (Figure 5.5a). The remaining 18% of wild-type cells had MUTYH and RAD9 co-localisation within the peri-nuclear region of the cell. Conversely, in the three MUTYH deficient cell lines most staining for both proteins remained in the peri-nuclear region of the cytoplasm (~60–70%; $P \leq 0.008$) whilst significantly less nuclear staining was observed ($P \leq 0.01$) (figure 5.5b and 5.5c). A small proportion of cells contained MUTYH that appeared to have remained completely in the 'pre-exposure' cytoplasmic localisation (3-7%; $P \leq 0.004$).

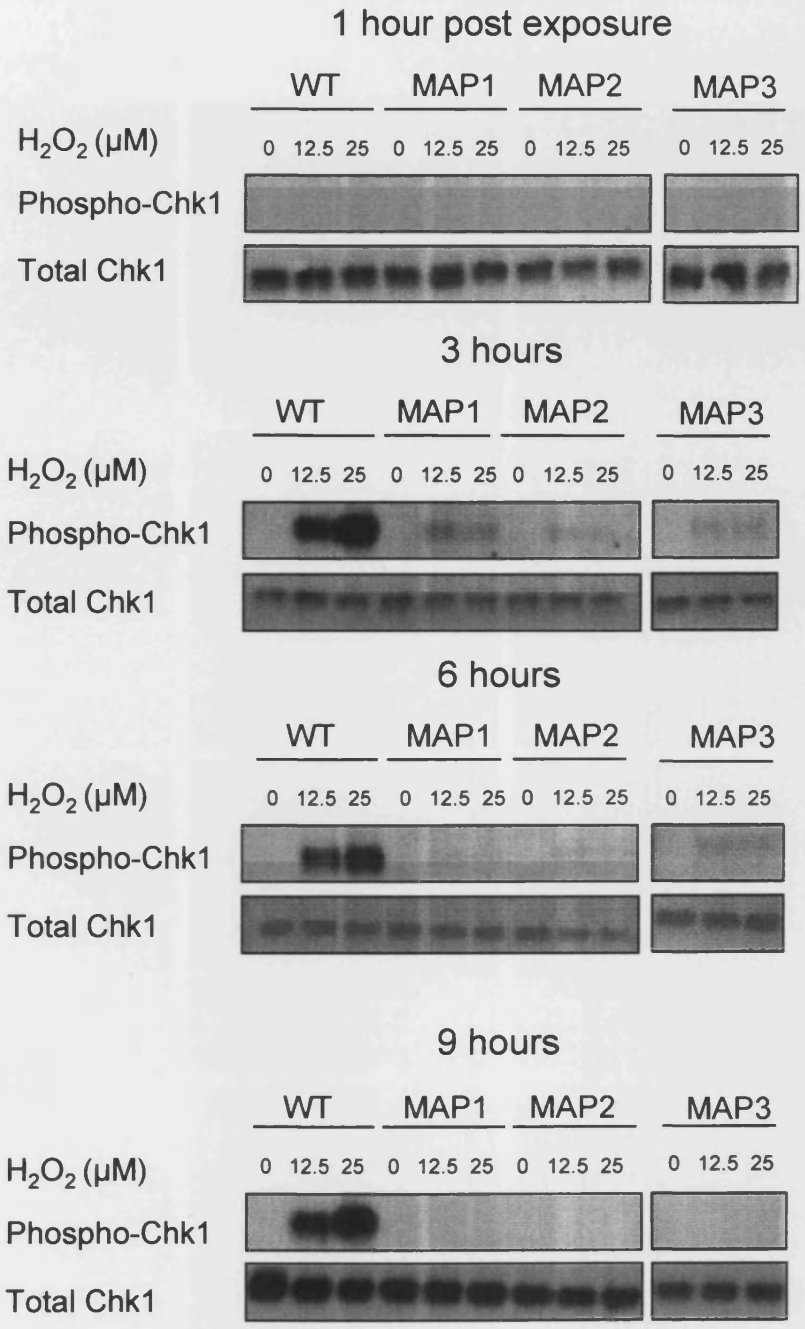


Figure 5.4 Phosphorylation status of Chk1 following exposure to oxidative damage. *MUTYH* proficient (WT) and *MUTYH* deficient (MAP1, MAP2 and MAP3) cell lines were exposed to 0, 12.5 and 25μM of H_2O_2 and Chk1 phosphorylation was determined at 1, 3, 6 and 9 hours after exposure by western blot analysis. Endogenous total Chk1 was determined by the same method to act as a positive control ensuring that differential phosphorylation was not due to differences in total protein expression.

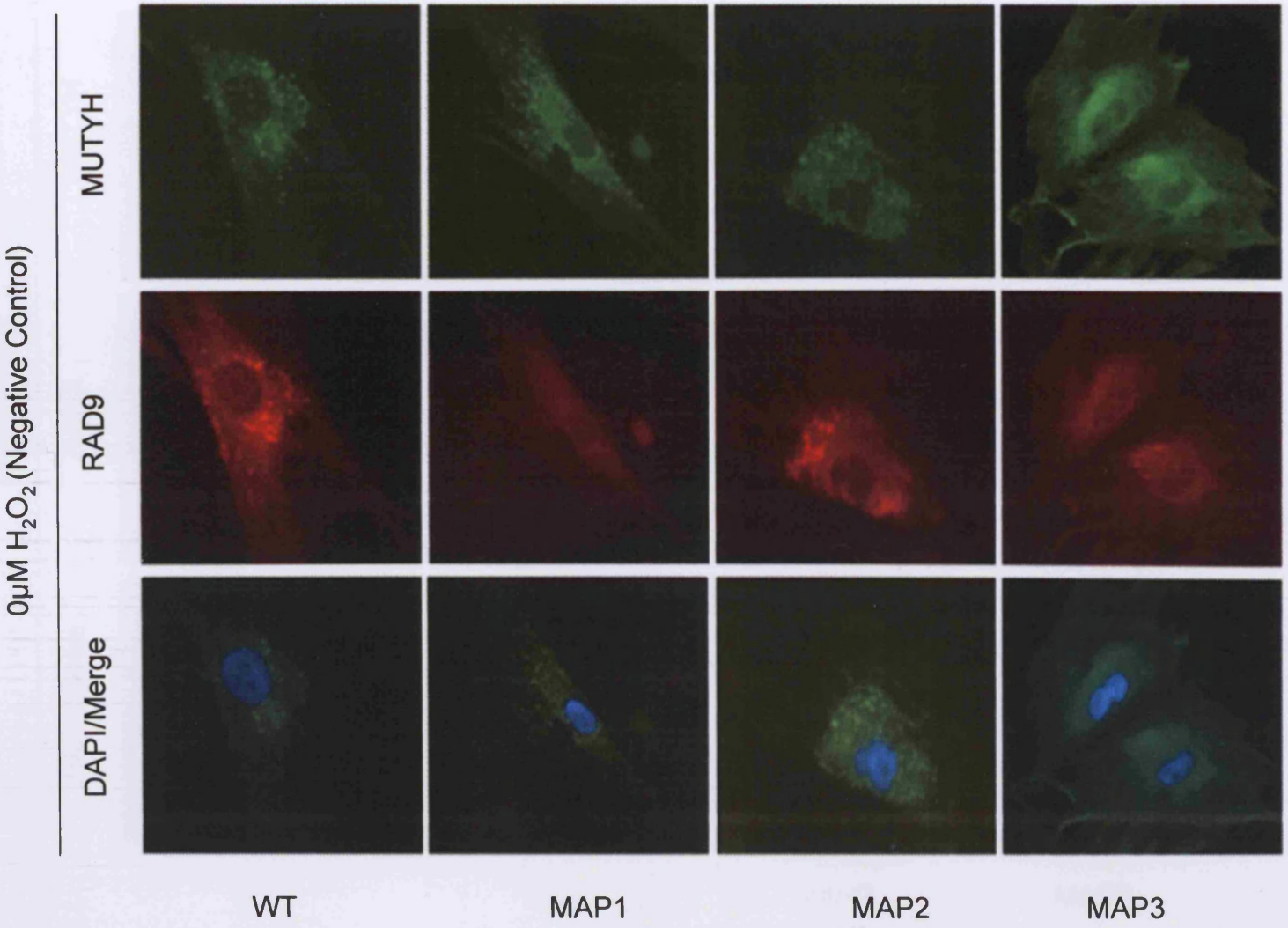


Figure 5.5a MUTYH and RAD9 localisation in MUTYH proficient (WT) and MUTYH deficient (MAP1, MAP2 and MAP3) cell lines (control)

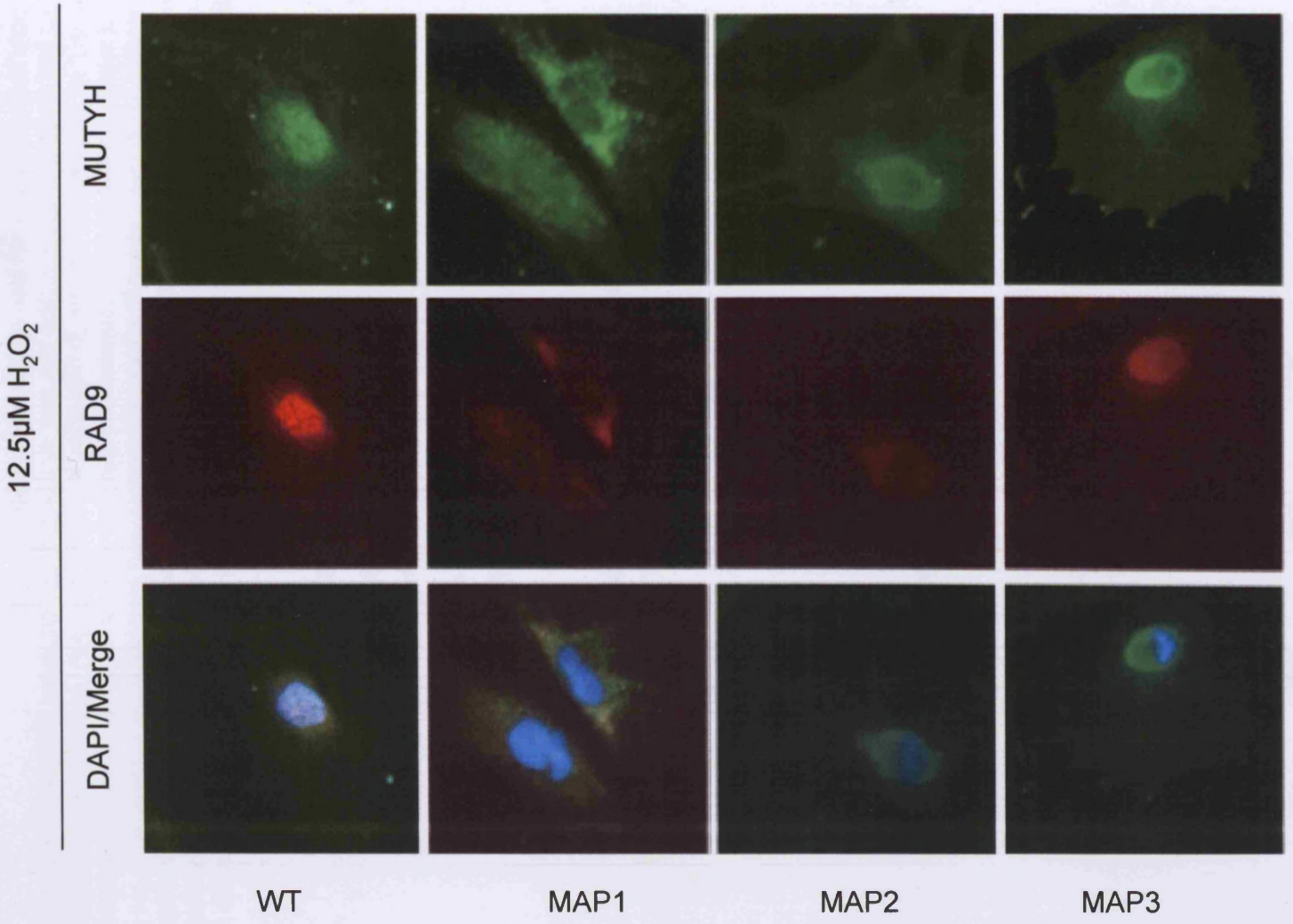


Figure 5.5b MUTYH and RAD9 localisation in MUTYH proficient (WT) and MUTYH deficient (MAP1, MAP2 and MAP3) cell lines after peroxide treatment.

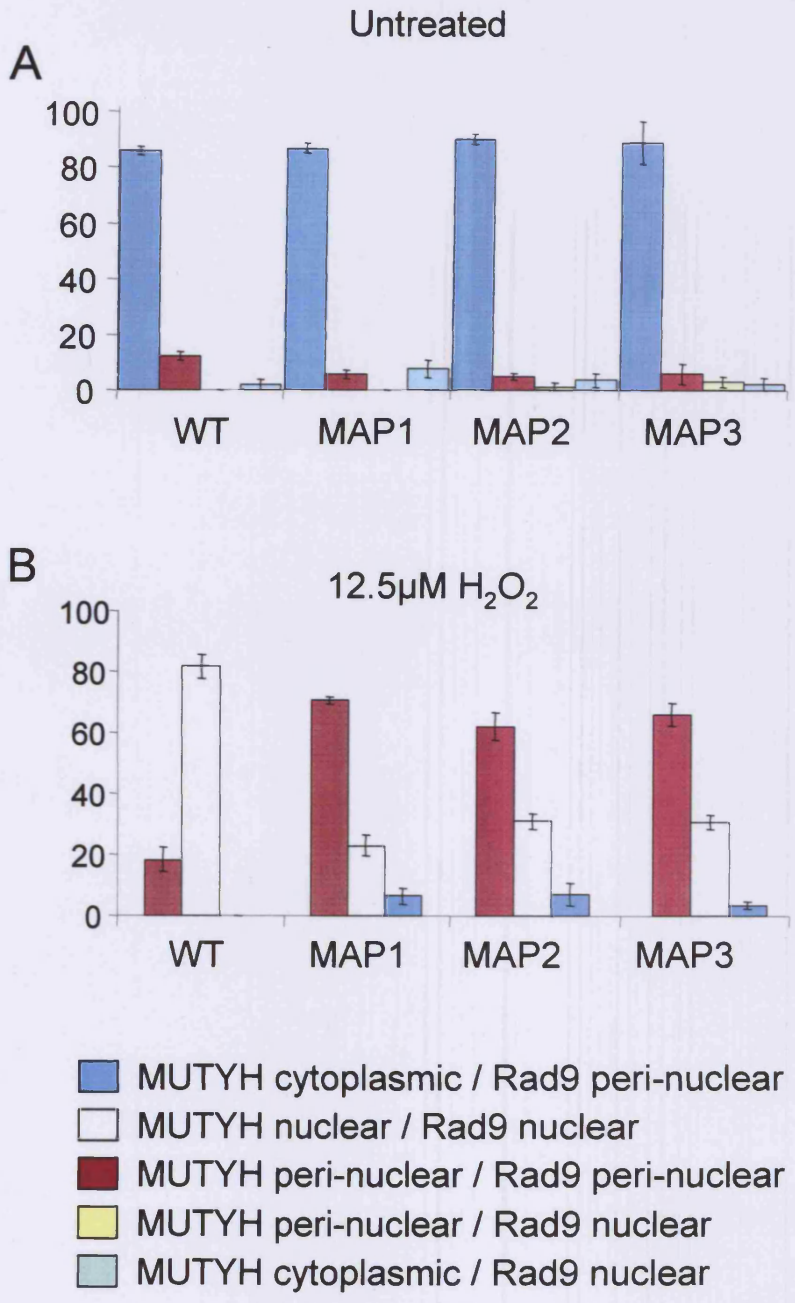


Figure 5.5c Intracellular localisation of MUTYH and Rad9. Data obtained 3 hours post exposure to 12.5µM H_2O_2. Cells were cultured on chamber slides and following oxidative damage were fixed and stained with antibodies against MUTYH and RAD9. The nucleus is visualised using DAPI staining. A) All cell lines treated with negative control. MUTYH is found predominantly in the cytoplasm and RAD9 is located within the peri-nuclear space (~80%;). B) Following exposure to hydrogen peroxide both MUTYH and RAD9 co-localised within the nucleus in the wildtype cell line. In the MUTYH deficient cell lines however staining for both proteins remained peri nuclear (P=0.008).

5.4 Discussion

5.4.1 MUTYH deficiency results in decreased apoptosis in response to peroxide mediated oxidative damage

We have used patient derived *MUTYH* deficient epithelial fibroblast primary cell lines to demonstrate a role for MUTYH in mediating an apoptotic response to the peroxides H_2O_2 and TBH. We found that apoptotic response of *MUTYH* deficient cells to the cytotoxic effects of oxidants was significantly decreased compared to the normal control. One *MUTYH* deficient cell line (MAP3; figure 5.2C) had a more marked apoptotic response to 50μM H_2O_2 compared to the two other MUTYH deficient cell lines. However, mean viability of this cell line seven days after exposure to H_2O_2 was still significantly higher than the wildtype cell line. All three *MUTYH* deficient cell lines had the same germline *MUTYH* mutations so differences in genotype cannot explain the variability seen. Cell lines were maintained at similar passage numbers to eliminate this acting as a variable. Active caspase measurement and Chk1 activation were similar for each cell line also so variability in cell viability appears to be determined by other factors

Interestingly, previous studies did not find any difference between H_2O_2 induced cytotoxicity in Mutyh$^{-/-}$ and wild type murine embryo fibroblasts that had spontaneously immortalised (Xie *et al,* 2008) or in Epstein–Barr virus transformed lymphocytes from patients with MAP and controls (Parker *et al,* 2001). However, our studies are based on primary cultures of human fibroblasts and are likely to represent a better *in vitro* model of MAP. The decrease in cell death in response to peroxides was accompanied by a lack of caspase-3 activation and lack of phosphorylation of the cell cycle checkpoint protein Chk1, suggesting that cell survival in MUTYH-deficient cells is due to an inability to execute programmed cell death in parallel with a failure to initiate cell cycle arrest.

5.4.2 Overlapping roles for DNA repair proteins in cell cycle progression

A potential role for MUTYH, or other members of the BER system, regulating cell cycle has not been demonstrated before. Interaction between proteins in other repair pathways and checkpoint activation proteins at DNA lesions has been previously demonstrated in other model organisms. (Meister *et al*, 2003; Giannattasio *et al,*

2004; Kai and Wang, 2003). DNA damage checkpoints ensure a proper cellular response to a range of genotoxic insults. Malfunctioning of such systems is linked to premature aging, uncontrolled cellular proliferation, genome instability and ultimately tumourigenesis (Hartwell 2002). Failure to detect the primary lesion results in defective activation of the checkpoint response and failure to arrest cell cycle progression and DNA replication. Moreover, if the primary damage is not detected by the checkpoint, it may be channelled into other cellular processes (e.g. aberrant recombination) that can cause DNA instability. The biochemical mechanisms involved in the sensing step of the checkpoint response are still relatively poorly understood. How do cells identify that their DNA has been damaged? How are the lesions found within the genome and how is the first protein kinase in the cascade activated?

Yoshioka *et al* (2006) showed that the MMR proteins MutSα and MutLα act as direct sensors of methylation damage and help recruit ATR-ATRIP to sites of cytotoxic O^6-methyl-G (O^6-meG) adducts to initiate ATR checkpoint signalling in yeast, *In vitro* assays showed that following DNA methylation, cell cycle checkpoint machinery is engaged during S-phase and that after DNA replication O^6-meG adducts are regularly mispaired with thymine residues and that these mispairs are a target of MutSα. ATR-ATRIP is preferentially recruited to O^6-meG/T mismatches in a MutSα and MutLα dependent manner. Furthermore, ATR kinase is activated to phosphorylate Chk1 in the presence of O^6-meG/T mispairs and MMR proteins. MMR and the corresponding antimutator role can be separated functionally from the DNA damage response role of MMR proteins. For example, cells having reduced levels of MSH2 or MLH1 protein are competent for MMR but fail to carry out checkpoint activation or undergo apoptosis in response to DNA alkylation (Lettieri *et al*, 1999; Cejka *et al*, 2003; Claij and te Riele, 2004). Even more intriguing has been recent report of two separation of function alleles in murine Msh2 and Msh6 that encode the two subunits of MutSα. These mutations in or near the nucleotide binding site of MSH2 and MSH6 disable mismatch repair but leave intact the apoptotic response to DNA damaging agents, including MNNG (Yang *et al*, 2004; Lin *et al*, 2004). The separation of function alleles argue strongly that MMR and damage signalling involve distinct pathways. This notion is supported by protein-protein interactions between Msh6p and Mek1p, the ATR homolog in budding yeast (Gavin *et al*, 2006), and

between human Msh2 and the checkpoint proteins Chk1 and Chk2 (Adamson *et al*, 2005; Brown *et al*, 2003).

One caveat not explored by this study is the upregulation of catalase in response to oxidative stress. Catalase is responsible for degradation of hydrogen peroxide encountered from exogenous sources or produced intracellularly as a result of celular metabolism. Two enzymes, glutathione peroxidase (GpX) and catalase are the two principal scavengers of hydrogen peroxide. Exposure to oxidative stress results in the upregulation of the enzymatic activity of the antioxidant enzymes such as GpX, catalase and the superoxide scavenger superoxide dismutase (SOD). Increased activity, enhanced protein and higher mRNA levels have been reported for the enzyme catalase in cells subjected to oxidative stress (Rohrdanz and Kahl, 2009; Rohrdanz *et al,* 2001). It is well-known that pre-exposure to stress upregulates cellular anti-oxidant defense mechanisms resulting in adaptation to stress (Rohrdanz and Kahl, 2009; Rohrdanz *et al,* 2001; Lai *et al*, 1996; Luo and Rando, 2003). The characterisation of the MUTYH deficient cells would not be complete without investigating the 'catalase upregulation' response to oxidative damage under similar experimental conditions and should be proposed as future work.

Having an established role for MMR proteins in checkpoint signalling sets the precedent for DNA repair enzymes behaving as genotoxic stress 'sensors' and 'adaptors'. This supports further examination of the role of MUTYH in response to oxidative damage outside of the remit of a classic 'repair protein'. A model using data generated in this study is described in detail in chapter 6. By identifying a suitable panel of *in vitro* model systems, future studies of damage signalling mediated by BER proteins will aid and facilitate the identification of the full complement of proteins required to elicit the damage response. In the context of inherited colorectal polyposis syndromes this would allow deeper insight into the complex mechanisms of pathogenesis required to initiate adenoma development and subsequent tumourigenesis.

Chapter six: General discussion

6.1 High and low penetrance alleles predisposing to CRC

Approximately 5% of cases of CRC are associated with dominantly inherited, familial Mendelian susceptibility. FAP, MAP and HNPCC are caused by highly penetrant mutations in *APC, MUTYH* and the MMR genes respectively. An additional 15 – 30% of cases are believed to be due to inherited susceptibility that is polygenic i.e. associated with much lower penetrance variants that do not give rise to clear cut familial patterns of inheritance. The genetic basis of most inherited CRC susceptibility is thought to be due to low penetrance alleles and these are now being identified. Genes in which highly penetrant CRC susceptibility alleles have been described may also harbour incompletely penetrant changes. In collaboration with Myriad Genetic Laboratories and Genzyme Genetics we comprehensively sequenced the entire ORF of *APC* in order to identify the full repertoire of nonsynonymous variants within the gene. We have reported that multiple rare (MAFs <2%) inherited *APC* nonsynonymous variants may predispose to CRAs. A higher proportion of patients without truncating *APC* mutations or biallelic *MUTYH* mutations (non-FAP, non-MAP) carried rare *APC* nonsynonymous variants than did the FAP or MAP cases. The highest over representation of these rare nonsynonymous variants was in non-FAP non-MAP patients. Compared to healthy controls, significantly more non-FAP non-MAP cases carried rare nonsynonymous variants in the β-catenin down-regulating domain of *APC* and again the highest over-representation was in patients with 11 – 99 CRAs. When only variants with a MAF<0.5% were considered (thereby excluding I1307K, E1317Q and G2502S), there was still an excess of rare *APC* missense variants in non-FAP non-MAP cases. Rare synonymous variants and common polymorphisms were detected at similar frequencies in non-FAP non-MAP patients, FAP or MAP cases and healthy controls (all of whom were of similar ethnic backgrounds) so the findings are unlikely to be the result of population stratification. Not all nonsynonymous variants would be expected to have a detrimental effect on APC function but *in silico* analysis predicted 39-46% of the variants were likely to be damaging.

Functional analysis of 16 nonsynonymous variants found in the β-catenin down regulating domain showed that 7 altered β -catenin transcription *in vitro*. N1026S, a novel *APC* nonsynonymous mutation has recently been reported to be associated with AFAP in a single family. The variant co-segregated with the disease in the reported family and functional analysis showed that it altered β -catenin-regulated transcription (Menendez *et al,* 2008).

The effect the rare nonsynonymous variant E1317Q had on a genetic level was examined by comparing the somatic mutation spectra of two AFAP families. Family S carried an early germline truncating mutation at codon 163 and Family B carried a germline truncating at codon 159 as well as the E1317Q variant. Several genetic studies of CRAs from FAP patients have shown that somatic *APC* mutations are dependent on the position of the germline *APC* mutation (reviewed in more detail in chapter 1). The APC protein contains seven 20AARs which are involved in degrading the transcriptional co-factor β-catenin and so negatively regulates Wnt signalling. In colorectal polyps, germline mutations between codons 1285 and 1378 leave only one 20AAR intact and are strongly associated with somatic loss of the wildtype *APC* allele. LOH usually occurs through mitotic recombination, thus leaving two identical alleles and a total of two 20AARs in the tumour cell (Sieber *et al*, 2002). FAP patients who carry germline mutations before codon 1285 (no 20AARs) tend to have somatic mutations which leave one or, more commonly two 20AARs in the protein. Finally, patients with germline mutations after codon 1398 (two or three 20AARs) tend to have somatic mutations before codon 1285 resulting in a truncated protein with no 20AARs. The same associations have also been found in sporadic colorectal tumours (Rowan *et al,* 2000).

This interdependence of 'first' and 'second' hits shows that selective constraints on *APC* mutations are active and that an optimum level of β-catenin signalling must be achieved for a nascent tumour cell to grow (Albuquerque *et al*, 2000). We sequenced the entire ORF of *APC* from DNA extracted from tumour biopsies from all family members to establish any differences in the somatic mutation spectra of each family. With this established interdependence in mind we would expect the majority of 'second' hit mutations to result in a truncated protein predicted to retain one 20AARs but there was a significant difference between the families; approximately 60% of

tumours from Family S (without the E1317Q variant) had somatic mutations that were predicted to result in truncated APC retaining a single 20AAR compared to <10% from family B (with the E1317Q variant). Conversely a larger proportion of tumours from Family B were predicted to result in a truncated APC protein retaining two 20AARs. These data suggest that mutant polypeptides retaining two or three 20AARs offer the necessary growth advantage when found in combination with a mutant allele expressing E1317Q at a low level.

6.2 Genetic susceptibility to disease

Two models of susceptibility to complex human disease have been proposed. The 'common disease-common variant' model states that genetic susceptibility to common disease is conferred primarily by common alleles (within the general population) that have very modest phenotypic effects (Lander 1996; Chakravati 1999). The alternative 'common disease-rare variant' model proposes that susceptibility to common diseases is due to the effect of multiple rare alleles, each conferring a more substantial increase in relative risk and their mechanisms are complementary, rather than mutually exclusive. Although individually rare, these alleles may be collectively common in the population. Despite the fact that, theoretically, such variants may have diverse effects on protein function, disease-phenotype biological fitness, many rare nonsynonymous variants are believed to elicit insignificant functional effects (Glazier *et al,* 2002).

6.3 Common variants and CRC

Genome-wide association studies (GWAS) are aiding the identification of common low penetrance CRC predisposition alleles at loci previously unassociated with an inherited CRC syndrome or colorectal tumourigenesis. Such studies have recently identified a CRC susceptibility locus at 8q24.21 (Tomlinson *et al*, 2007; Zanke *et al*, 2007). Two common SNPs (rs6983267 and rs10505477) that are in linkage disequilibrium were strongly associated with colorectal neoplasia (Tomlinson *et al*, 2007; Zanke *et al*, 2007) and mapped to a haplotype block which and been previously associated with prostate cancer risk (Haiman *et al*, 2007; Yeager *et al*, 2007). This locus does not contain any known genes but does have two predicted ORFs. One appers to be an uncharacterised gene with multiple alternatively spliced transcripts and harbours rs10505477 within an intron. The other is a processed

psuedogene of *POU5F1* (which encodes a transcription factor) and is found 20kb telomeric to rs6983267 (Tomlinson *et al*, 2007; Zanke *et al*, 2007). The *MYC* oncogene lies 116kb telomeric to rs6983267 and outside the haplotype block but its function could be influenced by the risk variant through genomic instability or long-range regulation of expression. The CRC susceptibility locus at 8q24.21 influences the risk of CRAs and so may be affecting tumour initiation rather than progression (Tomlinson *et al*, 2007). Three common *SMAD7* SNPs which influence CRC risk were also recently identified through a GWAS. This gene is involved in TGF-β and Wnt signalling and all three variants map to the same block of linkage disequilibrium located in intron 3 of this gene. Similar to the 8q24.21 locus, the *SMAD7* SNPs significantly influence the risk of CRAs so may be affecting tumour initiation rather than progression (Broderick *et al*, 2007).

Hereditary mixed polyposis syndrome (HMPS) reported in Ashkenazi families is associated with the highly penetrant CRAC1 locus and is characterised by multiple colorectal polyps (which are not classical adenomas) and CRC. A GWAS of UK CRC cases (selected for family history and/or early onset) and unaffected controls found that common SNPs within the *CRAC1* locus were strongly associated with an increased CRC risk suggesting that common low penetrance alleles at this locus can contribute to CRC predisposition in the general population (Jaeger *et al*, 2008).

However, despite the power of large population association studies, rare nonsynonymous variants will not be detectable because of their low allele frequency and individually small contributions to the overall inherited susceptibility of a disease. Current literature suggests that the most successful strategy for identifying rare nonsynonymous variants implicated in disease involves comparing the overall frequencies of such variants identified in candidate genes in disease and control groups. In such studies the patient groups may be shown to contain genes 'enriched' with rare variant. Critically, this approach is heavily biased since investigators are ultimately only able to identify variants they are searching for and this approach does not highlight true associations between 'unknown' rare nonsynonymous variants within these genes other than the one(s) of interest.

Significantly, the technology used to resquence (or 'deep' resquencing) genes is becoming relatively cheaper and more accessible. Genes implicated in disease predisposition can now have all variation comprehensively identified in both disease and control groups ensuring a thorough and systematic investigation, when before, limitations such as cost and time will have caused biased analysis.

The potential consequences for protein function of each rare variant can also be assessed for features suggesting pathogenicity i.e. occurrence in conserved regions, charge changes, and bulky changes likely to affect protein structure and also by direct biochemical or functional assays. In the context of these criteria, a potentially pathogenic variant is one that shows a significant difference in frequency between disease and control population either singly or more often, as a member of a group of variants affecting the same gene or a set of genes with related functions. Ideally, such variants will be characterised functionally by suitable *in vitro* assays to assess their eventual effect on the function of the relevant gene product.

6.4 Applications of CRC predisposition alleles in clinical practice

High penetrance CRC predisposition alleles can be used by medical genetics services to improve the counselling and surveillance of families at high risk. If a patient carries a pathogenic mutation(s), they can undergo colonic surveillance and prophylactic surgery if necessary whereas family members who do not carry the mutant allele(s) do not require additional surveillance. However, genetic counselling and management of patients who carry variants of unknown pathogenicity such as missense changes is difficult, with a requirement for unequivocal genetic and functional data before the variant can be used with confidence in the setting of pre-symptomatic testing.

Testing for low penetrance alleles should not be applied in a clinical setting at present but this situation is likely to change as the risks associated with such alleles are clarified. In practice, family history is currently used as an indicator of risk in families in which no high penetrance genetic defect can be identified. It is my opinion that testing for low penetrance alleles should be used in a diagnostic setting only when they have been determined to result in a pathogenic effect and are clearly shown to compromise the function of a protein, given the degree of academic

uncertainty surround their effects it would be irresponsible to see them tested for in the present day. Large scale GWAS have identified 11 loci that are associated with CRC incidence but only two of these studies have been followed up with functional studies to determine the mechanism that results in pathology. I feel this is the start of a process that will lead to the inclusion of low pentrance alleles in clinical diagnostics.

In the future, rare SNPs associated with increased CRC risk may well be used to identify individuals in the general population for whom additional CRC screening is appropriate – this prospect would appear to fit in with the ideal of personalised medicine that has been born out of the 'genomic era' , however this concept seems a million miles away from current public health priorities and cancer screening strategies. One would also be mindful of the psychological aspects this type of screening and would not want to burden the individual with a positive diagnosis without good reason.

6.5 DNA repair mechanisms and cancer predisposition

MUTYH is one of many DNA repair genes implicated in cancer predisposition (Vogelstein and Kinzler, 2004). Inherited defects in these genes are believed to cause tumourigenesis by increasing the rate of somatic mutations in key tumour suppressor genes, perfectly illustrated by the synergy of biallelic inactivating *MUTYH* mutations resulting in somatic inactivation of *APC* (Thibodeau *et al*, 1993; Al – Tassan *et al*, 2002). In contrast with other major repair pathways, inherited defects in just a single component of the BER system has so far not been found to predispose to cancer. Inactivation of the other BER proteins could result in programmed cell death, rather than leading to tumourigenesis.

Mutations of genes within identical repair pathways can also result in different phenotypes. Inactivation of ATM, the homologous recombination repair pathway (HRR) protein, causes ataxia telangiectasia which predisposes to lymphomas and leukaemias (Rotman and Shiloh, 1998). Similar cancer susceptibilities are observed in patients with germline mutations in the *NBS1*, *MRE11,* and Fanconi anaemia genes which are also found in this pathway. However, Rothmund-Thompson and Bloom's syndromes are both caused by defects in RecQ like-helicases that

recognise and promote branch migration of Holiday junctions, but the former leads to bone tumours and the latter is characterised by cancers of the haematopoietic and lymphatic systems (Hoejimakers, 2001). BRCA1 and BRCA2 also have roles in recombination repair; BRCA1 is a substrate for ATM and interacts with RAD50 (Zhong, 1999), BRCA2 is involved in the nuclear translocation of RAD51 (Davies *et al*, 2001) and both may assist HRR through histone acetylation and deacetylation (Scully, 2001). Mutations in these genes predispose to breast and ovarian cancers (Narod and Foulkes, 2004).

In contrast to mouse models of nucleotide excision and recombination repair deficiency, severe BER knockout mice do not display significantly enhanced susceptibility to DNA damage or cancer. (Tanaka *et al*, 2001; Moynahan, 2002; Takao *et al*, 2002; Klungland *et al*, 1999). In addition MUTYH$^{-/-}$ Ogg1$^{-/-}$ mice exhibit a more severe phenotype than their single knockout counterparts, suggesting that there is a functional overlap (Russo *et al*, 2004). A higher level of functional redundancy may exist between the BER system than in other repair pathways and MUTYH may be unusual in that its role cannot be adequately filled by other glycosylases.

6.6 Is MAP due to defective repair and failure to initiate correct cell-cycle surveillance?

The biochemical responses recorded from our *MUTYH* deficient cell lines provides novel data and suggests some overlap between the BER system and the replication checkpoint response. This is typified by a significant lack of Chk1 phosphorylation in *MUTYH* deficient cell lines and subsequent failure to engage in apoptosis as compared to a wildtype cell line. The primary question arising out of this is at what stage does checkpoint signalling become activated and where does MUTYH participate in this pathway? It is established that 8-oxoG lesions induced by oxidative damage, if left unrepaired, result in 8-oxoG/A mispairs following DNA replication. We can therefore state that such lesions have no effect on DNA replication i.e. stalling, and would not bring about a checkpoint signalling response. Another facet of oxidative damage is therefore implicated; ROS are also known to cause single-strand breaks in DNA that are blocked with 3'-phosphoglycolate (Barzilai and Yamamoto, 2004). This would inevitably result in stalled replication if left alone and a

role in replication coupled repair has previously been proposed for MUTYH (Boldogh *et al*, 2001). By examining the normal response to stalled replication could we speculate a model for BER mediated checkpoint signalling?

The mammalian replication checkpoint response system slows down cell cycle progression and stabilises stalled replication forks so that further DNA damage is prevented. At the heart of this checkpoint are two protein kinases, ataxia telangiectasia mutated and Rad3 related (ATR) and Chk1 (Chen and Sanchez, 2004; Zhang *et al*, 2006; Paulsen and Cimprich, 2007). A model proposing how these proteins mediate repair of stalled replication forks is as follows (figure 6.1). Stalled replication forks activate ATR which subsequently phosphorylates and activates Chk1. Because ATR is critical in replication stress response, the biochemical mechanism governing ATR activation has been extensively examined. During replication, if a DNA polymerase stalls, the minichromosome maintenance helicase continues to unwind DNA ahead of the stalled polymerase (Byun *et al*, 2005). This produces long tracts of single-stranded DNA (ssDNA), which become coated with replication protein A (RPA). Additional factors are required i) ATR-interacting protein (ATRIP), an ATR binding partner that interacts directly with RPA to load the ATR–ATRIP complex onto ssDNA (Cortez *et al*, 2001; Zou and Elledge, 2003; Unsal-Kacmaz and Sancar, 2004; Ball *et al*, 2005; Namiki and Zou, 2006), and ii) the 9-1-1 complex is loaded onto DNA by a clamp-loader protein consisting of Rad17 and four subunits of the replication factor C (RFC) complex (Parrilla-Castellar *et al*, 2004). What is less clearly understood is how components of the 9-1-1 complex are recruited to sights of DNA damage.

The response of DNA glycosylase enzymes to oxidative damage is relatively well characterised in both bacterial and mammalian cells. In several studies involving both eukaryotic and yeast systems MUTYH has been shown to interact and/or associate with proteins that are not directly implicated in BER; Chang and Lu (2002) showed in *S. pombe* that DNA damage induced phosphorylation of *Sp*Hus1 is dependent on *Sp*MYH expression. Subsequent studies in HeLa cell lines by Shi *et al* (2006) showed they MUTYH physically interacted with Hus1 and Rad1 following oxidative damage induced by hydrogen peroxide. It would appear that the relationship between MUTYH and the separate components of the 9-1-1 complex

merits further investigation and may explain the results generated from our biochemical characterisation of patient derived MUTYH deficient cell lines. Significantly, other enzymes in the BER system have been shown to interact with the 9-1-1 complex. These include NEIL1 (Guan *et al*, 2007), APE1 (Gembka *et al*, 2007), Polβ (Toueille *et al*, 2004), FEN1 (Friedrich-Heineken *et al*, 2005; Wang *et al*, 2004) TDG (Guan *et al*, 2007) and DNA ligase 1 (Smirnova *et al*, 2005; Wang *et al*, 2006).

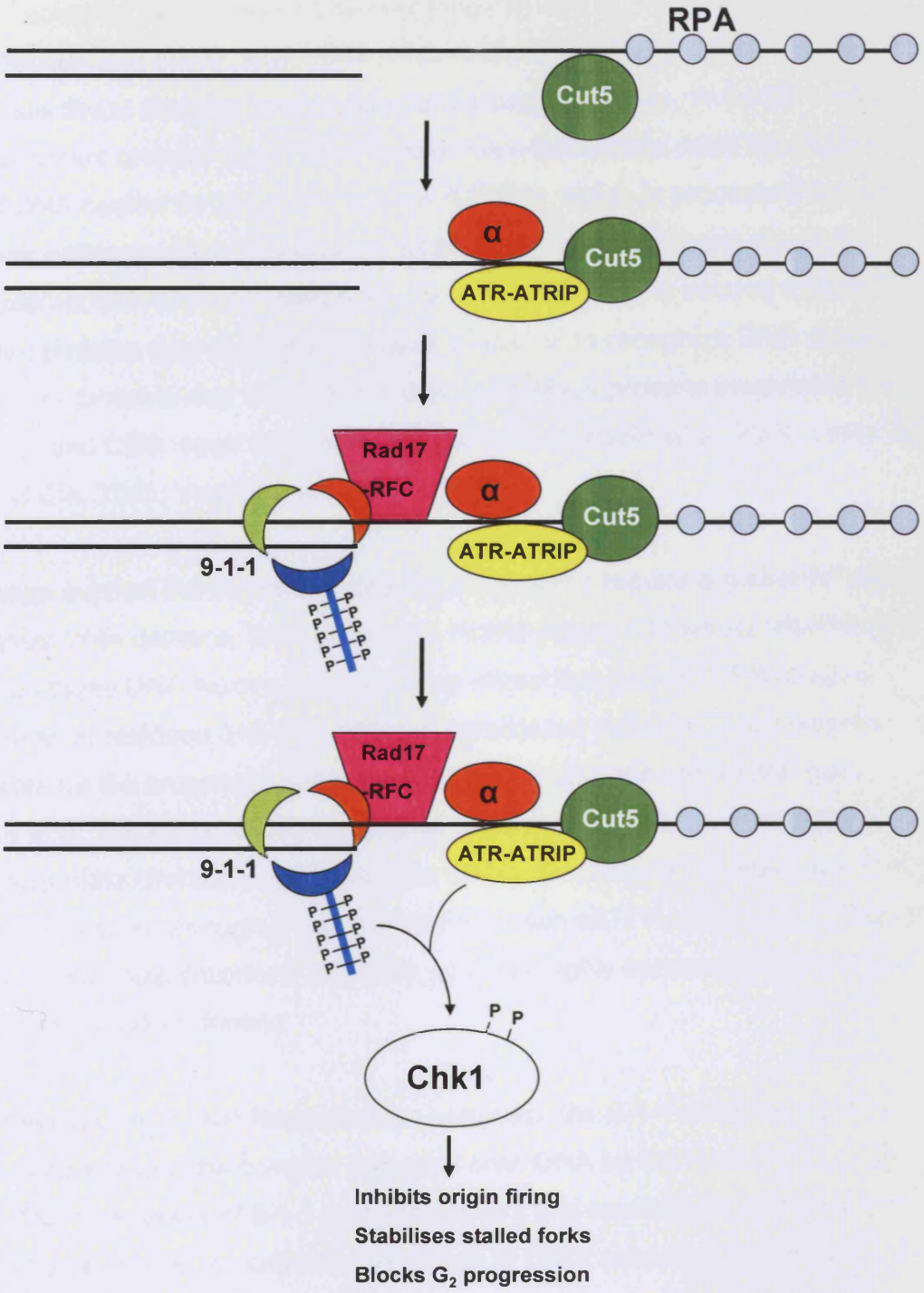


Figure 6.1 Step-wise assembly of the checkpoint signalling machinery at stalled replication forks. Replication fork stalling leads to the production of ssDNA, which is then coated by RPA. The RPA coated DNA then recruits Cut5, which in turn facilitates the binding of ATR and DNA polymerase α. DNA polymerase α is then required for the recruitment of the Rad17-RFC clamp loader and the loading of the 9-1-1 complex onto DNA. The chromatin bound 9-1-1 complex via the Rad9 phospho-tail, then facilitates the ATR-mediated phosphorylation of Chk1.

The 9-1-1 complex, Rad17 and ATM/ATR are proposed to act at an early step to sense DNA damage (Zhou *et al*, 2000; Melo *et al*, 2001). There are two models to address how these sensors are recruited to the damaged sites. In the first model, these checkpoint proteins may detect a common intermediate, such as single-stranded DNA coated by replication protein A (RPA), which is processed by various DNA repair pathways (Zou and Elledge, *et al*, 2003). RPA has been shown to directly interact with the 9-1-1 complex (Wu *et al*, 2005). In the second model, these checkpoint proteins may require a series of 'adaptors' to recognize DNA damage. Such adaptor proteins may be DNA damage recognition proteins involved in BER, MMR, NER and DSB repair (Brown *et al*, 2003; Giannattasio *et al*, 2004; Lavin 2004; Wang and Qin, 2003; Yoshioka and Hsieh, 2006).

Our findings support the model that checkpoint proteins require a series of 'adaptors' to recognize DNA damage. We speculate a model (figure 6.2) where MUTYH first recognizes these DNA lesions because of an interaction with APE1 through a binding motif at residues 318-395. APE1 is considered to be the major protein responsible for the enzymatic removal of 3'-phosphoglycolate in human cells (Parsons *et al*, 2004) and ssDNA arising as a result of ROS is blocked with 3'-phosphoglycolate (Barzilai and Yamamoto, 2004). Recruitment of Hus1 and Rad1 proteins could occur through physical interaction with MUTYH (Shi *et al*, 2006) at the Hus1 binding domain (residues 295-350), which is highly conserved and overlaps with the APE1 binding domain.

Whilst, the signal to recruit Rad9 remains unknown, the 9-1-1 complex forms near the damaged site and the complex is loaded onto DNA by the Rad17-RFC clamp loader. The recruitment of 9-1-1 to the lesion is a pre-requisite for ATR activation and could explain why our cell lines show lack of Chk1 activation and as a result, continued growth.

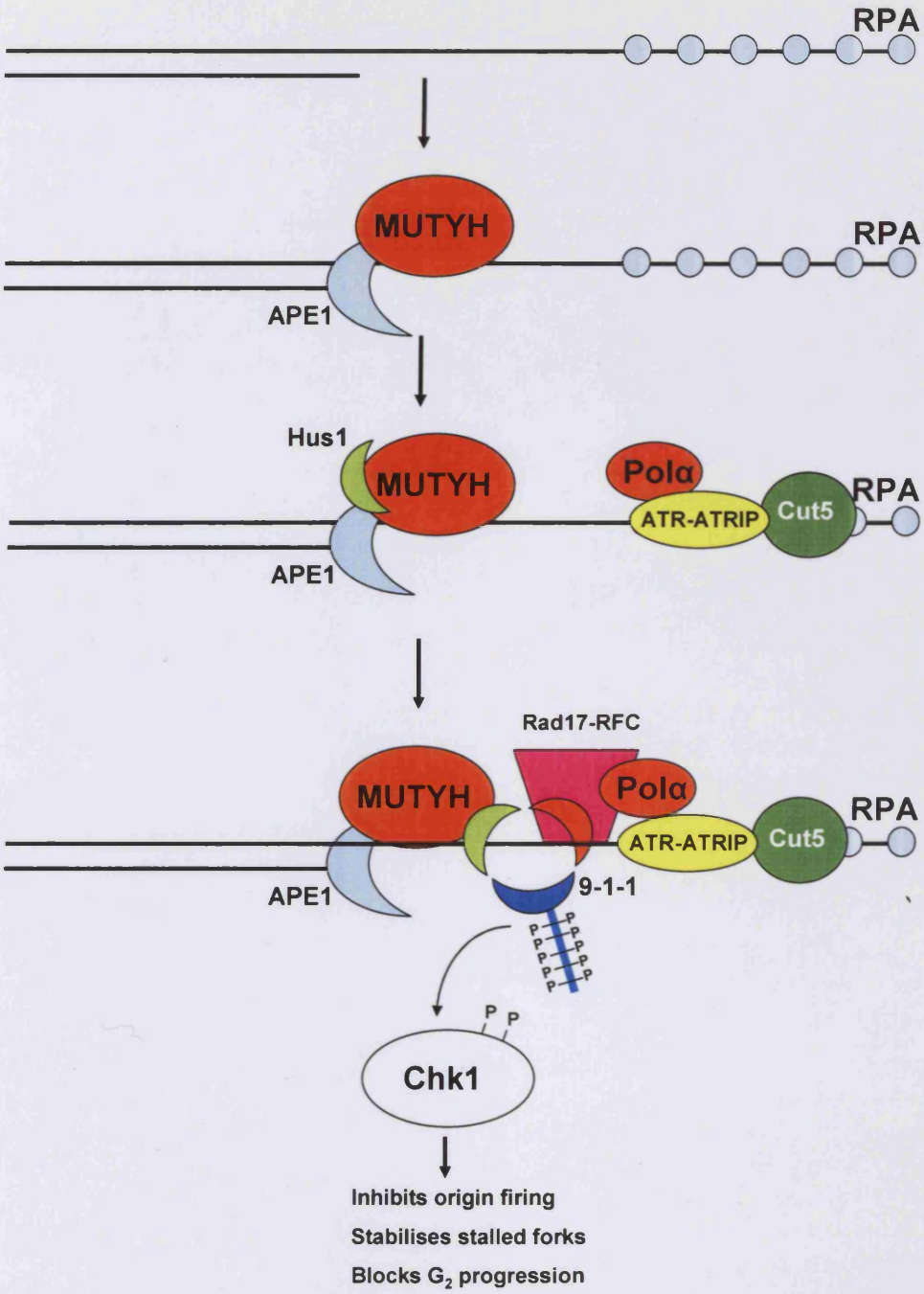


Figure 6.2 Step-wise assembly of the checkpoint signalling machinery at single strand breaks caused by ROS. Single strand breaks are blocked with 3'-phosphoglycolate which is enzymatically cleared by APE1. MUTYH is targeted to the site via a direct interaction with APE1 and recruits Hus1 and Rad1 to the site. The Rad17-RFC clamp loads the assembled 9-1-1 complex onto the DNA. The chromatin bound 9-1-1 complex via the Rad9 phospho-tail, then facilitates the ATR-mediated phosphorylation of Chk1. Similar to figure 6.1, the presence of RPA recruits DNA polymerase α, ATR-ATRIP complex and Cut5 to enable new DNA synthesis where possible.

Our results therefore, indicate that there is an uncharacterised relationship between MUTYH and components of the 9-1-1 complex. These initial functional experiments indicate that *MUTYH* deficient cells avoid induction of cell death and checkpoint mediated cell-cycle arrest. We propose that this feature, coupled with their inability to repair 8-oxoG:A mismatches (leading to a G:C→T:A mutator phenotype), underlies the tumourigenic process in MAP.

Publications resulting from this work

Azzopardi D, Dallosso AR, Eliason K, Hendrickson BC, Jones N, Rawstorne E, Colley J, Moskvina V, Frye C, Sampson JR, Wenstrup R, Scholl T, Cheadle JP. (2008). Multiple rare nonsynonymous variants in the adenomatous polyposis coli gene predispose to colorectal adenomas. **Cancer Res.** Jan 15; 68(2):358-63

Dallosso A, Jones S, Azzopardi D, Moskvina V, Al-Tassan N, Williams GT, Idziaszczyk S, Davies DR, Milewski P, Williams S, Beynon J, Sampson JR, Cheadle JP. The *APC* variant p.Glu1317Gln predisposes to colorectal adenomas by a novel mechanism of relaxing the target for tumourigenic somatic *APC* mutations. **Hum Mutat.** 2009 Oct;30(10):1412-8.

Azzopardi D, Sampson JR and Cheadle JP. A failure to engage apoptosis drives MUTYH-associated tumourigenesis. *Manuscript submitted.*

Appendix

B-catenin regulated transcription assay results

Variant	Non-normalised Luciferase Value	Normalised Luciferase Value	Standard Error	Independent T-Test P-value
APC 1309Δ	32	4.9	0.6	4.9±0.6
APC 1450Δ	24	1.9	0.20	0.00026
APC 1517Δ	27	1.7	0.10	0.00035
APC 1914Δ	26	1.5	0.20	0.034
P1442	16	1.1	0.12	0.725
T1493	21	1.2	0.15	0.379
G1678	16	0.7	0.08	0.836
A1755	25	1.2	0.12	0.438
S1756	23	1.0	0.11	0.738
P1960	22	1.1	0.17	0.647
I1307K	27	1.5	0.07	0.008
E1317Q	25	1.5	0.11	0.014
V1352A	15	1.0	0.14	1.000
M1413V	27	1.4	0.14	0.024
T1445A	17	0.8	0.08	0.118
A1446T	15	0.8	0.08	0.283
K1454E	16	1.1	0.13	0.794
P1476S	24	1.0	0.14	0.342
A1474T	29	1.0	0.06	0.813
I1572T	27	1.3	0.18	0.171
C1578G	26	1.2	0.10	0.256
I1579V	29	1.5	0.16	0.012
R1676G	24	1.4	0.11	0.033
D1714N	30	2.0	0.20	0.000
G1921S	43	2.0	0.13	0.000
P1934L	26	0.8	0.11	0.234

Data shown is the result of three independent assays.

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