THE INTERACTION OF ADENOMATOUS POLYPOSIS COLI (APC) AND BASE EXCISION REPAIR PROTEINS: ITS IMPLICATIONS IN COLORECTAL CARCINOGENESIS

By

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To my parents, Eser and Mayra, for always believing in me; In memory of my Grandmother Maria, for inspiring me and teaching me to always have faith; In memory of my Grandfather Victor whose battle with cancer inspired me to pursue my doctorate

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Mutations in the *adenomatous polyposis coli (APC) gene* are among the earliest events which lead to the development of colorectal cancer. The APC protein is critical for the maintenance of the dynamics of colonic epithelial cells. APC plays a role in the regulation of β-catenin, cell cycle control, cell-cell adhesion, chromosomal stability, chromosomal segregation, and apoptosis. Previous studies have shown that the APC gene is upregulated in response to DNA damage. However, the biological significance of the DNA damage induced APC levels is not yet known. We hypothesize that the DNA damage-induced APC levels may play a role in DNA repair and hence may be involved in the early stages of initiation of colorectal carcinogenesis. Interestingly, it has recently been found that APC can interact with two base excision repair (BER) proteins, DNA polymerase β (pol-β) and Flap endonuclease 1 (Fen-1), and block pol-β-directed BER. In the present study, the critical amino acid residues of pol-β and Fen-1 required for their interaction with APC have been identified. Furthermore, two mechanisms for blockage of long patch (LP)-BER by APC through its interaction with Fen-1 have been discovered.

CHAPTER 1
INTRODUCTION

Colorectal Cancer

Colorectal cancer is the second to fourth most common cancer in the industrialized world. It is estimated that 148,810 new cases of colorectal cancer will occur in 2008. Of these, 49,960 will result in death (Society, 2008American Cancer Society). Colorectal cancer is usually divided into two forms: familial or hereditary cases and sporadic cases. Of the hereditary cases there are two types which are characterized: hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). HNPCC is caused by mutations in genes encoding for DNA mismatch repair proteins. At least four genes have been shown to cause HNPCC: MSH2, MLH1, MSH6, and PMS2 (Baglioni and Genuardi, 2004). FAP arises almost exclusively from germline mutations in the *adenomatous polyposis coli* (*APC*) gene. Interestingly, loss of APC function is an extremely early event in the development of sporadic colorectal tumors. This suggests that APC is critical in the maintenance of the colon (Nathke, 2004).

Adenomatous Polyposis Coli (APC)

The *APC* gene encodes a large multi-domain protein which plays integral roles in many aspects of the cell. Traditionally known as a tumor suppressor, APC plays a role in many functions that are critical for cell survival. Interestingly, somatic mutations in the *APC* gene are found in approximately 80% of all sporadic colorectal tumors. These mutations almost always result in a truncated APC protein product which lacks some of the critical tumor suppressor functions of APC such as β-catenin regulation.

The APC gene

The *APC* gene is located on chromosome 5q21-q22, consists of 8535 nucleotides and spans 21 exons. The *APC* gene encodes a 310 kDa protein which is composed of 2843 amino

acids in its common isoform (Jaiswal et al., 2004). Approximately 75 percent of the coding sequence is located on exon 15, which appears to be the most common target for both germline and somatic mutations of *APC* (Beroud and Soussi, 1996).

Hypermethylation of the *APC* gene promoter has been observed in colorectal, melanoma, lung, oesophogeal, gastric, pancreatic, and hepatic cancers (Clement et al., 2004; Esteller et al., 2000; Grote et al., 2004; Tsuchiya et al., 2000). Methylation occurs at CpG sites in the promoter region of the *APC* gene and is mediated by the enzyme DNA methyl transferase 1 (DNMT1). DNMT1 induces methylation of the *APC* promoter by methylating the CpG region around the CCAAT-box. Once methylated, chromatin conformational changes occur which block the binding of the transcription factor CBF to the CCAAT box, silencing *APC* gene expression (Deng et al., 2004). Intriguingly, it has been found that *DNMT1* gene expression is regulated by APC mutations in an indirect manner (Campbell and Szyf, 2003). The above findings indicate that hypermethylation of the *APC* promoter may provide an alternative mechanism of *APC* gene inactivation during early stages of carcinogenesis.

The APC Protein and its Many Functions

APC is a large multi-domain protein, which is present in both the cytoplasm and the nucleus. It serves many functions in the cell as demonstrated by its structure (Fig. 1-2). Along the N-terminus an oligomerization domain, a homology domain, an armadillo repeat region and two nuclear export signals are present. The oligomerization domain has been shown to be a binding site for APC mutants. Studies have shown that APC mutant proteins that retain at least the first 171 amino acids are able to bind to this site and produce a dominant negative effect (Dihlmann et al., 1999; Su et al., 1993). However, a study involving the overexpression of a truncated APC protein in a mouse model seemed to contradict these findings when the mice did not form tumors (Smits et al., 1999). The armadillo repeat has been designated a protein-protein interaction site

and has been shown to bind PP2A, Asef, and KAP 3 (Jimbo et al., 2002; Kawasaki et al., 2000; Seeling et al., 1999). A number of 15-and 20-amino acid repeats are found in the center of the protein which have been shown to bind β-catenin. Nestled in between the amino acid repeats are axin binding sites, nuclear export signals, and the mutation cluster region. The mutation cluster region (MCR) is located between codons 1280-1513 and harbors over 60% of all somatic mutations in the *APC* gene (Miyoshi et al., 1992). Mutations that occur in the MCR result in a truncated protein that lacks most of the C-terminus, which is home to several important sites on the APC protein. It contains the basic domain, which is the site of microtubule binding, and binding sites for the proteins EB1 and hDLG (Bienz, 2002; Erdmann et al., 2000; Matsumine et al., 1996; Polakis, 1997; Su et al., 1995). Due to its numerous interactions with a variety of proteins it is expected that APC plays various roles in the cell (Figure 1-1).

APC and β-catenin regulation

The involvement of APC in the regulation of β-catenin occurs through several routes. APC is part of the destruction complex, which is activated in the absence of a Wnt signal, along with axin and glycogen synthase kinase-3-β(GSK3-β). APC causes phosphorylation and destruction of β-catenin through the ubiquitin proteasome pathway (Peifer and Polakis, 2000). A recent study suggests that different domains of APC are involved in the regulation of phosphorylation and ubiquination of β-catenin (Yang et al., 2006). The destruction of β-catenin keeps it from activating the T cell factor (Tcf) family of transcription factors. Targets of this family of transcription factors include the oncogene c-myc, cyclin D1, the oncogene c-jun, and the matrix metalloproteinase matrilysin (Crawford et al., 1999; Nateri et al., 2005; Orford et al., 1997; Shtutman et al., 1999; Tetsu and McCormick, 1999). When APC is mutated, it prevents GSK3β-mediated phosphorylation of β-catenin, sparing β-catenin from ubiquitination and

proteasomal degradation. The stable β-catenin accumulates in the nucleus and binds with the Tcf family of transcription factors leading to aberrant gene expression, which is associated with cell proliferation and cell cycle progression (Henderson and Fagotto, 2002). Furthermore, APC has been shown to be involved in the export of β-catenin from the nucleus, thus reducing its ability to interact with Tcf transcription factors (Henderson, 2000). APC has also been shown to sequester β-catenin in the nucleus with the aid of C-terminal binding protein (CtBP), and once again, prevent its binding to Tcf transcription factors (Hamada and Bienz, 2004). Similarly, cytoplasmic APC has been shown to sequester cytoplasmic β-catenin. When levels of cytoplasmic β-catenin are excessively elevated, it induces phophorylation of APC by casein kinase1, GSK3β and unknown kinases. Once phosphorylated, APC is able to retain β-catenin in the cytoplasm by keeping it bound in a complex preventing the entry of β-catenin into the nucleus and the activation of Tcf signaling (Seo and Jho, 2007). Moreover, APC's role in β-catenin regulation suggests a role for APC in cell cycle progression. A pathway has been elucidated which shows that APC can regulate Cdk4 expression as a result of its interaction with β-catenin. Through the use of an inducible *APC* gene in HT29 colon cancer cells, He and colleagues (1998) discovered that *c-myc* was a target gene of the APC pathway. They found that when APC was induced *c-myc* expression was repressed (He et al., 1998), and that *c-myc* expression was activated through Tcf-4 which is activated by β-catenin. These results lead to a proposed pathway for *c-myc* expression in colorectal epithelial cells. In normal colorectal epithelial cells, wild-type APC prevents β-catenin from forming a complex with Tcf-4 and activating *c-myc*. However, in colorectal tumors which have an *APC* mutation or activating β-catenin mutation, β-catenin/Tcf-4 complex formation is increased leading to overexpression of *c-myc* and the promotion of neoplastic growth (He et al., 1998).

Further studies proved that Cdk4 is the target of the aforementioned pathway (Hermeking et al., 2000). After *c-myc* overexpression was carried out in human umbilical vein cord cells (HUVEC) using adenovirus, a transcript encoding Cdk4 was found to be induced. This proved to be most intriguing since Cdk4 is involved in cell cycle regulation. To further confirm these results a mutant *c-myc* was overexpressed in HUVEC cells to see if the Cdk4 transcript was still induced. As expected, Cdk4 failed to be induced in the presence of the mutant *c-myc*. These results suggest that *c-myc* is involved in the direct regulation of Cdk4 mRNA. Moreover, a concordant increase in *c-myc* and Cdk4 expression is found in colorectal cancers when compared to normal colon cells (Hermeking et al., 2000). Based on these data the aforementioned model with APC has been amended. In normal colorectal epithelial cells, wild-type APC prevents β-catenin from forming a complex with Tcf-4 and activating *c-myc*. Since *c-myc* is not activated, Cdk4 will not be activated and this prevents cdk4 from phosphorylating and down-regulating important tumor suppressor genes such as the *retinoblastoma (Rb)* gene. However, in colorectal tumors which have an *APC* mutation or an activating β-catenin mutation, β-catenin/Tcf-4 complex formation is increased leading to over expression of *c-myc*. Over expression of c-myc leads to an overexpression of Cdk4, which can phosphorylate the Rb gene causing it to become down-regulated and promote neoplastic growth.

APC and its role in cell adhesion

APC is a part of the cell-cell adhesion complex with E-cadherin, since it directly binds to γ-catenin and actin filaments as well as β-catenin. In *Drosophila* mutational inactivation of APC causes delocalization of β-catenin, gaps between adhesive plasma membranes, and a mislocalization of oocytes resulting from a failure of cadherin based adhesion (Hamada and Bienz, 2002). Moreover, Hughes et al., found that enterocytes from C57BL/6J-min/+ (Min/+)

mice showed a reduced association between E-cadherin and β-catenin in adherens junctions (Hughes et al., 2002). Min/+ mice serve as an animal model for familial adenomatous polyposis. These mice have one APC allele which carries a truncation mutation at codon 850 and one normal APC allele (Su et al., 1992). Furthermore, Faux and colleagues were able to show that restoration of full length APC into SW480 cancer cells, which contain truncated APC, resulted in enhanced cell adhesion (Faux et al., 2004). The enhanced cell adhesion was attributed to the involvement of APC in post-translational regulation of E-cadherin localization. When full length APC was restored E-cadherin localization changed from the cytoplasm to the cell membrane resulting in tighter adherens junctions (Faux et al., 2004).

APC and its role in cell migration

Wild-type APC is necessary for the upward movement of colonic epithelial cells along the crypt villus axis. During migration colonic epithelial cells are differentiated into absorptive, secretory, paneth, and endocrine cells. Mutation or loss of APC causes aberrant migration of these cells and the formation of polyps as well as altered differentiation in colonic epithelial cells (Moss et al., 1996; Sansom et al., 2004). Kroboth and colleagues showed that loss of APC results in immediate defects in cell migration in cells that constitutively lack APC and in cells where APC loss was induced conditionally using a Cre-Flox system (Kroboth et al., 2007). Migration was affected in both cell monolayers and in single cell movements.

Furthermore, APC can regulate cell polarity and migration through the actin cytoskeleton. APC can interact with a Rac-specific guanine nucleotide exchange factor (Asef1) through its armadillo repeats. Once activated, Asef activates Rac 1, leading to increased cell spreading, membrane ruffling and lamellipodia formation (Hanson and Miller, 2005; Kawasaki et al., 2000). Additionally, it has been shown that truncated APC constitutively activates Asef. The

constitutively active Asef causes a decrease in E-cadherin mediated cell-cell adhesion and promotes cell migration (Kawasaki et al., 2003). A recent study has shown that APC can also interact with Asef2, a relative of Asef1. Through this interaction it was found that APC activates Cdc42, which is involved in actin polymerization and cell polarity (Kay and Hunter, 2001). Moreover, it has been found that IQ-motif containing GTPase activation protein 1 (IQGAP1), a scaffold protein involved in the control of actin filaments and microtubules as well as an effector of Rac 1 and Cdc42, recruits APC to specific sites in migrating cells. A complex is then formed between IQGAP1, APC, Rac 1, Cdc42, and the microtubule stabilizing protein CLIP-170. If either APC or IQGAP1 is removed, polarized migration as well as formation of the actin meshwork is inhibited (Aoki and Taketo, 2007; Briggs and Sacks, 2003; Watanabe et al., 2004).

Role of APC in chromosomal segregation and mitotic spindle assembly

The ability of APC to interact with Bub kinases and microtubules suggests a role for APC in chromosomal segregation and mitosis. APC has been found at the ends of mictrotubules in a complex with the checkpoint proteins Bub1 and Bub3, suggesting a role of APC in mitosis. It has been shown that APC localizes to spindle poles, spindle microtubules, and kinetochores (Dikovskaya et al., 2004; Fodde et al., 2001; Kaplan et al., 2001; Louie et al., 2004) and the loss of APC or expression of APC truncation mutants, similar to those found in cancer cells, leads to defects in the mitotic spindle. Moreover, the spindle checkpoint is compromised allowing cells to progress through mitosis with incomplete chromosomal alignment or attachment (Dikovskaya et al., 2004; Draviam et al., 2006; Green and Kaplan, 2003; Green et al., 2005; Tighe et al., 2004). Incomplete chromosomal alignment or attachment can lead to aneuploidy and tetraploidy causing chromosomal instability.

Along with its roles in chromosomal segregation and mitosis, APC has also been found to bind the ends of microtubules and stabilize them. It is thought that stabilization of microtubules

by APC occurs through its ability to interact with EB1 (Morrison et al., 1998; Nakamura et al., 2001; Su et al., 1995). APC may form a complex with EB1 and the formin family protein, mDia, in order to regulate the microtubule network and its stabilization (Wen et al., 2004). Further evidence is seen in the fact that APC has been found to interact with the microtubule destabilizing protein kinesin (KIF2C). Moreover, loss of APC causes microtubule depolymerization to occur much faster than is seen in cells with wild-type APC. Compromised microtubule stability can lead to failures in mitotic spindle assembly and regulation of the microtubule network (Kroboth et al., 2007).

Role of APC in cell cycle regulation and apoptosis

Overexpression of APC has been shown to block cell cycle progression from G_0/G_1 to S phase by negatively affecting cyclin-cdk complexes. Increased APC has been found to arrest the cell cycle in the G_0/G_1-S phase and G_2/M phase (Baeg et al., 1995; Heinen et al., 2002). It is thought that the APC binding partner, human discs large (hDLG) forms a complex with APC that regulates the G_1/S phase transition. APC which lacks the hDLG binding site does not block cell cycle activity as strongly suggesting that the interaction with hDLG is necessary to down-regulate cell growth (Ishidate et al., 2000). However, the role of APC in cell cycle control with damaged DNA is not yet fully understood.

The role of APC in apoptosis is controversial. The precise mechanism is not yet clear. In the colon, increased levels of APC can induce cell death under certain circumstances. Studies have been conducted using the HT29 colon cancer cell line which show that expression of full length APC in this cell line leads to a decrease in cell growth and apoptosis (Morin et al., 1996). Additional studies with this cell line have shown that expression of full length APC down-regulated the anti-apoptotic factor Survivin (Zhang et al., 2001). In addition, studies have shown that APC induces apoptosis by accelerating apoptosis-associated caspase activities (Chen et al.,

2003; Steigerwald et al., 2005). Furthermore, Qian and colleagues have shown that caspase cleavage of APC and release of its amino terminal domain may be required for APC to accelerate apoptosis-associated caspase activities independent of transcription. Lastly, some studies have suggested that a decrease in APC levels is associated with apoptosis. It is possible that both increased and decreased levels of APC play a role in apoptosis and that different factors such as cellular signals play a role in whether increased or decreased levels of APC are involved in apoptosis (Brancolini et al., 1998; Browne et al., 1998; Browne et al., 1994; Jaiswal et al., 2002; Jaiswal and Narayan, 2004; Schmeiser and Grand, 1999; Williams et al., 1996).

APC and its involvement in DNA damage and repair

The basal level expression of APC occurs through Upstream Stimulating Factors 1 and 2 (USF-1 and USF-2) (Narayan and Jaiswal, 1997). However, *APC* gene expression is inducible and upregulated by p53 in response to DNA damage (Jaiswal and Narayan, 2001; Narayan and Jaiswal, 1997). The presence of damaged DNA usually leads to one of three possibilities. It can cause the cell to undergo DNA repair, apoptosis, or if the DNA damage persists and the cell survives it can lead to cell transformation. Since *APC* gene expression is upregulated in response to DNA damage, it is possible that APC is playing a role in one or all of these scenarios. However, it was decided to focus on the involvement of APC in DNA repair since a defect in DNA repair is one of the initial steps in carcinogenesis. Recently, a DNA Repair Inhibitory (DRI) domain was identified in APC that allowed it to bind with two base excision repair proteins DNA polymerase β (pol-β) and Flap endonuclease-1 (Fen-1). Through this interaction it appeared that APC was able to block pol-β deoxyribose phosphate (dRP) lyase activity,short-patch base excision repair (SP-BER), pol-β-mediated long-patch base excision repair (LP-BER), and Fen-1 cleavage activity (Balusu et al., 2007; Jaiswal et al., 2006; Narayan et al., 2005). In

addition to the DRI-domain, the APC protein also contains a mutation cluster region (MCR). Approximately, 60 percent of the APC mutations in colon cancer arise from the MCR. Mutations in this region lead to a more severe polyposis than patients with mutations outside the MCR (Narayan and Roy, 2003). It is of interest to note that the DRI-domain is not in this region, indicating that both wild-type and truncated APC retain the ability to interact with these proteins and block BER.

DNA Damage and Repair

The genomes of eukaryotic cells are under constant attack by endogenous and exogenous sources. Exogenous exposure arises from environmental agents such as ultraviolet (UV) radiation, ionizing radiation (IR), and various genotoxic agents while endogenous exposure arises as by-products of cellular metabolism and includes hydrolysis, oxidation, alkylation, and mismatch of DNA bases (Houtgraaf et al., 2006). Damaged DNA that is not repaired can lead to genomic instability, apoptosis, or mutagenesis which can eventually lead to carcinogenesis. Fortunately, the cell has developed various DNA damage checkpoints and DNA repair pathways to deal with the genomic insults it encounters.

DNA Damage Checkpoints

DNA damage triggers the activation of cell cycle checkpoints. These checkpoints are activated during the different phases of the cell cycle. The eukaryotic cell cycle contains the following phases, gap $(G)_1$, synthesis (S), gap $(G)_2$, mitosis (M) and one phase outside of the cell cycle G_0 (Kastan and Bartek, 2004; Nyberg et al., 2002). During G_1 the cell becomes larger and starts synthesizing RNA and proteins which are needed for transcription and translation. The synthesis of RNA and protein continues through G_2. In S phase, the cell replicates its DNA so that one copy can be passed on to each daughter cell. During G_2 the cell continues to grow and makes extra proteins to ensure that two viable cells can be formed. Lastly, the cell will enter M

phase where the chromosomes will be aligned and two genetically identical daughter cells will be formed. As long as cells are dividing, the cycle will continue. When cells stop dividing they are said to be in G_0. DNA damage checkpoints generally occur during the transition of each phase. They cause cell cycle arrest and allow the cell's DNA repair machinery to repair the damaged DNA. However one checkpoint occurs during a cell cycle phase. The checkpoints are the G_1/S checkpoint, the intra-S checkpoint, and the G_2/M checkpoint (Ishikawa et al., 2006).

G_1/S checkpoint

In response to DNA damage the G_1/S checkpoint serves to prevent the transition between the G_1 phase and the S phase in the cell cycle. Once DNA damage is sensed a myriad of signaling cascades serve to cause G_1 arrest. Briefly, ATM (ataxia-telangiectasia) or ATR (ATM and RAD related) which are both known as DNA damage sensors, directly phosphorylate the p53 in its transactivation domain on Ser 15. Moreover, MDM2 is also targeted by ATM/ATR and CHK2/CHK1 kinases due to its involvement in p53 regulation. Activation of p53 and MDM2 allows the regulation of the transcription of target genes p21CIP1/WAF1. Once activated, p21CIP1/WAF1 inhibits the G_1/S cyclin/cdk promoting complexes (Wahl and Carr, 2001). Specifically, the p21CIP1/WAF1 signal inhibits the RB/E2F pathway and thus prevents G_1/S promotion.

S-phase checkpoint

During the S-phase of the cell cycle DNA replication occurs. In addition to DNA damage, S-phase checkpoints can be triggered as a result of difficulties with DNA replication such as stalled replication forks. There are three types of S-phase checkpoints: 1) the replication dependent checkpoint, 2) the replication independent checkpoint, and 3) the S-M checkpoint. The replication dependent checkpoint and the S-M checkpoint are dependent upon DNA replication while the replication independent checkpoint is triggered by double strand breaks. All

three of these checkpoints share components (Bartek et al., 2004). Key components in all three of the S-phase checkpoints include, RAD17, and the RAD9-RAD1-HUS1 complex, replication protein A, ATR, the ATR interacting protein (ATRIP), the ATR activator Top BP1, and the mediator protein CLASPIN. In response to DNA damage all three checkpoints have a similar response. Once DNA damage is detected, the RAD9-RAD1-HUS1 complex is loaded onto the area surrounding the DNA lesion by RAD17. This action allows ATR-mediated phosphorylation and activation of CHK1 kinase, causing cell cycle arrest (Mordes et al., 2008; Zou et al., 2002).

G_2/M checkpoint

The main function of the G_2/M checkpoint is to prevent cells with unrepaired DNA damage from entering mitosis. Cells are prevented from entering mitosis in CHK1 kinase-mediated manner. Once activated, the G_2/M checkpoint begins with ATM/ATR-mediated phosphorylation of CHK1/CHK2 kinases and p38 kinase, causing degradation, inhibition, or sequestration of cdc25 phosphatases. Once inhibited, the cdc25 phosphatases can no longer activate CDK1 which allows the cell to proceed into mitosis. Thus, the cell cycle is arrested (Nyberg et al, 2002; (Kastan and Bartek, 2004).

DNA Repair

If left unrepaired, DNA damage interferes with replication and transcription leading to apoptosis. Furthermore, continuous DNA damage can cause accumulation of genetic mutations that lead to carcinogenesis. However, the cellular system is equipped with repair mechanisms to deal with damaged DNA. There are six main DNA repair mechanisms or pathways: DNA damage reversal, nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination repair (HR), non-homologous end-joining (NHEJ) repair, and base excision repair (BER). The type of DNA damage present determines the pathway of DNA repair that is used.

Direct damage reversal

In direct damage reversal, the damage to the base is repaired by methyltransferases or oxidative demethylases. In general, O-alkylations are repaired by alkyltransferases and N-alkylations tend to be repaired by oxidative demethylases. These enzymes work by transferring the alkyl or methyl group from the damaged base onto the enzyme. This action auto-inactivates the enzyme. Thus, the enzyme is limited by the amount available in the area of the damaged base (D'Incalci et al., 1988; Gerson, 2004; Middleton and Margison, 2003; Sabharwal and Middleton, 2006).

Nucleotide excision repair

DNA adducts that form large bulky lesions and covalent linkages between adjacent pyrimidines resulting from UV exposure are repaired through nucleotide excision repair (NER). NER has two direct pathways termed transcription-coupled repair (TCR) and global genomic repair (GGR). TCR serves as a system that detects and removes DNA damage that blocks the progression of RNA polymerase II (Hanawalt, 2002). GGR is slower than TCR and serves as a system that surveys the entire genome for damage. Both pathways follow a similar process. Briefly, DNA damage is recognized by the XPC-HR23B complex in GGR and RNA polymerase II in TCR and the damage is verified by XPA. Subsequently the DNA is unwound by XPB and XPD helicases that are in a complex with TFIIH basal transcription initiation factor. The DNA is then incised by XPF and XPG on the damaged strand. Lastly, the damaged portion of the DNA is removed and the repair is completed by a DNA polymerase followed by DNA ligase. Two additional proteins are required for TCR, CSB and CSA (Hanawalt, 2002).

Mismatch repair

The mismatch repair (MMR) system removes base-base mismatches and insertion and deletion loops that are formed during DNA replication (Jiricny, 2006). It is also important in the

maintenance of repeated sequences. Mutations in the MMR system are associated with a pre-disposition to cancer (Ben Yehuda et al., 2000; Karran, 1996; Skinner and Turker, 2005). Briefly, base mispairs and small insertion or deletions are recognized and the recognized lesions are excised. Lastly, the DNA is resynthesized and repair is complete (Neri et al., 2005).

Double-strand break repair

Double strand breaks are repaired by the cell's homologous recombination (HR) and non-homologous end-joining (NHEJ) repair systems. Repair of these lesions is important because these lesions can lead to genomic instability, apoptosis and/or mutations due to deletions, insertions, translocations, and aneuploidy. HR is an error-free repair pathway. In this pathway homologous sequences are aligned on the sister chromatid (Hefferin and Tomkinson, 2005; Khanna and Jackson, 2001; Scott and Pandita, 2006). The sister chromatid serves as a template so that the correct information can be copied to the missing information on the locus where the break occurred. This process is mediated by RAD5, is aided by proteins in the RAD52 epistasis group (Helleday, 2003; Jackson, 2002), and usually occurs during the G_2 phase of the cell cycle (Hefferin and Tomkinson, 2005; Khanna and Jackson, 2001; Scott and Pandita, 2006). On the other hand NHEJ is an error prone process that usually occurs during the G_o/G_1 phase of the cell cycle. In NHEJ, the two broken ends are fused together irrespective of homology. The process begins with Ku70/Ku80 heterodimer binding to the broken ends of the DNA. Then the broken ends are processed for ligation with the aid of the Artemis-DNA-PKCs complex which is recruited by the Ku heterodimer. Next, gap filling by a DNA polymerase of the pol-X family occurs and the resulting nicks are covalently joined by the XRCC4-DNA ligase IV complex (Lieber, 1999; Lieber et al., 2003). Due to the fact that this process disregards homology, NHEJ can lead to insertions or deletions of filler DNA making it error prone (Gorbunova and Levy, 1997; Sargent et al., 1997; Seluanov et al., 2004)

Base excision repair

Base excision repair (BER) is a pathway that removes damaged DNA, specifically abasic DNA (Fig. 1-3). It is composed of two sub-pathways "single nucleotide base excision repair which is also referred to as short-patch (SP)-BER and "multi-nucleotide base excision repair" which is also referred to as long-patch (LP)-BER. The pathway used depends on the size of the repair gap and the type of DNA damage. For example, if the damaged DNA base is reduced or oxidized it would go through LP-BER (Klungland and Lindahl, 1997; Podlutsky et al., 2001).

In both SP-BER and LP-BER the process begins with the removal of the damaged base. A DNA glycosylase recognizes the damaged base and cleaves the glycosidic bond between the sugar and the base leaving an apurinic/apryrimidinic (AP) or abasic site. AP-endonuclease 1 (APE-1) then comes in and makes an incision at the 5'end of the AP-site of the DNA backbone. The decision is then made whether to use the SP-BER or LP-BER depending on the type of DNA damage. During SP-BER, the deoxyribose phosphate (dRP) lyase activity of DNA polymerase β (pol-β), which is located in the 8 kDa domain of pol-β, removes the 5'dRP left behind by APE-1 resulting in a 5' phosphorylated nicked DNA strand (Matsumoto and Kim, 1995). Pol-β then uses its polymerase activity, which is located in its 31 kDa C-terminal domain, to synthesize the correct base at the site of the damaged base. The remaining nick is sealed by DNA ligase I (Mitra et al., 2001).

When the AP-site is oxidized or reduced it becomes chemically resistant to the dRP lyase activity of pol-β and must be repaired through LP-BER. In this case, pol-β can synthesize up to 13 nucleotides creating a 5' overhang or flap that must be removed to complete LP-BER. The flap is removed by flap endonuclease-1 (Fen-1) leaving behind a nick which is sealed by DNA ligase I (Lieber, 1997; Podlutsky et al., 2001). In addition to pol-β, pol-δ and pol-ε can also

participate as the main polymerase that synthesizes the 13 nucleotides in strand displacement synthesis (Fig. 1-3).

Alternate BER pathways. Recently, a different class of sub-pathways of BER have been discovered. The common denominator in all these sub-pathways is a single strand break. These pathways have also been termed alternate BER pathways (Almeida and Sobol, 2007). The main difference in these pathways is in the initiation of BER. The sub-pathways that have been found are: nucleotide incision repair, NEIL2 DNA glycosylase initiated repair, radiation-induced DNA damage or single strand break repair (SSBR), and strand breaks generated by topoisomerase poisons. In response to the type of damage that causes the single strand break, one of the sub-pathways may be initiated (Almeida and Sobol, 2007).

Nucleotide incision repair occurs when APE1 acts directly on the damaged DNA bypassing the glycosylase step. It makes an incision upstream of the oxidatively damaged base leaving a 3'OH and 5' phosphate containing the damaged base at the ends. It is thought that this specifically happens in response to a specific type of oxidative damage, 5-hydroxy-2'-deoxycytidine (5OH-C). The resulting product is a substrate for LP-BER and APE1 goes on to stimulate Fen-1 activity as well as DNA ligase I activity (Dianova et al., 2001; Ischenko and Saparbaev, 2002; Ranalli et al., 2002).

NEIL2 DNA glycosylase-initiated repair occurs through the use of the bi-functional DNA glycosylases NEIL1/NEIL2. In addition to removing the oxidized base similarly to mono-functional glycosylases such as OGG1 and NTH1, NEIL1 and NEIL2 further process the AP site to yield a 3' phosphate and 5'phosphate at the margins. Due to the fact that APE 1's 3' phosphatase activity is not very strong, this pathway utilizes another enzyme to process the resulting NEIL1/NEIL2 product. Instead, polynucleotide kinase phosphatase (PNKP) is required

to remove the 3' phosphate. A complex then forms between NEIL1/NEIL2, pol-β, XRCC1, DNA ligase III, and indirectly with PNKP. Repair then proceeds in a manner similar to SP-BER (Caldecott, 2002; Wiederhold et al., 2004).

Radiation-induced DNA damage causes single stranded breaks in the DNA. The pathway begins with PARP-1 recognizing and binding to single strand breaks. XRCC1 is then recruited and repair proceeds in the traditional manner of SP-BER. On the other hand, strand breaks generated by topoisomerase poisons are repaired in a different manner. The lesion in this case begins as a DNA:topoisomerase 1 (Top1) complex creating a toxic single strand break. The enzyme tyrosyl DNA phosphodiesterase (Tdp 1) catalyzes the hydrolysis of Top1 from the DNA leaving a gap with a 3' phosphate and a 5' hydroxyl. PNKP can then transfer the 3' phosphate to the 5' end. At this point, gap filling can proceed through pol-β and it is thought that XRCC1 also participates (Plo et al., 2003).

APC's proposed role in BER. As previously mentioned it was found that APC was able to interact with pol-β *in vivo* and that APC was able to block pol-β-directed strand-displacement synthesis during LP-BER (Narayan et al., 2005). Furthermore, in 2006, Jaiswal and colleagues found that APC also interacted with Fen-1 *in vivo* and *in vitro* (Jaiswal et al., 2006). Combined, these findings indicate that APC is playing an important role in modulating BER activity. Specifically, these data suggest that APC is playing a role in LP-BER.

DNA Polymerase β (pol-β)

DNA polymerase β is one of the smallest eukaryotic DNA polymerases known to date. It is one of several known mammalian cellular DNA polymerases. Its major role is in DNA repair, specifically, BER. It has been shown to participate in both SP-BER and LP-BER (Narayan et al.,

2005). Furthermore, it is embryonic lethal indicating that pol-β is necessary for survival (Niimi et al., 2005).

Pol-β Structure

Pol-β is a 39 kDa protein composed of 335 amino acids (Prasad et al., 1998b). It contains two domains and one protease sensitive hinge region. The first domain is the 8 kDa lyase domain and the second domain is the 31 kDa polymerase domain. The 8 kDa lyase domain is located at the amino terminus and contains the lyase activity that is responsible for removing the 5'-deoxyribose phosphate that is generated after incision of the damaged DNA by APE1 in SP-BER. The active site of the lyase domain has been identified as Lys72 (Prasad et al., 1998a). Moreover the residues that form the active site pocket have been identified. The active site pocket is defined by the following residues: Glu26, Ser30, His34, Lys35, Tyr39, Lys68, Lys72 (Batra et al., 2005; Prasad et al., 2005). The lyase reaction proceeds through a β-elimination reaction with the active site pocket residues participating in the lyase reaction itself. Tyr39 is proposed to perform nucleophilic attack. Lys35 is proposed to be involved in enzyme mediated hydrolysis while Glu 26 and Ser 30 are proposed to participate in proton abstraction. However, the reaction is not well understood (Prasad et al., 2005). In addition to lyase activity, the 8 kDa lyase domain contains single stranded DNA binding activity (Prasad et al., 1993). The 8kDa lyase domain is alpha-helical in nature and forms one of two helix-hairpin-helix (HhH) structural motifs present in pol-β located in residues 55-79 of the lyase domain (Krahn et al., 2004; Mullen and Wilson, 1997; Pelletier et al., 1996). It has been observed to aid in the stabilization of the 90^{o} bend in nicked DNA and/or in the stabilization of the incised DNA strand by facilitating the proper positioning of the two free DNA ends (Matsumine et al., 1996; Sawaya et al., 1997).

Like other DNA polymerases, pol-β contains a two metal ion catalytic site found in the 31 kDa polymerase domain which is responsible for the catalysis of the nucleotidyl transferase reaction (Pelletier et al., 1994; Sawaya et al., 1997). It is located at the C terminus of pol-β. The 31 kDa domain can be broken down into three sub-domains: 1) the D-sub-domain (6 kDa) which is responsible for dsDNA binding; 2) the C-sub-domain (10 kDa) which houses the catalytic activity needed for the nucleotidyl transferase reaction; 3) the N-sub-domain (12 kDa) which is involved in dNTP selection and nascent base pair binding (Beard et al., 2002). The D- and N-sub-domains are situated on opposite sides of the C-sub-domains (Sobol and Wilson, 2001). The D-sub-domain contains the other HhH structural motif in pol-β. It is located at residues 92-118 (Beard et al., 2002; Krahn et al., 2004; Mullen and Wilson, 1997; Pelletier et al., 1996; Sobol and Wilson, 2001). The HhH motif serves the same function as the aforementioned HhH motif located in the 8 kDa lyase domain. The two HhH motifs work together to facilitate the proper positioning of the two free DNA ends of the DNA substrate. The C-sub-domain possesses critical amino acids that are important for the catalytic activity of the 31 kDa domain. Residues Asp190, Asp192, and Asp256 have been shown to coordinate the two divalent metal cations, Mg^{2+} that assist in the nucleotidyl transferase activity and they are known as active site residues (Date et al., 1991; Menge et al., 1995; Sobol et al., 2000). Two other residues Ser180 and Arg183 have also been found to play a significant role in catalysis (Date et al., 1990; Kraynov et al., 2000). The N-sub-domain contains residues involved in pol-β fidelity. Residues Tyr265 and Ile260 have been shown to be important in maintaining the fidelity of pol-β. It has been observed that the N-sub-domain closes around the nascent base pair once the correct nucleotide binds (Clairmont et al., 1999; Opresko et al., 2000; Opresko et al., 1998; Shah et al., 2001; Shah et al., 2003; Starcevic et al., 2005). Pol-β is thought to have an induced-fit mechanism for fidelity. In

the induced fit model of fidelity after initial binding of the correct dNTP, the ternary complex undergoes conformational changes which allow catalytic residues to be aligned for proper activation of the catalytic site. However, if the incorrect dNTP is incorporated, it is a poor fit and the alignment of the catalytic residues does not occur correctly which causes sub-par activation of the catalytic site (Johnson, 1993; Showalter and Tsai, 2002). Additional amino acid residues shown to be involved in pol-β fidelity are found in a loop under the C-sub-domain. Mutation of Asp246, Glu249, and Arg253 leads to a decrease in polymerase fidelity (Dalal et al., 2004; Hamid and Eckert, 2005; Kosa and Sweasy, 1999). Lastly, pol-β contains a protease sensitive hinge region which connects the 8 kDa lyase domain and the 31 kDa polymerase domain (Prasad et al. 1998). This region is sensitive to proteases and allows separation of the 8 kDa and 31 kDa domains when it is exposed to proteases.

Roles of Pol-β

Pol-β plays a dual role in BER. In SP-BER, pol-β uses its lyase activity to remove the 5'-deoxyribose phosphate (dRP) left behind after APE1 incision (Matsumoto and Kim, 1995). It then uses its polymerase activity to fill the 1-nt gap left behind with the correct corresponding nucleotide. While in LP-BER, pol-β uses its polymerase activity to perform strand-displacement synthesis. Moreover, in studies performed using pol-β deficient cells or in the presence of a neutralizing antibody, lack of pol-β caused a reduction in DNA repair capacity suggesting a role for pol-β in LP-BER (Dianov et al., 1999).

Pol-β has been reported to be involved in meiosis and double stranded break repair. Specifically, pol-β has been shown to be associated in the meiotic events of synapsis and recombination (Plug et al., 1997). It has also been shown to be involved in non-homologous end joining in yeast, where Wilson and colleagues discovered a pol-β yeast homologue-dependent

pathway for non-homologous end joining, suggesting that a similar pathway may exist in mammals although none have been found to date (Wilson and Lieber, 1999).

Post-translational Modifications of Pol-β and its Effect on Pol-β Function

Post-translational modifications are known to affect the activity of the target protein. It stands to reason that post-translational modifications serve to modulate the desired activity of a target protein. In the case of pol-β, it would seem that post-translational modification would play a large role in affecting pol-β's activity with regard to BER. Studies have now confirmed this thought. Pol-β has been found to undergo acetylation as well as methylation. In 2002, Hasan and colleagues found that pol-β was able to form a complex with the transcriptional co-activator p300, and that p300 was able to acetylate pol-β *in vitro* and *in vivo* (Hasan et al., 2002). Interestingly the target for acetylaton by p300 was Lys 72 of pol-β. Lys 72 is the active site for pol-β lyase activity. Not surprisingly, this acetylation caused a block to pol-β's lyase activity and it was not able to function properly in a reconstituted BER assay.

More recently, El-Andaloussi and colleagues discovered that Arg 83 and Arg 152 of pol-β are targets for methylation by protein arginine methyltransferase (PRMT6) *in vitro* and *in vivo* (El-Andaloussi et al., 2007; El-Andaloussi et al., 2006). This methylation stimulates pol-β's activity. Specifically, it causes an increase in pol-β's polymerase activity but not the lyase activity. Thus, the methylation of pol-β plays a role in increasing the activity of BER.

Flap Endonuclease 1 (Fen-1)

Fen-1 is a structure specific nuclease that has many roles. It has been found to be involved in maintaining genome stability through its involvement in DNA replication and repair. It is embryonic lethal indicating that Fen-1 is critical for survival of the organism. Moreover, mutations in Fen-1 have been implicated in various diseases including Huntington's disease,

myotonic dystrophy, and cancer (Zheng et al., 2007 [53]). It is thought that Fen-1 may be a novel tumor suppressor (Liu et al., 2004; Shen et al., 2005; Henneke et al., 2003).

Fen-1 Structure and Biochemical Characteristics

Fen-1 is a 43 kDa protein that houses three domains: The N-domain, I-domain, and C-domain. The N-and I-domains are important for catalytic activity and are highly conserved among Fen-1 homologues (Zheng et al., 2002). The C-domain houses the proliferating cell nuclear antigen (PCNA) motif and the nuclear localization signal and is only found among higher organisms (Frank et al., 2001; Qiu et al., 2001; Shen et al., 1998). The nuclear localization signal is critical for eukaryotic organisms because Fen-1 must be in the nucleus to perform its various roles.

Three individual biochemical activities have been attributed to Fen-1. Harrington and colleagues discovered the 5' flap endonuclease activity and the 5' to 3' exonuclease activity of Fen-1 (Harrington and Lieber, 1994). Both of these activities are housed in the same catalytic domain of the protein. Fen-1 efficiently cleaves DNA or RNA flaps using its 5' endonuclease activity and this activity is essential for its role in DNA replication where it is involved in Okazaki fragment maturation (Bambara et al., 1997; Lieber, 1997; Liu et al., 2004). The exonuclease activity of Fen-1 can cleave nicks, gaps, and 5'end recessed double stranded DNA. It can also cleave blunt-ended DNA, albeit less efficiently (Lindahl, 1971; Murante et al., 1994). The role of Fen-1's exonuclease activity is not as well defined, but current thought suggests a role in Okazaki fragment maturation and apoptosis (Liu et al., 2004; Parrish et al., 2003). The most recent biochemical activity attributed to Fen-1 is a gap endonuclease (GEN) activity. Human Fen-1 was found to cleave the template strand of a gapped DNA fork and bubble substrates which simulate those substrates found in a stalled replication fork (Zheng et al., 2005).

The cleavage was able to occur without the presence of a single stranded 5' DNA flap indicating that GEN activity and endonuclease activities are separate.

Residues and Stuctural Motifs of Fen-1 Important for Fen-1 Function

Several residues and structural motifs have been identified that are important for the function of Fen-1 (Fig. 1-4). Amino acids D86, E158, E160, D179, D181 and D233 have been reported to be critical for the catalytic activity of Fen-1. Mutations of these residues caused a complete loss of Fen-1 endonuclease activity. The aforementioned residues are thought to be involved in the coordination of the two Mg^{2+} or Mn^{2+} ions to create the nuclease active site of the protein (Shen et al., 1997; Zheng et al., 2002). In addition, two more amino acid residues were identified (K93 and R100) that played a crucial role in catalysis. Mutation of these amino acids caused a loss of flap endonuclease activity (Qiu et al., 2004). It is thought that these amino acids may participate in the catalytic reaction itself possibly through activation of a water molecule that participates in nucleophilic attack.

Fen-1 also contains a H3TH motif which is proposed to mediate the binding of ssDNA and dsDNA portions of the flap substrate (Hosfield et al., 1998). Other important amino acids involved in Fen-1 function include: 1) L340, D341, F343, and F344 which are important for the interaction of PCNA with Fen-1, and 2) K354, R355, K356, K365, K366, and K367 which are critical for the nuclear localization of Fen-1 in eukaryotic cells (Frank et al., 2001; Qiu et al., 2001).

Roles of Fen-1

Fen-1 has roles in DNA replication, DNA repair, apoptosis, and in maintaining genome stability. In DNA replication Fen-1's main role is in Okazaki fragment maturation. In eukaryotic organisms, DNA synthesis is initiated with short RNA primers that must ultimately be removed. When the DNA replication proteins encounter a down stream Okazaki fragment, some of the

RNA primer is displaced forming a 5' flap, that must be removed for DNA replication to continue (Arezi and Kuchta, 2000). The basic thought is that Fen-1 is required to remove or degrade the RNA primers. Three models have been proposed for Okazaki fragment maturation and Fen-1 is a major player in all three models. In the first scenario, Fen-1 cooperates with RNase H to remove the RNA primer leaving a nick which is sealed by DNA ligase I (Turchi et al., 1994). However, it has been found that RNase H is not required for removal of the RNA primer (Qiu et al., 1999). Thus, a Fen-1 only pathway has been proposed. In this case, Fen-1 alone removes the entire RNA primer (Murante et al., 1995; Siegal et al., 1992; Turchi and Bambara, 1993). Lastly, a pathway involving another endonuclease, Dna2p, has been proposed. In this pathway Dna2p physically interacts with Fen-1 and removes the DNA portion of the RNA primer flap. Dna2p's activity is stimulated by replication protein A (RPA). However, RPA inhibits Fen-1 activity. Once cleavage by Dna2p is complete, RPA leaves, and a shorter flap remains that is cleaved by Fen-1 (Kang et al., 2000).

Fen-1's role in DNA repair has been discussed in the previous section on BER. Briefly, Fen-1 is required in LP-BER for removal of the flap formed from strand-displacement synthesis. Moreover, Fen-1 activity in LP-BER is regulated through its interaction with other BER proteins such as pol-β and PCNA (Gary et al., 1999; Prasad et al., 2000). Additionally, Fen-1 is involved in another mechanism of LP-BER termed the "Hit-and-Run" mechanism. In this mechanism, pol-β recognizes and binds to the nicked DNA generated by Fen-1 as a result of the removal of the flapped DNA. Then pol-β extends the 3'OH displacing the dRP-moiety and fills the 1-nt gap leaving a nick. Pol-β dissociates from the DNA and Fen-1 binds to the nicked DNA and removes 1-nt using its 5'-3' exonuclease activity. The removal of 1-nt creates a gap and Fen-1 dissociates

from the DNA. This cycle of LP-BER is repeated several times until DNA ligase seals the nick (Liu et al., 2005).

Interestingly, another role for Fen-1 in DNA repair has been discovered in yeast. Yeast Fen-1 was found to interact with Pol4 and Dnl4/Lif1, which are a part of the non-homologous end joining (NHEJ) double stranded break DNA repair pathway (Tseng and Tomkinson, 2004).

Mutations in Fen-1 have been found to increase genomic instability by causing chromosome loss and accumulation of toxic flaps that may then anneal forming bubbles in the DNA. Furthermore, it has been shown that abnormal expression of Fen-1 may cause defective BER and thus, accumulation of mutations that can lead to genomic instability (Liu et al., 2004; Shibata and Nakamura, 2002). Lastly, another way in which Fen-1 may contribute to genomic stability is in its role in preventing tri-nucleotide repeat expansion and contraction. Studies in yeast have shown that mutations in Fen-1 lead to an expansion of tri-nucloeotide repeats through flap misalignment suggesting that Fen-1 is involved in their prevention (Schweitzer and Livingston, 1998; White, 1999).

Post Translational Modifications of Fen-1

To date only two types of post translational modifications have been identified for Fen-1: acetylation and phosphorylation. Both of these modifications have an effect on Fen-1's endonuclease and exonuclease activities. Acetylation of Fen-1 is accomplished through complex formation with the transcriptional co-activator p300. Specifically, Lys 354, Lys 375, Lys 377, and Lys 380 are acetylated *in vitro* and *in vivo*. Acetylation of Fen-1 causes a reduction in the endonuclease and exonuclease activities of Fen-1 without affecting PCNA binding. Thus, acetylation may provide a means of regulating Fen-1 activity and as a result of Fen-1 down regulation, affecting DNA metabolic events (Hasan et al., 2001; Henneke et al., 2003).

Phosphorylation of Fen-1 also has an effect on its endonuclease and exonuclease activities. The phosphorylation of Fen-1 is mediated by cyclin dependent kinase (Cdk) 1-cyclin A complex. Phosphorylation occurs at Ser 187 which is located in the internal nuclease domain. In contrast to acetylation, phosphorylation abolishes PCNA binding, and thus PCNA-mediated stimulation of Fen-1 endonuclease and exonuclease activities. However, phopshorylation does not affect the DNA binding activity of Fen-1. The phosphorylation of Fen-1 by Cdk1-cyclin A suggests a critical role for phosphorylation as a cell cycle regulatory mechanism for Fen-1 activity (Henneke et al., 2003).

Purpose/Significance

As previously stated, APC was able to interact with two BER proteins pol-β and Fen-1 and interfere with pol-β-directed strand-displacement synthesis (Jaiswal et al., 2006; Narayan et al., 2005). Some cancer therapeutic drugs are DNA-alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and temozolomide (TMZ) which create lesions that can be repaired by the BER pathway. Through a more complete understanding of APC's blockage of BER it may be possible to improve the efficacy of these chemotherapeutic drugs. Another significance of these studies is in the basic science aspect of BER. As I discussed above, there are two types of BER pathways: SP- and LP-BER. How these two pathways are selected by cells is not very clear. There is some evidence suggesting that the type of DNA damage is responsible for this distinction. Our studies suggest that APC may play an additional role in distinguishing the SP- versus LP-BER pathway along with the type of DNA damage present in the cell. By determining where the interaction of APC with pol-β and Fen-1 is occurring, it will be possible to elucidate the mechanism that APC is using to block BER. This knowledge will shed more insights into

APC's involvement in BER and how to exploit this involvement to increase the efficacy of DNA alkylating agents used as chemotherapeutic drugs. This study will focus on determining the interaction sites of APC with pol-β and Fen-1 with particular emphasis on the APC and Fen-1 interaction and the mechanism by which APC is blocking BER through its interaction with Fen-1.

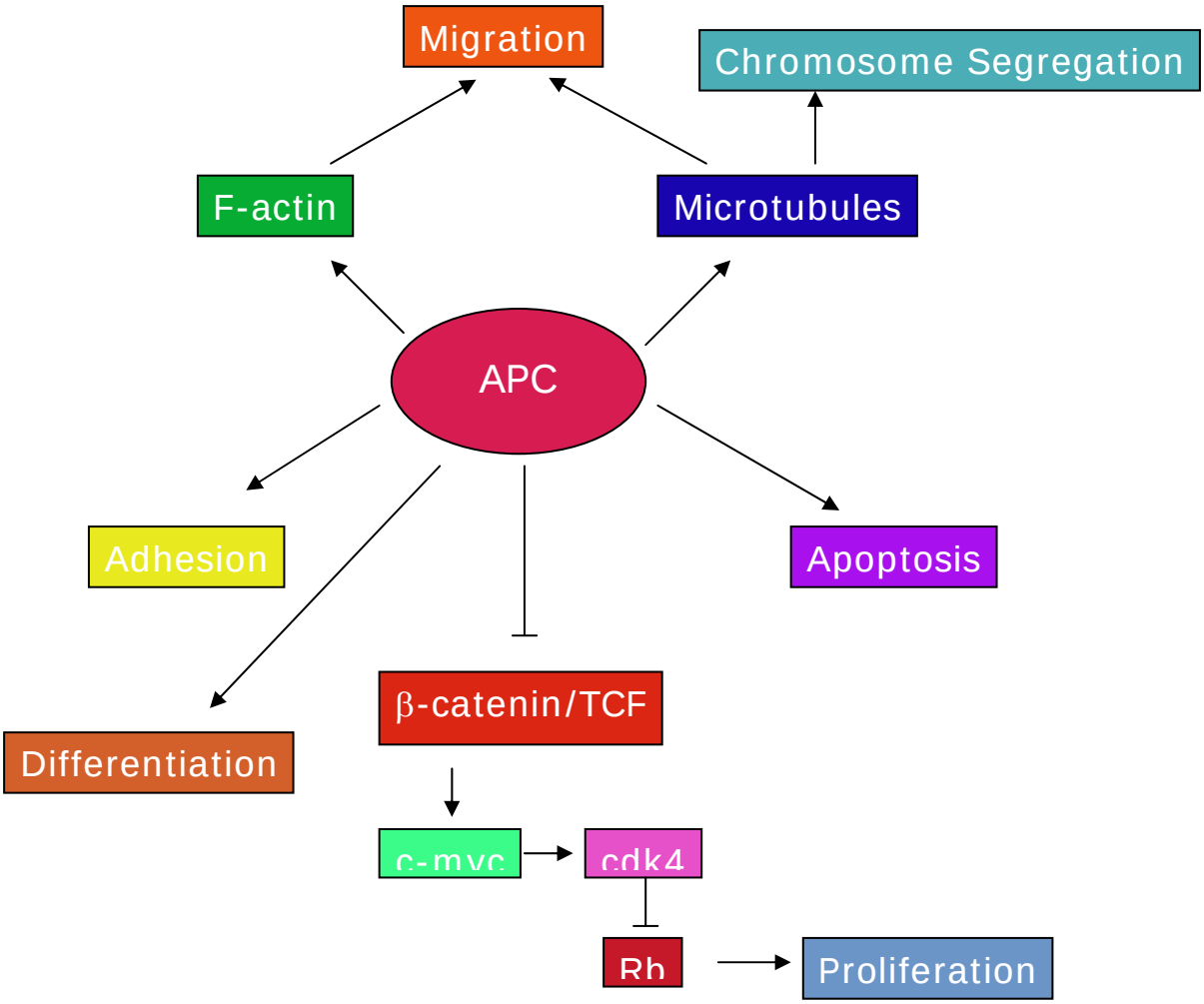


Figure 1-1. Roles of APC. Examples of the many cellular functions that APC has been implicated in are depicted in the above diagram.

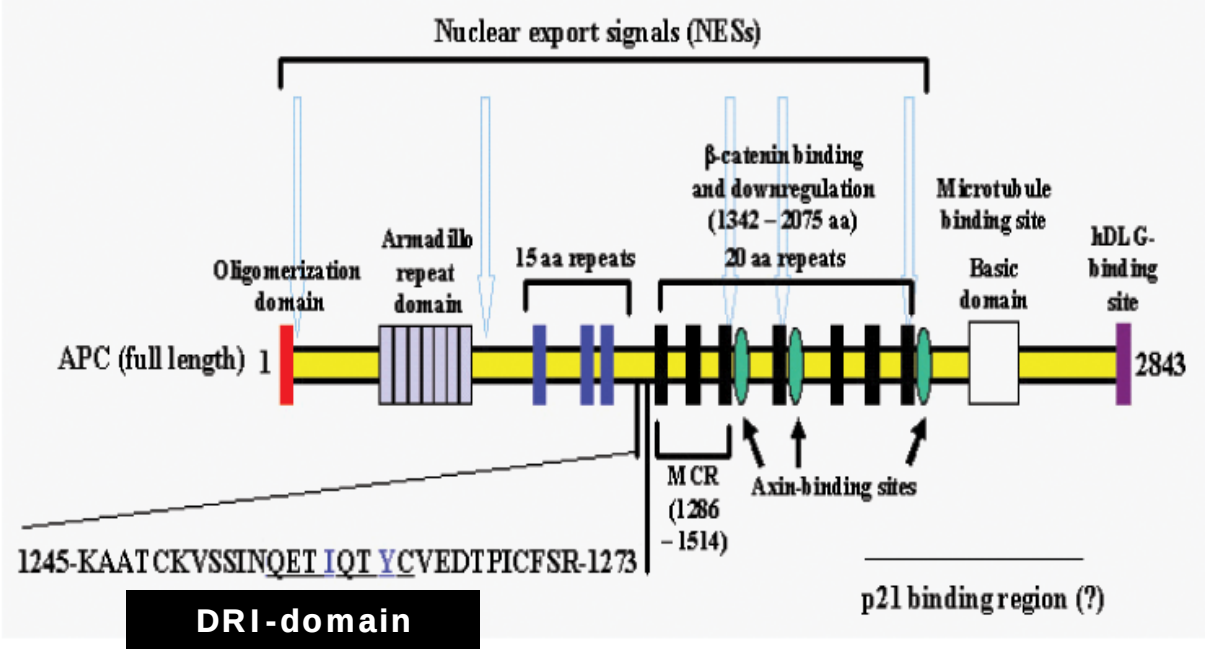


Figure 1-2. Structure of the APC protein. Structure of the APC protein showing its β-catenin binding and downregulation sites, its microtubule binding site, the mutation cluster region, and the DRI-domain.

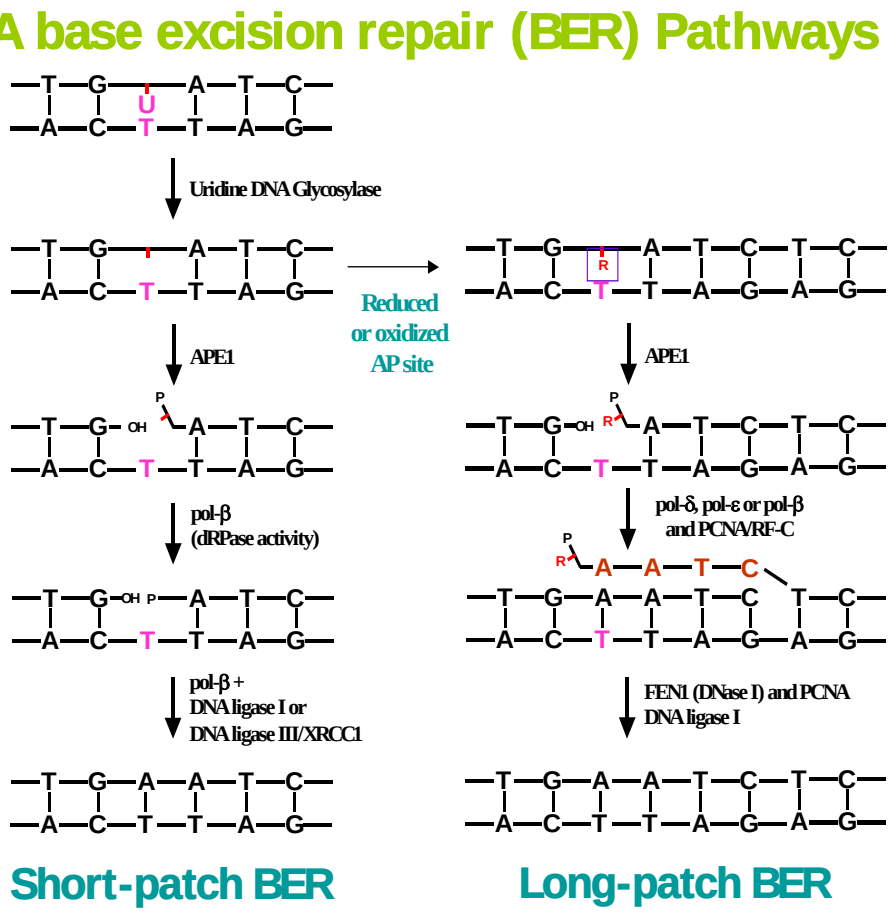


Figure 1-3. Base excision repair. BER repair has two main pathways- "Short-patch" BER and "Long-patch" BER.

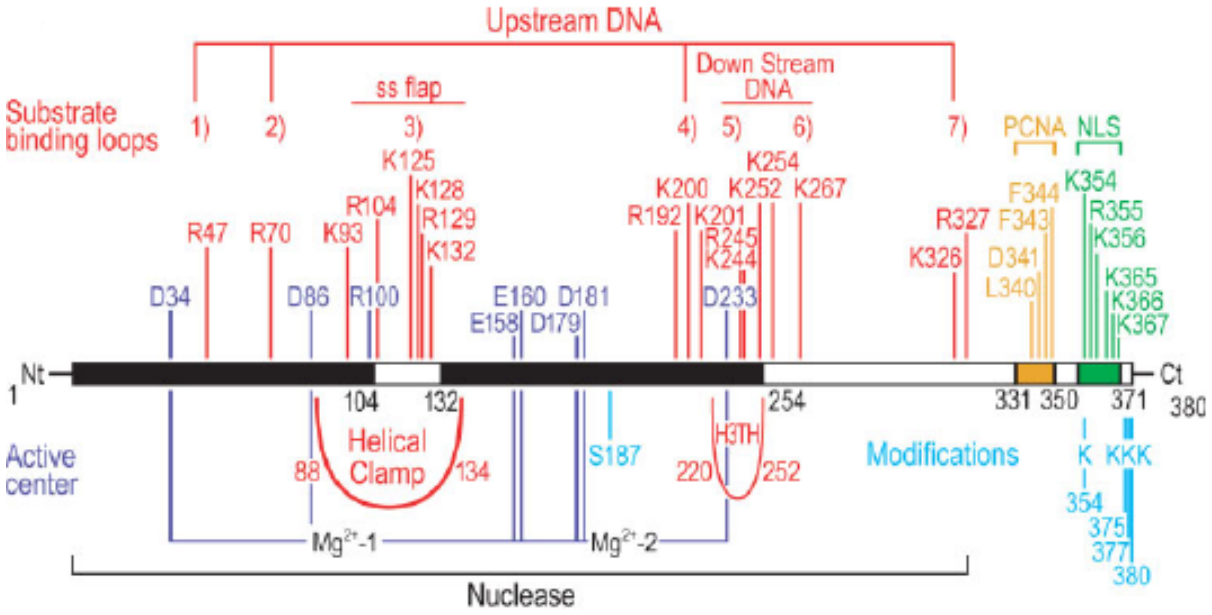


Figure 1-4. Structure of Fen-1. Diagram depicting important amino acids of Fen-1. Residues in dark blue show the amino acids involved in the active center of Fen-1. Residues shown in red are part of the seven loops important for substrate binding while residues shown in blue are important for post translational modifications. Amino acid residues shown in yellow are involved in the PCNA interactions while those in green are involved in nuclear localization of Fen-1. Taken with permission from Shen et al., 2005.

CHAPTER 2
MATERIALS AND METHODS

Yeast Cell Maintenance

The yeast strain PJ694-A was used for all yeast two-hybrid experiments. The yeast strain PJ69-4A (*MAT*a *trp1-901 leu2-3,112ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2*::*GAL1-HIS3 met2*::*GAL7-lacZ*) was grown on synthetic dropout medium lacking lysine [(0.17% Difco yeast nitrogen base without amino acids, ammonium sulfate (5.0 g/L), complete supplemental amino acid mixture minus appropriate amino acid containing 2% glucose)]. For long term storage the yeast cells were maintained in a glycerol stock composed of yeast peptone dextrose media (YPD) and 40% glycerol and stored at -80^{o}C.

Yeast Two-Hybrid Assays of the Interaction of APC with Pol-β and Fen-1

The yeast two-hybrid system was used to determine the functional interaction of APC with pol-β and Fen-1 *in vivo*. The APC DNA fragments containing the wild-type (amino acids 1190-1328) or the DRI-domain mutant (amino acids 1200-1324, in which amino acids Q1256, I1259, and Y1262 were replaced with alanine) for protein-protein interaction studies were fused to the yeast Gal4 DNA binding domain (BD) in plasmid pGBDU-C3. Single mutant APC cDNA fragments of the DRI-domain of APC(Q1256-A), APC(I1259-A), or APC(Y1262-A) were used to further characterize the pol-β and Fen-1-interactions and also fused to the yeast Gal4-BD in plasmid pGBDU-C3. The interacting proteins such as full length pol-β and Fen-1 were fused to the yeast Gal4 activation domain (AD) in plasmid pGADC3. Adapters were included as needed for the in-frame insertion of APC, pol-β, and Fen-1 DNA sequences relative to the Gal4-BD or Gal4-AD plasmids. The yeast strain *S.cerevisiae* PJ69-4A was co-transformed with pGBDU-C3

and pGAD-C3 derived plasmids and spread on plates containing yeast synthetic dropout (SD)-UL medium lacking only vector markers Ura for pGBDU-C3 derived plasmids and Leu for pGAD-C3 derived plasmids. To test for potential protein-protein interactions, transformed cells were screened for growth on yeast SD-ULH medium, which lacked Ura, Leu, and His, but contained 5 mM His3 inhibitor, 3-amino-1,2,4-triazole to prevent His3-reporter gene autoactivation.

Maintenance of Mammalian Cells

Human colon cancer cell lines HCT-116-APC(WT) (wild-type APC expression), HCT116 APC(KD) (knockdown APC expression by pSiRNA-APC) were grown in McCoy's 5a medium, and LoVo cells (truncated APC expression lacking DRI-domain) were grown in Ham's F12 medium at 37 °C under a humidified atmosphere of 5% CO2. In each case, the medium was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Generation of Pol-β Deletion Constructs

Plasmids were constructed to encode various segments of pol-β (amino acids 60-120, 80-170, 140-200, and 160-250) to identify the amino acids of pol-β that interact with the DNA repair inhibitory (DRI) domain of APC. These deletion fragments were subcloned into the pGAD-C3 vector between the *Pst*I and *Bam*HI restriction sites. The following primers were used to generate various pol-β deletion constructs: pol-β (60-120) (sense primer 5′CGCGGATCCAAGAAATTGCCTGGAGTA-3′ and antisense primer 5′ CCAATGCATTGGTTCTGCAGTTTAATTCCTTCATCTAC-3′), pol-β (80-170) (sense primer 5′-CGCGGATCCGGAAAATTACGTAAACTG-3′and antisense primer 5′-CCAATGCATTGGTTCTGCAGATCCACTTTTTTAACTT-3′), pol-β (140-200) (sense primer

5′-CGCGGATCCCTGAAATATTTTGGGGAC-3′ and antisense primer 5′-CCAATGCATTGGTTCTGCAGGAAGCTGGGATGGGTCAG-3′), and pol-β (160-250) (sense primer 5′-CGCGGATCCGATATTGTTCTAAATGAA-3′ and antisense primer 5′-CCAATGCATTGGTTCTGCAGATATTCTTTTTCATCATT-3′).

Site Directed Mutagenesis of Pol-β

Two different sets of pol-β mutants, Set-1 mutant (T79A/K81A/R83A) and Set-2 mutant (R89A/Q90A/D92A), were generated by use of the Quick Change site-directed mutagenesis kit from Stratagene (La Jolla, CA). The following primer pairs were used for Set-1 and Set-2 mutants: Set-1, sense primer 5′GAAAAGATTGATGAGTTTTTAGCAGCCGGAGCGTTAGCTAAACTGGAAAAGATTCGGCAG-3′ and antisense primer 5′CTGCCGAATCTTTTCCAGTTTAGCTAACGCTCCGGCTGCTAAAAACTCATCAATCTTTTC-3′; Set-2, sense primer 5′GGAAAATTACGTAAACTGGAAAAGATTGCCGCGGATGCTACGAGTTCATCCATCAATTTCCTG-3′ and antisense primer 5′CAGGAAATTGATGGATGAACTCGTAGCATCCGCGGCAATCTTTTCCAGTTTACGTAATTTTCC-3’

Yeast Two-Hybrid Assay of Pol-β Deletion Constructs, Pol-β Mutants, and APC

The yeast two-hybrid assay was employed to identify critical amino acids of pol-β to define the functional interaction with APC *in vivo*. APC cDNA fragments containing the wild-type (residues 1190-1328) or the mutant DRI domain (residues 1200-1324, in which amino acids Ile1259 and Tyr1262 were replaced with alanine) were fused to the yeast Gal4 DNA-binding domain (BD) in plasmid pGBDU-C3. The interacting pol-β protein fragments such as full-length

and various segments (residues 60-120, 80-170, 140-200, and 160-250) were fused to the yeast Gal4 activation domain (AD) in plasmid pGAD-C3. Set-1 (T79A/K81A/R83A) and Set-2 (R89A/Q90A/D92A) pol-β mutants also were cloned into plasmid pGAD-C3. Yeast two-hybrid analysis was performed as described earlier.

APC Peptides

An APC peptide, 20-amino acids in length, (1250-KVSSINQET***I***QT***Y***CVEDTPI-1269) was synthesized at the Protein Chemistry and Biomarkers core facility at the Interdisciplinary Center for Biotechnology Research (ICBR) of the University of Florida. They represent the DRI-domain of the wild-type APC (APCwt) or a mutated form of the APC, APC(Q-A,I-A,Y-A), or APC(I-A,Y-A) in which amino acids Q1256, I1259, and Y1262 were replaced with alanine (A) (1250-KVSSIN***A***ET***A***QT***A***CVEDTPI-1269 or 1250-KVSSINQET***A***QT***A***CVEDTPI-1269).

Synthesis and Labeling of the Fen-1 Substrate

The Fen-1 substrates for 5'-flap endonuclease activity were made by annealing an upstream 23-mer (5'-CTAGATGCCTGCAGCTGATGCGC-3') and a downstream 45-mer or (5'-FTTTTTGTACGGATCCACGTGTACGGTACCGAGGGCGGGTCGACA-3') or 51-mer (5'-AACATTFTTTTTGTACGGATCCACGTGTACGGTACCGAGGGCGGGTCGACA-3') oligonucleotide to a 63-mer complementary template (5' AGATGCCTGCAGCTGATGCG CGGTACGGATCCACGTGTACGGTACCGAGGGCGGGTCGACA-3'). The 45-mer downstream oligonucleotide has a flap of 5-nts while the 51-mer has a flap of 11-nts. Both flaps are cleaved by Fen-1. The Fen-1 substrate for 5'-3'exonuclease activity was made similarly except that the 5-nt flap from the 45-mer oligonucleotides was removed. After annealing with a 63-mer complementary template as described above, these oligonucleotides created a nick in the DNA suitable for Fen-1 exonuclease activity. The 45-, 51- and 40-mer downstream oligonucleotides were radiolabeled at the 5'-end with [γ-32P]ATP and T4 polynucleotide kinase

(New England Bio Lab, Woburn, MA). The labeled probe was purified by using a nick column (GE Healthcare, Piscataway, NJ). All three oligonucleotides were annealed at a molar ratio of 1:1:1.

Fen-1 Activity *in vitro*

The assays for 5'-flap endonuclease and 5'-3' exonuclease activities for Fen-1 were performed in a final volume of 20 μl. The reaction mixture contained 30 mM Hepes at pH 7.5, 30mM KCl, 8 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 200μg/mL bovine serum albumin (BSA), and indicated amounts of Fen-1 and APCwt or DRI-domain mutant APC [APC (I-A, Y-A)] peptides. After addition of the APC peptides, the reaction mixture was incubated at room temperature for 5 min. Then 2.5 nM ^{32}P-labeled flapped-DNA or nicked-DNA substrates were added to the mixture and further incubated at 37°C for 30 min. Reactions were terminated with stop solution containing 0.4% (w/v) SDS and 5 mM EDTA. The DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation. The 5-nt or 11-nt DNA products from the 5'-flap endonuclease activity and 1-nt product from the 5'-3' exonuclease activity were separated on a 15% acrylamide and 7 M urea gel and quantitated by electronic autoradiography (InstantImager; Packard Instrument Co.,Meriden, CT). Statistical analysis (n=3) was performed using a t-test on the Sigma Plot computer software.

PCR of Fen-1 Protein Fragments for use in *in vitro* Transcription/Translation

To identify the APC-binding site on Fen-1, different Fen-1 cDNA constructs were made, Fen-1 protein fragments were expressed using the *in vitro* transcription/translation system and then the interaction of APC and Fen-1 was analyzed by Far-Western Analysis. Primers for each protein fragment were designed to include a T7 promoter and a kozak sequence in the sense primer and a stop codon and poly A tail in the anti-sense primer. Five fragments of Fen-1 were

designed (amino acids 1-115, 78-192, 154-268, 231-345, and 269-380). The following primers were used to generate the protein fragments: Fragment 1-115: (sense primer: 5’ TAATTTTAATACGACTCACTATAGGGAACAGCCACCATGGGAATTCAAGGCCTGGCC 3’ and anti-sense primer: 5’TTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTGAGCCTGCTGCAGCTGCTTCTCTGCCT C3’), Fragment 78-192: (sense primer: 5’TAATTTTAATACGACTCACTATGGGAACAGCCACCATGGGCATCAAGCCCGTGTAT 3’ and anti-sense primer: 5’TTTTTTTTTTTTTTTTTTTTTTTTTTTTTATCGCATTAGCACAGGGCTGCCGAACCT 3’), Fragment 154-268: (sense primer: 5’TAATTTTAATACGACTCACTATAGGGAACAGCCACCATGGATGCACCCAGTGAGG CA3’ and anti-sense primer: 5’TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAGGGTACTTGTTGGGGTCAAGTCGCA C3’), Fragment 231-345: (sense primer: 5’ TAATTTTAATACGACTCACTATAGGGAACAGCCACCATGGGCAGTGACTACTGTGAG AGT3’ and anti-sense primer: 5’ TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTTGAAGAAATCATCCAGGCGGCCCTG GGT3’), and Fragment 269-380: (sense primer: 5’ TAATTTTAATACGACTCACTATAGGGAACAGCCACCATGGTGCCAGAAAATTGGCTC CAC3’ and anti-sense primer: 5’ TTTTTTTTTTTTTTTTTTTTTTATTTTCCCCTTTTAAACTTCCCTGCTGCCCCAGTCTTT GC3’).

The full length protein was synthesized using the sense primer from 1-115 and the anti-sense primer from 269-380. The PCR of these fragments was carried out using Herculase, a combination of Pfu DNA polymerase and Taq polymerase, according to the manufacturer's recommendations (Stratagene, La Jolla, CA).

Site-Directed Mutagenesis of Fen-1

Two sets of Fen-1 mutants (set 1: K354A/R355A/K356A and set2: K365A/K366A/K367A) were generated using the Quick Change site directed mutagenesis kit (Stratagene, La Jolla, CA). To generate each mutant two pairs of primers were used. One pair was used first to generate two amino acid mutations. Then the resultant mutant protein was used as template and other primer pair was used to generate the last amino acid mutation. The following primer pairs were used to generate the set 1 and set 2 mutants: Set-1 primer pair 1, sense primer: 5' GGCTCACTCTCTTCAGCTGCGCGCGCAGAGCCAGAACCCAAGGGATCC3' and anti-sense primer: 5' GGATCCCTTGGGTTCTGGCTCTGCGCGCGCAGCTGAAGAGAGTGAGCC3' and Set-1 primer pair 2, sense primer: 5' GGCTCACTCTCTTCAGCTGCGGCCGCAGAGCCAGAACCCAAGGGATCC3' and anti-sense primer: 5' GGATCCCTTGGGTTCTGGCTCTGCGGCCGCAGCTGAAGAGAGTGAGCC3';
Set-2 primer pair 1 , sense primer: 5' GAACCCAAGGGATCCACTGCGAAGGCCGCAAAGACTGGGGCAGCA3' and anti-sense primer: 5'TGCTGCCCCAGTCTTTGCGGCCTTCGCAGTGGATCCCTTGGGTTC3'; Set-2 primer pair 2, sense primer: 5'

GAACCCAAGGGATCCACTGCGGCAGCCGCAAAGACTGGGGCAGCA3' and anti-sense primer 5' TGCTGCCCCAGTCTTTGCGGCTGCCGCAGTGGATCCCTTGGGTTC3'.

Synthesis of Fen-1 Protein Fragments, Fen-1 wt, and Fen-1 Mutant Proteins Using *in vitro* Transcription/Translation

Full-length Fen-1, Fen-1 protein fragments, and Fen-1 set-1 and set-2 mutant proteins were synthesized using the TNT PCR Quick Kit from Promega (Madison, WI). PCR product was added directly to the rabbit reticulocyte mix along with the methionine provided with the kit and 50μM MG132. The reaction was run as per instructions provided with the TNT PCR Quick Kit from Promega. Synthesis of Fen-1 proteins and Fen-1 protein fragments was determined by 15% SDS-PAGE analysis followed by western blotting. Fen-1 proteins and protein fragments were detected using an anti-mouse Fen-1 antibody (Novus Biologicals, Littleton, CO) that was able to detect amino acids 1-380 of Fen-1. The signals were detected using the chemiluminescence technique (GE Healthcare, Piscatway, NJ).

Far-Western Blot Analysis

APCwt and APC(I-A,Y-A) peptides were slot-blotted (0-15μg) onto a polyvinylidene difluoride membrane (GE Healthcare, Piscatway, NJ) in a binding buffer containing 20 mM Tris-Cl at pH 7.4, 100 mM phosphate buffer at pH7.4, 60 mM KCl, and 0.25% (v/v) Nonidet P-40. After blotting, the membrane was blocked with 5% (w/v) bovine serum albumin and washed three times with Tris-buffered saline with 0.025% (v/v) Tween-20 prior to incubation with *in vitro* transcription/translation synthesized Fen-1 protein fragments (described above), and wild type, K354A/K355A/K366A mutant or K365A/K366A/K367A mutant human Fen-1 proteins. Binding was detected using mouse anti-Fen-1 antibody (Novus Biologicals, Littleton, CO). The signals were detected using the enhanced chemiluminescence technique (GE Healthcare, Piscatway, NJ).

Pull-Down of Fen-1 wild type and FenS-1 K365A/K366A/K367A from TNT Synthesis Reaction Mixture

Fen-1 wild-type (Fen-1wt) and Fen-1 K365A/K366A/K367A mutant (Fen-1 mutant) were pulled-down by incubating them with rabbit anti-human Fen-1 antibody in immuno-precipitation (IP) buffer (30 mM Hepes at pH 7.5, 30 mM KCl, 8 mM $MgCl_2$, 0.01% NP-40, 2% Glycerol) for 4 hours followed by Protein A sepharose beads (Promega, Madison, WI), that were blocked with 5% BSA (v/v), for an additional 2-3 h. Beads were washed with washing buffer (30 mM Hepes at pH 7.5, 30 mM KCl, 5% glycerol) 3 times and then beads were resuspended in reaction buffer and used in Fen-1 activity assays. The beads were then used in the indicated amount for the Fen-1 activity assays.

Immunohistochemistry

Human colon cancer cell lines LoVo, HCT-116-APC(WT), and stable knockdown HCT-116-APC(KD) were plated into 6-well plates containing sterilized cover slips. Each well contained about 80-100,000 cells. Cells were allowed to grow for 48 h and were then treated with 500 μM MMS for 24 h and fixed using 4% Paraformaldehyde in phosphate-buffered saline (PBS). For time course experiments, cells were treated with 500 μM methylmethane sulfonate (MMS) for 5, 15, or 24 h. At the appropriate time point cells were fixed using 4% Paraformaldehyde in PBS. Once fixed, cells were quenched using 20 mM ammonium chloride in PBS. Fixed cells were then incubated in mouse anti Fen-1 antibody (Abcam, Cambridge, MA) and rabbit anti APC antibody (Santa Cruz, Santa Cruz, CA) for 2hrs. The fixed cells were then washed four times for 5 min. in PBS and were subsequently incubated in anti mouse Alexa Flour 488 and anti-rabbit Alexa Flour 595 antibodies (Invitrogen, Carlsbad, CA) for 1 h. Cells were washed as previously described and were mounted onto slides using Vecta Shield (Vector

Laboratories, Burlingame, CA). Slides were viewed under a Zeiss Upright Microscope at wavelength 500 nm for Fen-1 staining and 640 nm for APC staining.

Western Blot Analysis of Fen-1 and APC Protein Levels in the Cytoplasm and the Nucleus in Cells Treated with DNA-Alkylating Agents

HCT-116-APC(WT) and HCT-116-APC(KD) cells were treated with 500 μM methylmethane sulfonate (MMS) for 5, 10, 15 or 25 h. Nuclear and cytosolic fractions from MMS-treated HCT-116-APC(WT) and HCT-116-APC(KD) cells were prepared as described in (Shapiro et al., 1988). In order to determine Fen-1 levels in the nucleus and the cytoplasm, 15 μg of the nuclear and cytosolic fractions were analyzed on a 10% SDS-PAGE followed by Western blotting. A mouse anti-Fen-1 antibody (Novus Biologicals, Littleton, CO) was used to detect the presence of Fen-1 in the nuclear and cytosolic fractions. The signals were then detected using the chemiluminescence technique (GE Healthcare, Piscataway, NJ). In order to determine APC levels in the nucleus and the cytoplasm, 50 μg of the nuclear fractions and 80 μg of the cytosolic fractions were analyzed on a 4% SDS-PAGE followed by Western blotting. The presence of APC was detected using a mouse anti-APC antibody (Millipore, Temecula, CA). The signals were then detected using the chemiluminescence technique (GE Healthcare, Piscataway, NJ).

CHAPTER 3
APC INTERACTS WITH DNA POLYMERASE BETA

Amino acids Thr79/Lys81/Arg83 of pol-β are Critical for its Interaction with APC

Previous studies have shown that APC and pol-β interact *in vivo* and that this interaction blocks pol-β-directed strand-displacement synthesis (Narayan et al., 2005). In order to map the critical amino acids of pol-β necessary for its interaction with APC, deletion constructs of pol-β were generated through PCR amplification and cloned into the pGAD-C3 yeast two-hybrid (Y2H) vector. A total of four deletion constructs were made (Figure 3-1A). These constructs were then used in a Y2H assay along with APC wild-type protein (APCwt). As seen in Figure 3-1B, the deletion constructs containing amino acids 60-120 and 80-170 were able to interact with APCwt while with the APC DRI-domain mutant [APC(I-A, Y-A)] construct there was no interaction. Known interactions of wild-type APC and pol-β, PCNA and pol-β were used as positive controls, while the pol-β-activating domain was used as a negative control for background colonies. Screening for interacting proteins was performed as described in the Materials and Methods.

In order to further define the important amino acids from the interacting region of amino acids 80-120, we examined solvent surface accessibility of the residues implicated in the interaction with APC. Since a complete structure of APC does not exist, it was not possible to identify possible interactions through docking modes. Interestingly, amino acids 80-120 are located in a region that connects the 8 kDa lyase domain and the 31 kDa polymerase domain of pol-β. From the structure of the ternary substrate complex two regions set-1 (amino acids Thr79, Lys81, and Arg83) and set-2 (amino acids Arg89, Gln90, and Asp92) were identified that exhibited high solvent accessibility (Fig. 3-2).

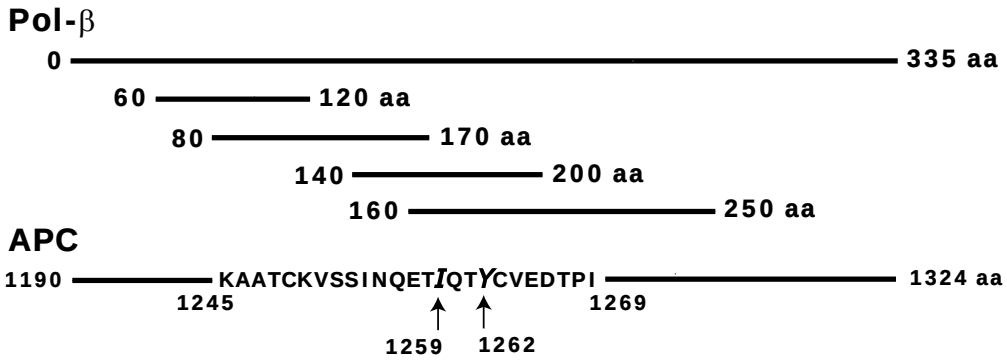
Site-directed mutagenesis was used to change each set of amino acids to alanine and generate two pol-β mutants. One mutant contains the amino acids of set-1 mutated to alanine and the other contains the amino acids of set-2 mutated to alanine. The two pol-β mutants were then cloned into the Y2H vector pGAD-C3 and a Y2H analysis with APCwt was used to determine if either set of mutants was able to disrupt the interaction between pol-β and APC. As seen in Figure 3-3, the pol-β set-1 mutant was able to disrupt the interaction while the pol-β set-2 mutant was unable to interrupt the interaction. This indicates that amino acids Thr79, Lys81, and Arg83 are critical for the interaction of pol-β with APC.

Amino Acids I1259A and Y1262A, but not Q1256A are Required for APC's Interaction with DNA Polymerase β (Pol-β)

It was originally thought that APC contained a PCNA interacting protein (PIP)-like box composed of Q1256, I1259, and Y1262 and all three amino acids were necessary for the interaction of APC with pol-β. In order to test this possibility, site-directed mutagenesis of the APCwt fragment used previously (Narayan et al., 2005) was carried out. Single mutation of each amino acid to alanine was carried out and each mutant's ability to disrupt the interaction of APC with the proteins was analyzed via Y2H assay (Fig. 3-4). After each single mutant was constructed, the peptide was cloned into the Y2H vector pGBDU-C3. Full length pol-β was already in the Y2H pGAD-C3 vector. Screening for interacting proteins was conducted in the same manner as described in the Materials and Methods. The effects that the single APC mutants Q1256A, I1259A, and Y1262A had on the ability of APC to interact with pol-β can be seen in Figure 3-3B. The single mutant Q1256A cannot impede the interaction between pol-β and APC. However, the single mutants I1259A and Y1262A can disrupt the ability of APC to interact with pol-β indicating that Y1259A and Y1262A are equally important in the ability of APC to interact

with pol-β. These findings indicate that the PIP-like box found in APC is actually not a PIP-like box. In order to be a PIP-like box all three amino acids involved must have been involved in the interaction. Clearly, this is not the case for APC. The PIP-like box was therefore renamed the DNA Repair Inhibitory (DRI)-domain after much consideration. Known interactions of wild-type APC and pol-β, PCNA and pol-β, and Daxx and p53 were used as positive controls, while the APC binding domain was used as a negative control for background colonies.

A. Deletion constructs of Pol-β



B. Yeast two-hybrid analysis

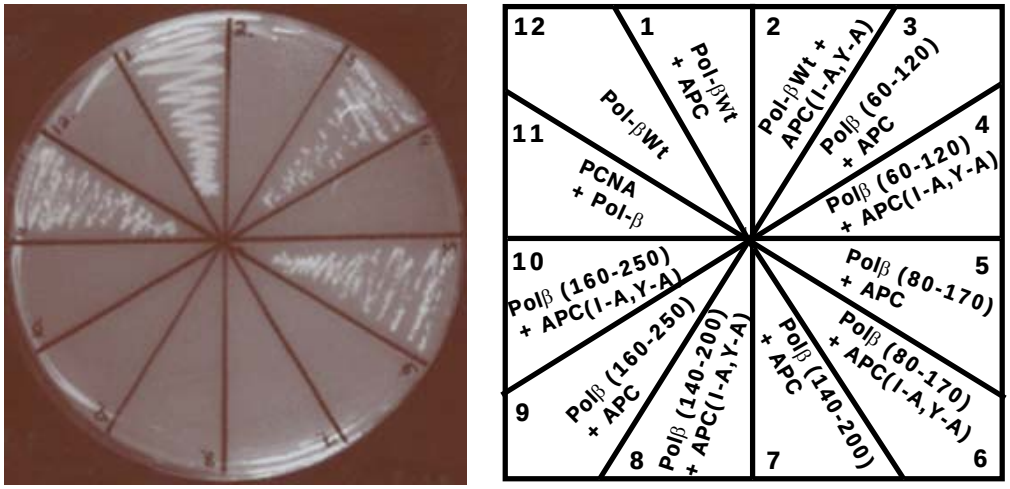


Figure 3-1. Interaction of APC with pol-β-deletion constructs. A) Deletion constructs of pol-β B) Y2H assay of pol-β-deletion constructs and APC; interactions used are depicted in the diagram. Taken with permission from Balusu et al., 2007.

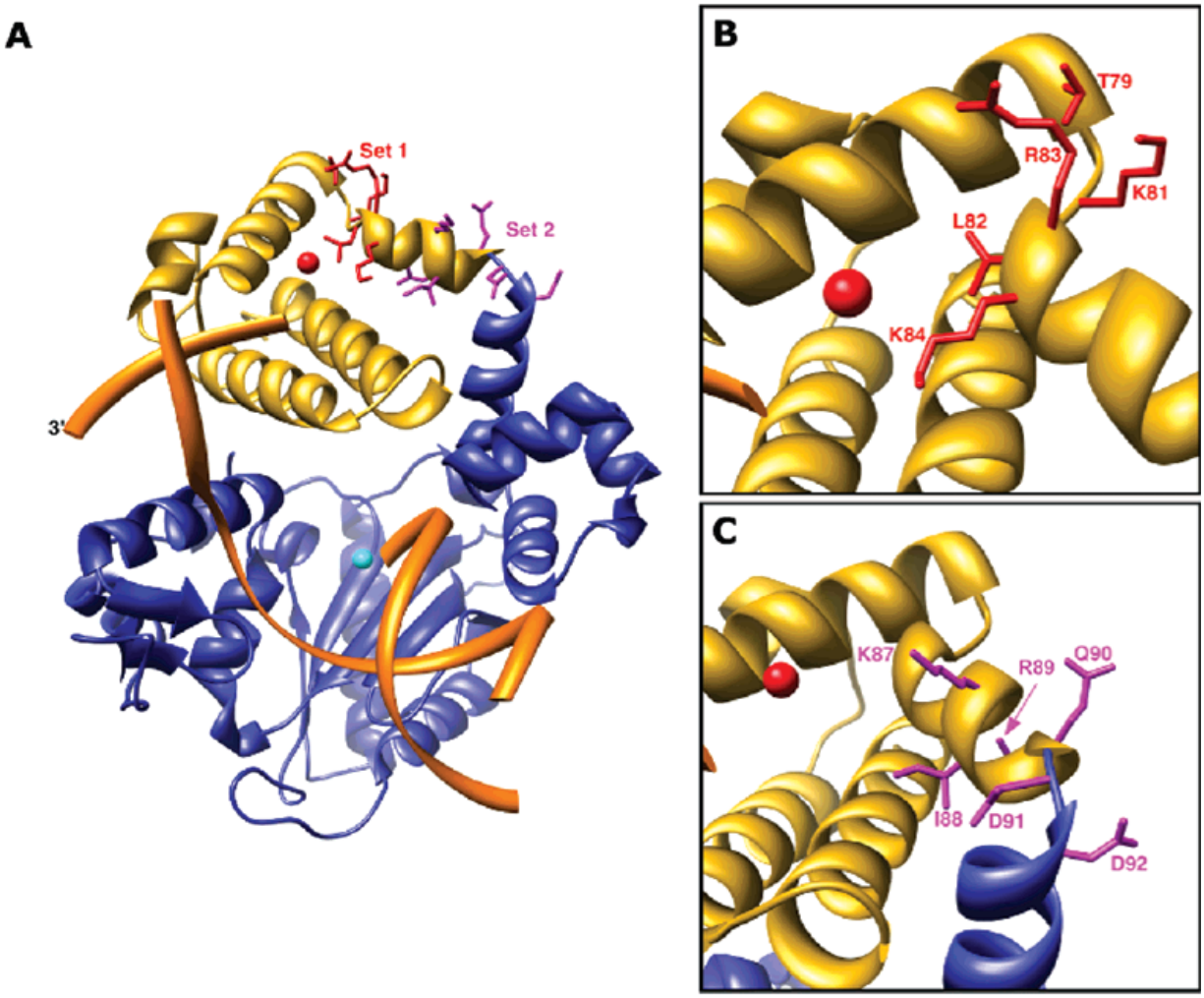
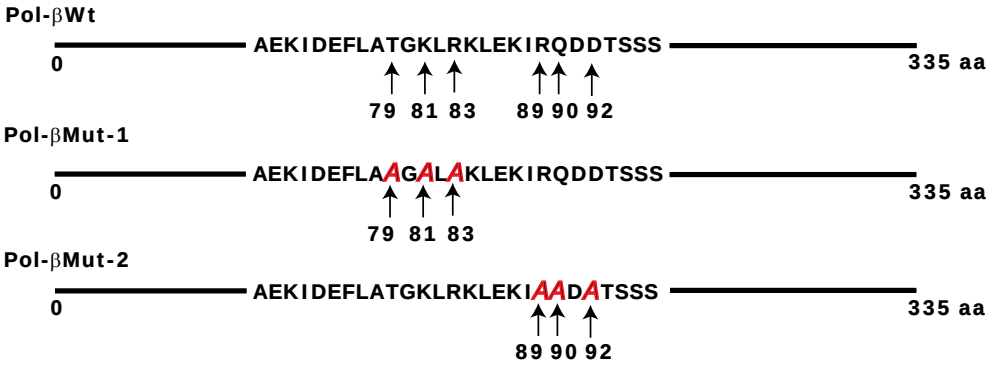


Figure 3-2. Ribbon representation of pol-β highlighting the position of key mutants. A) Set 1 (red) and Set 2 (purple) residues are displayed of a ternary substrate complex of pol-β. Lyase domain is shown in gold and the polymerase domain is shown in blue (PDB accession code 2MFS). The DNA backbone is shown in orange while a light blue sphere (Mg2+) identifies the polymerase active site and a red sphere (NZ of lyase 72) identifies the lyase active site. B) Set 1 (residues 79-84) side chains. C) Set 2 (residues 87-92) side chains. Taken with permission from Balusu et al., 2007.

A. Structure of peptides



B. Yeast two-hybrid analysis

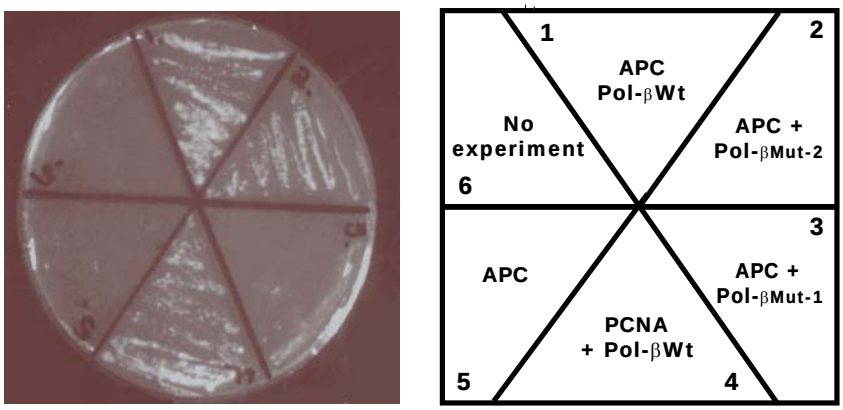


Figure 3-3. Interaction of APC with pol-β mutants. A) Structure of the peptides used in the Y2H assay. B) Y2H assay of the two pol-β mutants and APC; interactions used are depicted in the diagram. Adapted with permission from Balusu et al., 2007.

A. Structure of the APC peptides

APCwt(1190-1328)	1250-KVSSIN*Q*ET*I*QT*Y*CVEDTPI-1269
APC(Q-A,I-A,Y-A)	KVSSIN*A*ET*A*QT*A*CVEDTPI
APC(Q-1256A)	KVSSIN*A*ET*I*QT*Y*CVEDTPI
APC(I-1259A)	KVSSIN*Q*ET*A*QT*Y*CVEDTPI
APC(Y1262A)	KVSSIN*Q*ET*I*QT*A*CVEDTPI

B. APC/pol-β interaction

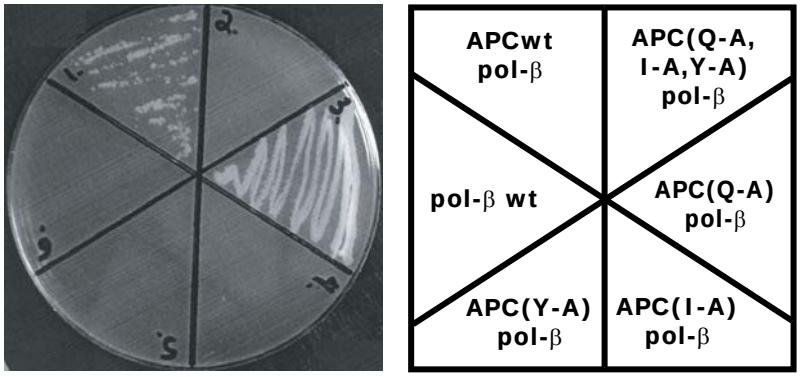


Figure 3-4. Interaction of APC mutants with pol-β. A) Structure of the peptides used in this assay. B) Y2H assay of APC mutant proteins with pol-β, interactions used shown in the diagram. Adapted with permission from Jaiswal et al., 2006.

CHAPTER 4
APC INTERACTS WITH FLAP ENDONUCLEASE 1 (FEN-1)

APC and Fen-1 interact *in vivo*

APC and Fen-1 were shown to physically interact by immuno-precipitation and Far-western analysis (Jaiswal et al., 2006). However, this needed to be confirmed *in vivo*. In order to accomplish this, a Y2H assay was used. Full length Fen-1 that was already cloned into the Y2H vector pGAD-C3, APCwt fragment which contains the DRI-domain (amino acids 1190-1328), and APC mutant (mut) fragment which contains a mutated DRI-domain (amino acids 1200-1324 with 1259I-A, and 1262Y-A mutations) that was already cloned into the Y2H vector pGBDU-C3 were used to run the Y2H assay. As Figure 4-1 clearly shows, APCwt is able to interact with Fen-1 *in vivo*. The known interaction of PCNA and pol-β was used as a positive control, while the APC binding domain was used as a negative control to check for background colonies. Lys^-, Leu^-, Ura^-, His-selection media was used to screen for interacting colonies. In order to grow, the yeast cells needed to make histidine and the only available method to do so is for the two proteins to interact.

The Interaction Site of APC is the Same for Fen-1 and Pol-β

Once the interaction site of APC and pol-β was determined, it was thought that the same amino acids that are necessary for APC's interaction with pol-β may be important for APC's interaction with Fen-1 since APC interacts with Fen-1 at the DRI-domain. In order to determine this, a Y2H analysis was carried out with Fen-1 and the single mutants of APC that were previously used in the yeast two-hybrid analysis with pol-β. It was found that the single mutants I1259A and Y1262A of APC were able to disrupt the interaction of APC and Fen-1.

However, the Q1256A single mutant of APC did not have an effect on the interaction of APC and Fen-1 (Fig. 4-2). These results are identical to that of the interaction site of APC and pol-β indicating that APC interacts with these BER proteins at the same site.

APC Blocks the Endonuclease and Exonuclease Activities of Fen-1

Since APC was found to interact with Fen-1 *in vivo* and *in vitro,* the effect of this interaction on the catalytic activities of Fen-1 was measured. Fen-1 contains two types of cleavage activity that are relevant for completion of BER: 5' endonuclease activity and 5'-3'exonuclease activity. The 5' endonuclease activity is important in strand-displacement during LP-BER where it removes the flap, while the 5'-3' exonuclease activity is important for "Hit-and-Run" synthesis of LP-BER. In hit-and-run synthesis, Fen-1 excises a nucleotide at the 3'-side of a nick to create a gap to be filled by pol-β (Klungland and Lindahl, 1997; Prasad et al., 2001; Qiu et al., 2001; Shibata and Nakamura, 2002). In order to test the effect of APC on Fen-1 cleavage activity, three *in vitro* assays were run: two which used substrates specific for the 5' endonuclease activity of Fen-1 and one that used a substrate specific for the 5'-3' exonuclease activity of Fen-1.

To test APC's effect on Fen-1 5' endonuclease activity, an *in vitro* 5' flap endonuclease assay in the presence or absence of APC peptide (20 amino acids long with DRI-domain in the middle) using a ^{32}P-labeled flapped substrate was conducted. As can be seen in Figures 4-3 and 4-4, cleavage of the substrate by Fen-1 results in a 5 or 11 nt product. When the APCwt peptide is added cleavage of the flap by Fen-1 is blocked in a dose-dependent manner (compare lane 2 with lanes 3-7 in both Fig. 4-3A and Fig. 4-4A, respectively).

However, when the APC(I-A,Y-A) mutant peptide is used cleavage of the flap by Fen-1 appears to be unaffected (compare lane 2 with lanes 8-12 in both Fig. 4-3A and Fig. 4-4A, respectively). A quantitative analysis of the data is shown in Figure 4-3B and Figure 4-4B.

In order to determine the effect of APC on Fen-1 5'-3- exonuclease activity, a nicked DNA substrate was used. The nicked DNA substrate was formed in the following manner: a 23-mer upstream and 40-mer downstream oligonucleotides were annealed with a 63-mer complimentary template oligonucleotide as described in the materials and methods section. The 40-mer oligonucleotide was labeled with γ- ^{32}P[ATP] before annealing at the 5'end. The 5'-3' exonuclease activity of Fen-1 will cleave the nick resulting in a 1-nt product. The results show a 1-nt cleavage product when Fen-1 is added to the substrate. However, in the presence of APCwt peptide cleavage of the nicked substrate by Fen-1 is blocked in a dose-dependent manner (Fig. 4-5A compare lane 2 with lanes 3-6). The 5'-3' exonuclease activity of Fen-1 appears to be unaffected in the presence of the APC(I-A,Y-A) mutant peptide (Fig. 4-5A compare lane 2 with lanes 7-10). A quantitative analysis of the data is shown in Figure 4-5B.

Amino Acids K365/K366/K367 are Important for the Interaction of Fen-1 with APC

In order to determine which amino acids of Fen-1 were important for its interaction with APC, five different protein fragments of Fen-1 were synthesized using the transcription coupled translation (TNT) Quick PCR kit from Promega (Madison, WI). The five protein fragments are depicted in Figure 4-6A. Synthesis of the protein fragments was determined via Western blot analysis. As seen in Figure 4-6B, all protein fragments as well as Fen-1wt were detected. Equal amounts of proteins were estimated by analyzing the densities of the bands from the Western blot. A non-specific band was seen with all expressed Fen-1 fragments, which was originating due to the cross-reactivity of anti-Fen-1-antibody with TNT extract.

Far-western analysis was then performed using the Fen-1 protein fragments and APCwt and APC(I-A,Y-A) mutant peptides to determine where the interaction of Fen-1 with APC was occurring. The only Fen-1 protein fragment able to interact with the APCwt peptide was the Fen-1 protein fragment which contained amino acids 269-380 indicating that this region of Fen-1 might be responsible for the interaction with APC (Fig. 4-7). The overlapping Fen-1 protein fragment which contained amino acids 231-345 was not able to interact with the APCwt peptide indicating that the region of Fen-1 that interacts with APC is between amino acids 346-380.

The interacting site was further narrowed down by analyzing the role of amino acids 346-380 in the structure and function of Fen-1, and by reviewing the literature for other proteins that interact with Fen-1 in this region. The most striking thing about amino acids 346-380 was that most of the amino acids are a part of the nuclear localization signal of Fen-1 (Shen et al., 2005). Furthermore, several proteins have been found to interact with Fen-1 at the C-terminus. Interestingly, three of the proteins were found to interact with Fen-1 between amino acids 353-380 which overlaps with the region of Fen-1 determined to interact with APC from the Far-western analysis (Guo et al., 2008; Sharma et al., 2005). Based on the above information two sets of Fen-1 amino acids (K354/R355/K356 and K365/K366/K367) were chosen to mutate to see if they were important for the interaction of Fen-1 with APC. Each set was chosen based on its importance in nuclear localization and on the fact that the amino acids chosen were in the range identified for the interaction of Fen-1 with other proteins such as Werner protein (WRN), Bloom's syndrome protein (BLM), and endonuclease G (EndoG). Each amino acid was mutated to alanine and the two Fen-1 mutants were synthesized using the TNT PCR Quick Kit from Promega (Madison, WI). Synthesis of the Fen-1 mutants was determined using Western blot analysis. As seen in Figure 4-8, Fen-1wt and mutant proteins were present after synthesis. Equal

amounts of proteins were estimated as described for the Fen-1 protein fragments and the Fen-1(K354A/R355A/K356A) and Fen-1(K365A/K366A/K367A) mutants were used in a Far-western analysis. Both Fen-1(K354A/R355A/K356A) and Fen-1(K365A/K366A/K367A) mutants were able to decrease the binding of APC and Fen-1. However, the Fen-1(K365A/K366A/K367A) mutant was able to decrease binding more than the Fen-1(K354A/R355A/K356A) mutant indicating that the Fen-1(K365A/K366A/K367A) mutant is more important for binding (Fig. 4-9A). Percent binding of the Far-western analysis can be seen in Figure 4-9B.

Fen-1(K365A/K366A/K367A) Mutant Retains Catalytic Activity

Due to the fact that the Fen-1(K365A/K366A/K367A) mutant was able to reduce the binding of APC with Fen-1 and that previous experiments had shown that APC was able to block Fen-1 catalytic activty, it was decided to investigate whether the Fen-1(K365A/K366A/K367A) mutant contained catalytic activity. In order to test the 5'endonuclease activity of the Fen-1 (K365A/K366A/K367A) mutant, a ^{32}P-labeled flapped substrate was used as previously described in the Results sections. In order to use the TNT synthesized Fen-1wt and Fen-1(K365A/K366A/K367A) mutant for the assays, the proteins were pulled-down from the TNT reaction mixture using an anti-Fen-1 antibody. The beads with bound Fen-1wt or Fen-1 (K365A/K366A/K367A) mutant were then used in the endonuclease reaction. Endonuclease activity of the Fen-1wt and Fen-1(K365A/K366A/K367A) mutant was determined by the presence of a 5-nt product. Interestingly, the Fen-1(K365A/K366A/K367A) mutant was able to cleave the 5' endonuclease substrate as efficiently as the wild-type protein (Fig. 4-10C compare lanes 7-9 with lanes 10-12) indicating that mutations in Fen-1 at amino acids K365/K366/K367 are not important for the endonuclease activity of Fen-1.

Additionally, the exonuclease activity of the Fen-1(K365A/K366A/K367A) mutant was also tested. In order to determine the effect of APC on Fen-1 5'-3- exonuclease activity, a nicked DNA substrate was used. Labeling of the nicked substrate with γ-^{32}P[ATP] was done as previously described in the Materials and Methods section. Fen-1wt and Fen-1(K365A/K366A/K367A) mutant proteins were pulled-down from the TNT reaction mixture as described for the endonuclease activity. The beads with bound Fen-1wt or Fen-1(K365A/K366A/K367A) mutant were then used in the exonuclease reaction. Exonuclease activity of the Fen-1wt and Fen-1(K365A/K366A/K367A) mutant was determined by the appearance of a 1-nt product. Surprisingly, the Fen-1(K365A/K366A/K367A) mutant had more exonuclease activity that the wild-type protein (Fig. 4-10D compare lanes 4 and 5 with lanes 6 and 7). This could be due to the fact that the mutations may have caused a conformational change within the protein that may have caused the active site to be more accessible. Combined, the results from the endonuclease and exonuclease assays indicate that the mutations in Fen-1 at amino acids K365/K366/K367 are not important for the catalytic activity of Fen-1. Therefore, the blockage of Fen-1 activity by APC after interaction with amino acids K365/K366/K367 could be due to the conformational change of Fen-1 structure affecting its activity. Alternatively, it could be due to steric hindrance caused by the APC protein since it is such a large protein.

APC Prevents the Entry of Fen-1 into the Nucleus after Exposure to DNA-Alkylating Agents

The fact that the Fen-1(K365A/K366A/K367A) mutant still contained catalytic activity prompted the idea that APC could be blocking Fen-1's participation in LP-BER through more than one mechanism. Since the Fen-1(K365A/K366A/K367A) mutant does not affect catalytic activity, the purpose of an interaction with APC at this site remained unclear. In order to get a better idea of the functional interaction of APC with Fen-1 at amino acids K365/K366/K367 an

immunohistochemical analysis was performed to determine the localization of these two proteins in cells after exposure to the DNA-alkylating agent, methylmethane sulfonate (MMS). In 2007, Kundu and colleagues, showed that the treatment of cells with 500 μM of MMS caused the upregulation of APC and that this upregulation of APC caused a blockage of DNA repair. Based on these data, HCT-116-APC(WT) cells were treated with 500 μM MMS to ensure that APC upregulation would occur and that it would interact with Fen-1. HCT-116-APC(WT) cells contain wild-type APC. Particular attention was paid to the localization of APC and Fen-1 with regards to the nucleus as DNA repair occurs in the nucleus and the identified APC interaction site on Fen-1 occurs in the nuclear localization signal. After 24 h of treatment with 500 μM MMS, an interesting phenomenon can be seen in the HCT-116-APC(WT) cells. Fen-1 nuclear localization is severely decreased while the cytoplasmic level of Fen-1 has increased as compared to control HCT-116-APC(WT) cells. Moreover, when the staining of Fen-1 (green) and APC (red) is merged a considerable amount of co-localization, as demarcated by a yellow color, is seen between Fen-1 and APC in the cytoplasm and more specifically around the nuclear membrane as compared to control HCT-116-APC(WT) cells (Fig 4-11). Interestingly, this phenomenon was not seen in HCT-116-APC(KD) cells subjected to the same treatment as the HCT-116-APC(WT) cells. The HCT-116-APC(KD) cell line has been stably transfected with siRNA against APC. Thus, APC levels are greatly reduced in this cell line. As seen in Figure 4-12, after 24 h of treatment the HCT-116-APC(KD) cells still contain the majority of Fen-1 in the nucleus indicating that APC's interaction with Fen-1 may be playing a role in the loss of nuclear Fen-1 in response to treatment with MMS.

In order to gain a more complete understanding of what was occurring during the 24 h treatment with MMS, a time course experiment was performed. The cells were treated with 500

μM MMS for a time period of 5, 15, or 24 h. In addition to the aforementioned cell lines HCT-116-APC(WT) and HCT-116-APC(KD), the LoVo cell line was also used. LoVo cells were used because they contain truncated APC (120 kDa) which lacks the DRI-domain and will not be able to interact with Fen-1. Thus, these cells serve as an additional method to ensure that the loss of nuclear Fen-1 after treatment with MMS for 24 h is dependent upon the APC and Fen-1 interaction. As expected, both LoVo cells and HCT-116-APC(KD) cells retained nuclear Fen-1 throughout the length of the time course experiment (Fig. 4-12 and 4-13). Moreover, co-localization (yellow) was not seen in the merged photographs of APC (red) and Fen-1 (green). On the other hand, a significant difference was observed between HCT-116-APC(WT) cells treated with MMS versus control cells as the time course experiment progressed. After 5 h of treatment, Fen-1 (green) is still localized in the nucleus and some Fen-1 is present in the cytoplasm while, APC (red) is seen in both the cytoplasm and the nucleus (Fig. 4-13). When the localization of APC (red) and Fen-1 (green) are merged, more co-localization (yellow) is seen in both the cytoplasm and the nucleus of the HCT-116-APC(WT) cells as compared to control cells (Fig. 4-14), indicating that in response to DNA damage there is an increased interaction between Fen-1 and APC. After 15 h of MMS treatment, about 50 percent of cells have developed a loss of nuclear Fen-1 (green) and an increase of cytoplasmic Fen-1 as compared to cells treated for only 5 h and HCT-116-APC(WT) control cells. Furthermore, APC (red) is localized more in the cytoplasm than in the nucleus. When APC (red) and Fen-1 (green) localization is merged a considerable amount of cytoplasmic co-localization (yellow) is seen. Interestingly, co-localization can also be seen in the nucleus where the remaining Fen-1 is present (Fig. 4-12). After 24 h of treatment, most of the cells have developed a loss of nuclear Fen-1 (green) and an increase in cytoplasmic Fen-1 as compared to control cells and cells treated with MMS for only 5

h. Furthermore, most of the APC (red) appears to be localized in the cytoplasm as compared to the nucleus although some nuclear APC also remains in HCT-116-APC(WT) cells (Fig. 4-12). Lastly, when the localization of Fen-1 (green) and APC (red) is merged a significant amount of co-localization (yellow) is seen in the cytoplasm especially around the nuclear membrane. Moreover, some co-localization can still be seen between the remaining nuclear Fen-1 and APC, which is much lower than the interaction between Fen-1 and APC which are co-localized in the cytoplasm after 24 h of MMS treatment (Fig. 4-12). Thus, it appears that APC is required for the loss of nuclear Fen-1 over time since neither LoVo cells nor HCT-116-APC(KD) cells exhibited this phenomenon (Fig. 4-12 to 4-13). This discovery provides a role for the interaction of APC with Fen-1 at its nuclear localization signal by suggesting that APC prevents Fen-1 from entering the nucleus after exposure to DNA-damaging agents.

In order to confirm the immunofluorescence results, nuclear and cytosolic levels in HCT-116-APC(WT) and HCT-116-APC(KD) cells treated with 500 μM MMS for 5, 10, 15, and 25 h were determined by Western blot analysis. The cytosolic fraction showed no significant change in the levels of Fen-1 in both HCT-116-APC(WT) cells and HCT-116-APC(KD) cells treated with 500 μM MMS (Fig. 4-14) while the nuclear fraction showed a decrease in the levels of Fen-1 in MMS treated cells over time in HCT-116-APC(WT) when compared to control HCT-116-APC(WT) cells (Fig. 4-14). However, no significant change was seen in the levels of nuclear Fen-1 in MMS treated HCT-116-APC(KD) cells (Fig. 4-15). These results are consistent with the findings of the immunofluorescence study, which showed similar results for nuclear Fen-1. Analysis of the APC levels in the cytosolic and nuclear fractions showed an increase in the level of APC in MMS-treated HCT-116-APC(WT) cells over time. As expected, APC levels were undetectable in MMS treated HCT-116-APC(KD) cells in both the nuclear and cytosolic

fractions. Furthermore, the increase in cytoplasmic APC correlates with the decrease in nuclear Fen-1 suggesting that the cytoplasmic APC is preventing Fen-1 translocation into the nucleus. In HCT-116-APC(WT) cells treated with MMS for 15 h cytoplasmic APC reaches its highest levels while nuclear Fen-1 reaches its lowest levels. At the 25 h time point, the cytoplasmic APC begins to decrease while nuclear Fen-1 shows a modest increase which further supports the hypothesis that APC may be blocking nuclear translocation of Fen-1 in response to DNA-alkylating agents.

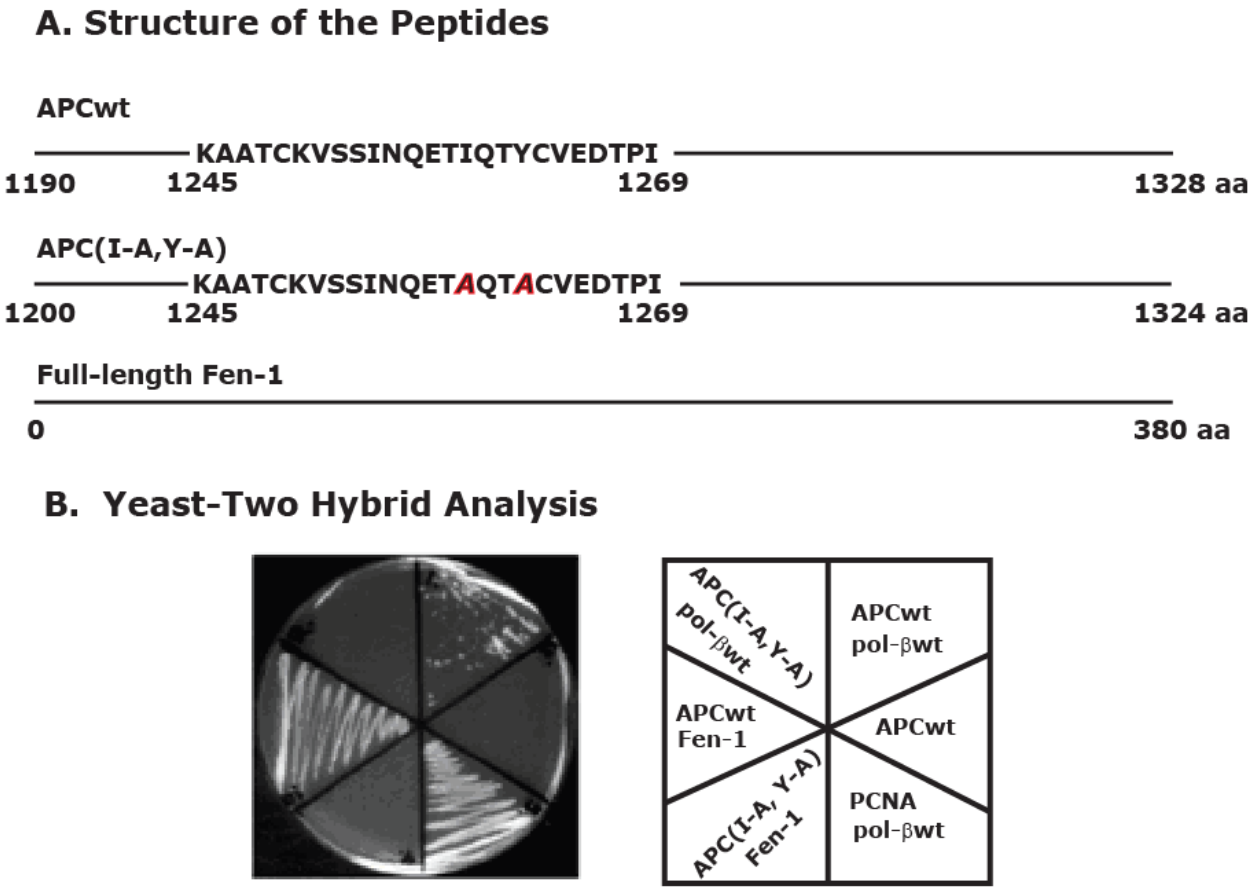


Figure 4-1. Interaction of Fen-1 and APC. Y2H assay showing the *in vivo* interaction of APCwt and Fen-1wt. A) Structure of the peptides used in the Y2H assay. B) Y2H assay of APC and Fen-1. Interactions used are shown in the diagram.

A. Structure of the APC peptides

APCwt(1190-1328)	1250-KVSSIN*Q*ET*I*QT*Y*CVEDTPI-1269
APC(Q-A,I-A,Y-A)	KVSSIN*A*ET*A*QT*A*CVEDTPI
APC(Q-1256A)	KVSSIN*A*ET*I*QT*Y*CVEDTPI
APC(I-1259A)	KVSSIN*Q*ET*A*QT*Y*CVEDTPI
APC(Y1262A)	KVSSIN*Q*ET*I*QT*A*CVEDTPI

B. APC/Fen-1 interaction

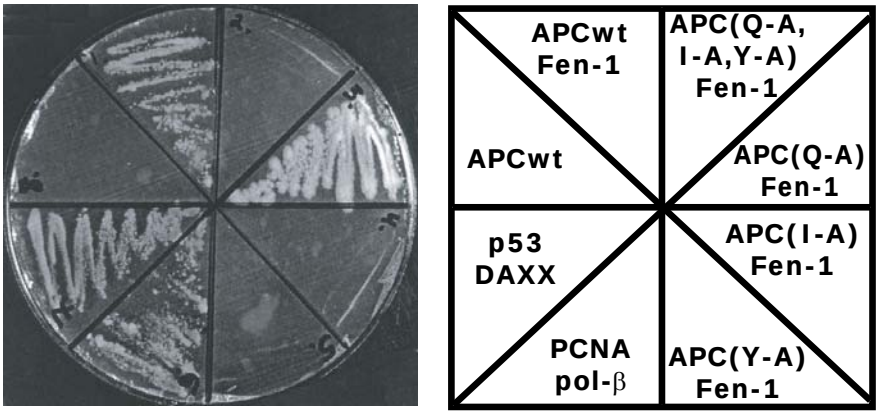
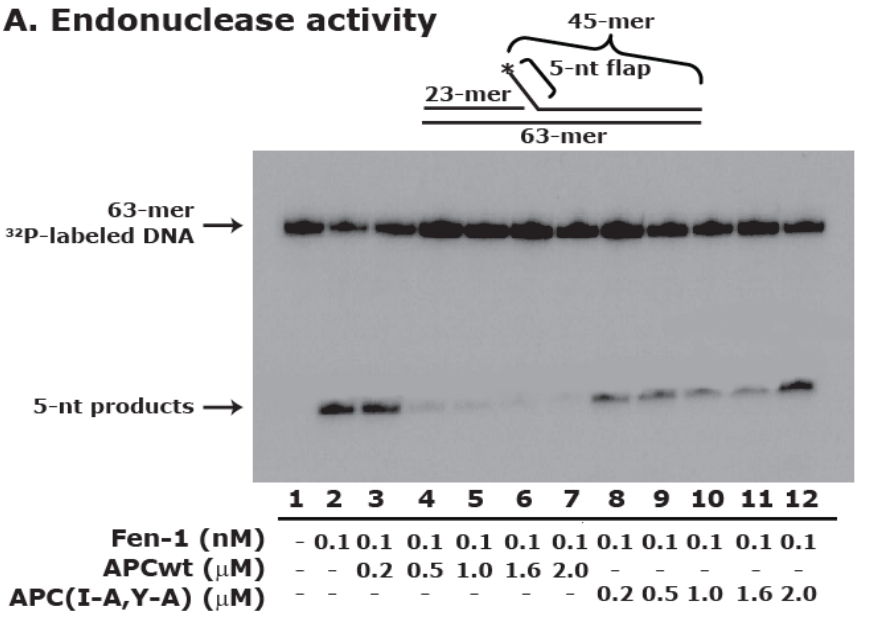


Figure 4-2. Interaction of APC mutants with Fen-1. A) Structure of the peptides used in this assay. B) Y2H assay of APC mutant proteins with Fen-1, interactions used shown in the diagram. Adapted with permission from Jaiswal et al., 2006.



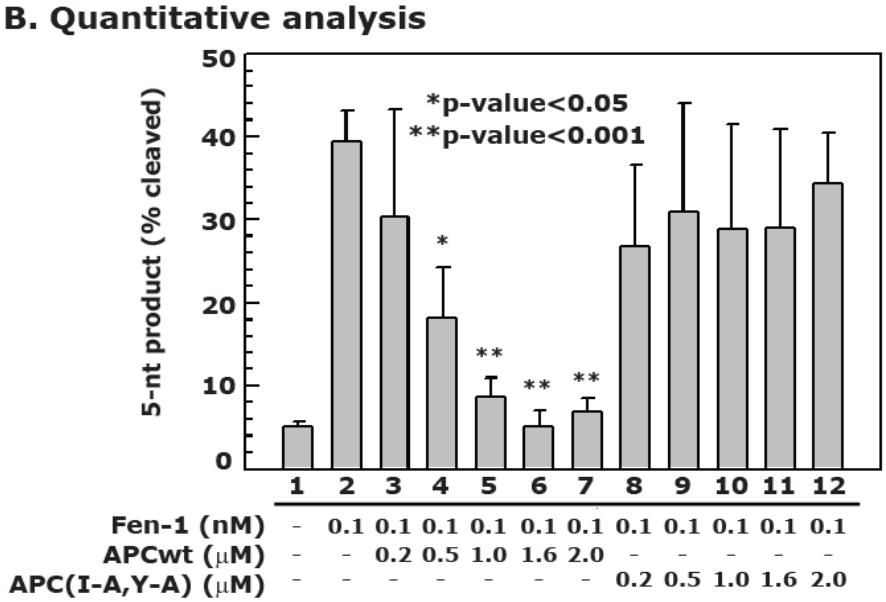
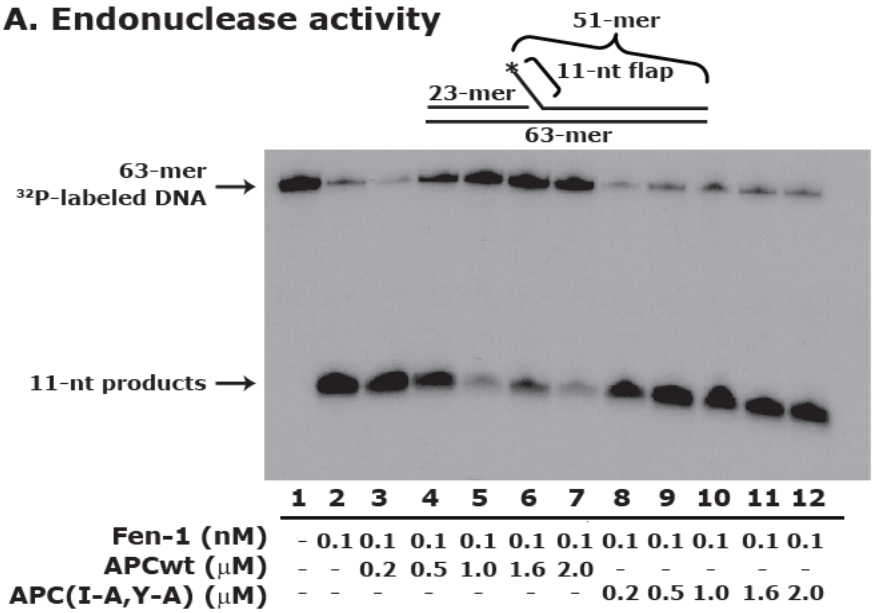


Figure 4-3. APC blocks the 5'-flap endonuclease activity of Fen-1. A) Autoradiogram of a representative experiment. Reaction mixtures in a 25 μL final volume contained a fixed (0.1 nM) concentrations of Fen-1 coupled with different concentrations (0.2, 0.5, 1.0, 1.6, and 2.0 μM) of either APCwt or mutant APC(I-A,Y-A) peptides. The mixture was incubated for 5 min on ice, and then 2.5 nM of the ^{32}P-labeled flapped-DNA substrate was added. The cleavage reaction was carried out at 37^{o}C for 30 min. X-ray film autoradiography determined the 5-nt cleaved product, and the electronic autoradiography measured the radioactivity (InstantImager; Packard Instrument Co., Meriden, CT). B) Quantitative analysis of the 5-mer cleaved product. The fraction of cleavage was calculated as the percent radioactivity present in the cleaved product as follows: % cleavage = [5-nt/(45-mer + 5nt)]-100. Data are the mean ± SE of three different experiments.



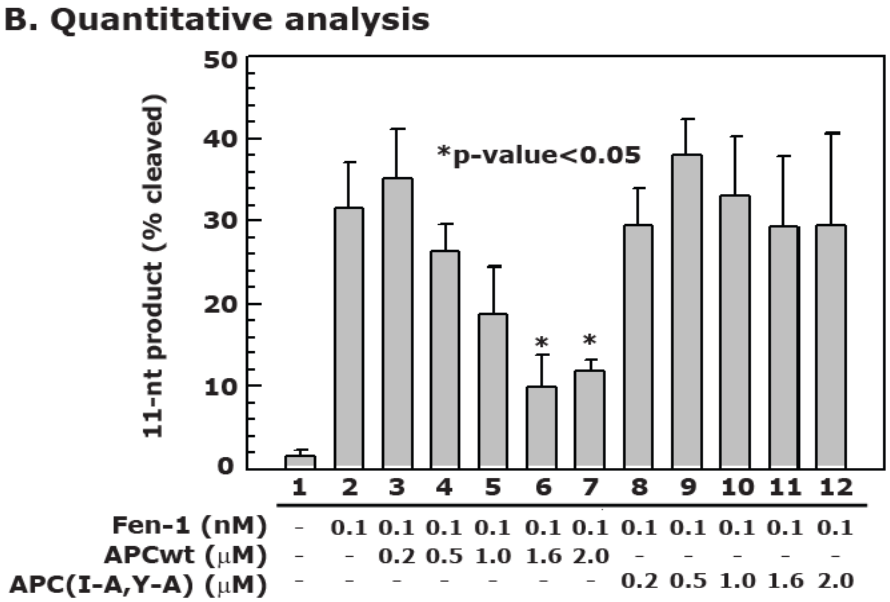


Figure 4-4. APC blocks the 5'-flap endonuclease activity of Fen-1 regardless of flap size. A) Autoradiogram of a representative experiment. Reaction mixtures in a 25 μL final volume contained a fixed (0.1 nM) concentrations of Fen-1 coupled with different concentrations (0.2, 0.5, 1.0, 1.6, and 2.0 μM) of either APCwt or mutant APC(I-A,Y-A) peptides. The mixture was incubated for 5 min on ice, and then 2.5 nM of the ^{32}P-labeled flapped-DNA substrate was added. The cleavage reaction was carried out at 37^{o}C for 30 min. X-ray film autoradiography determined the 5-nt cleaved product, and the electronic autoradiography measured the radioactivity (InstantImager; Packard Instrument Co., Meriden, CT). B) Quantitative analysis of the 11-mer cleaved product. The fraction of cleavage was calculated as the percent radioactivity present in the cleaved product as follows: % cleavage = [11-nt/(45-mer + 11-nt)] - 100. Data are the mean ± SE of three different experiments.

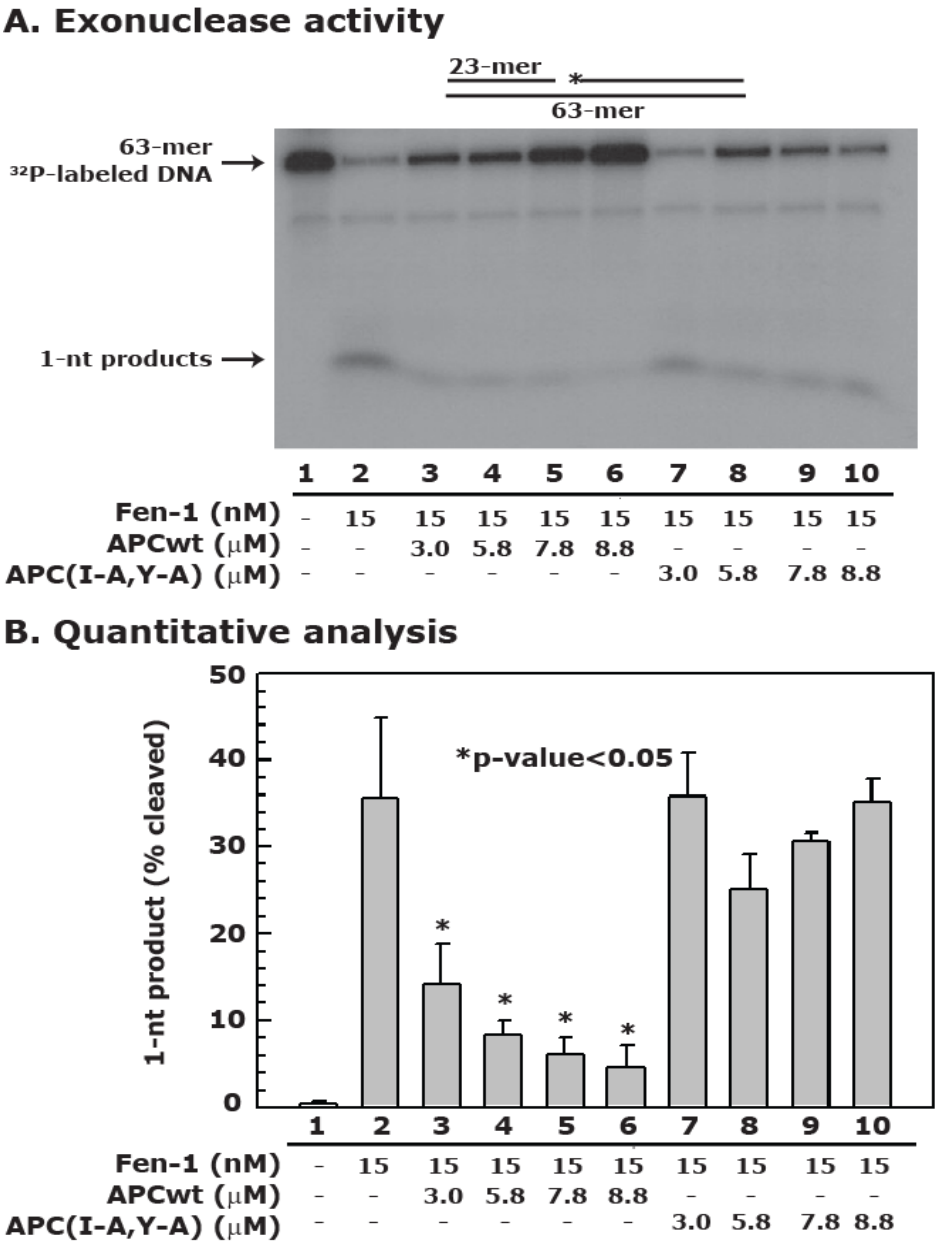


Figure 4-5. APC blocks the 5'-3' exonuclease activity of Fen-1. Exonuclease activity reaction mixture was assembled in a total of 25 μL final volume and contained fixed (15 nM) concentrations of Fen-1 along with (3, 5.8, 7.3 or 8.8 μM of either APCwt or APC(I-A,Y-A) peptides. The mixture was incubated for 5 min at 22°C and then 2.5 nM 63-mer oligonucleotide substrates containing the ^{32}P-labeled 40-mer downstream oligonucleotides were added. The exonuclease reaction was carried out at 37°C for 30 min. A) X-ray autoradiography determined the 1-nt cleaved product. B) Quantitative Analysis using electronic autoradiography to measured the radioactivity. (InstantImager; Packard Instrument Co., Meriden, CT). The fraction of cleavage was calculated as the percent radioactivity present in the cleaved product as follows: % cleavage = [1-nt/(40-mer + 1-nt)]-100. Data are the mean ± SE of three different experiments.

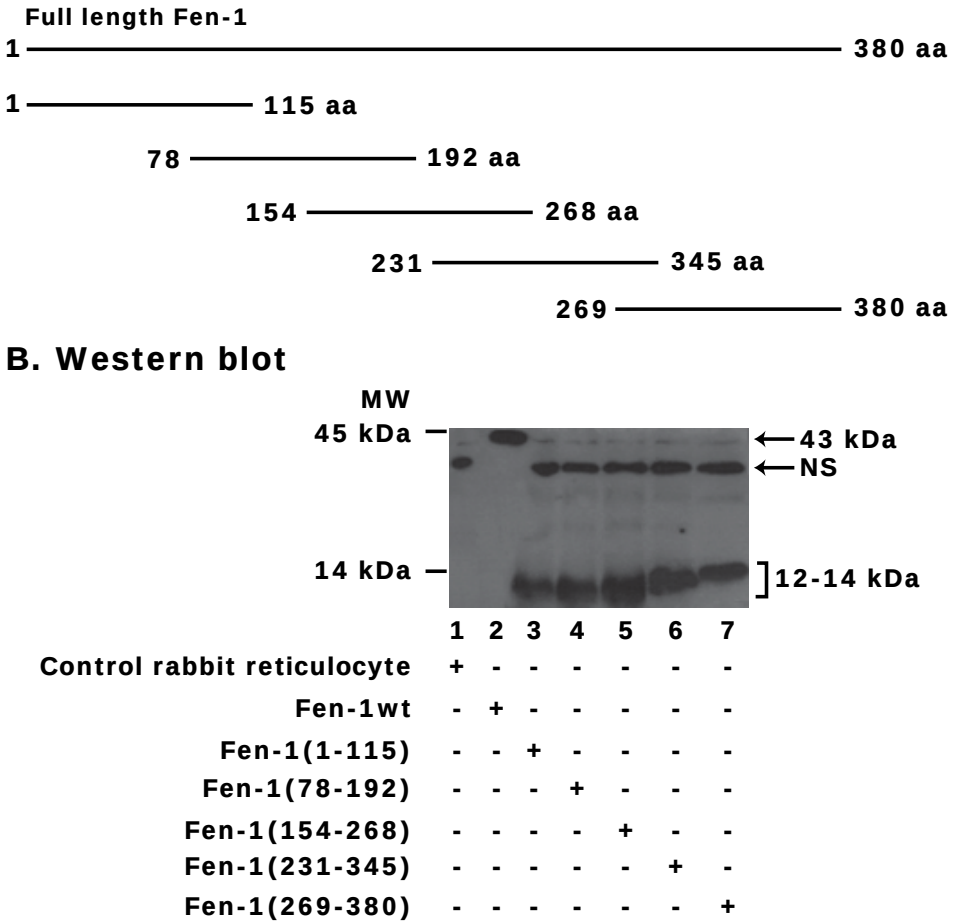


Figure 4-6. Protein levels of *in vitro* transcription/translation of wild-type and fragments of Fen-1. A) Depiction of protein fragments of Fen-1. B) TNT synthesis of Fen-1 wild- type and Fen-1 protein fragments. Proteins and protein fragments were run on a 15% SDS-PAGE followed by Western blot analysis. Fen-1 wild-type protein and protein fragments were detected using mouse anti-Fen-1antibody from Novus Biologicals.

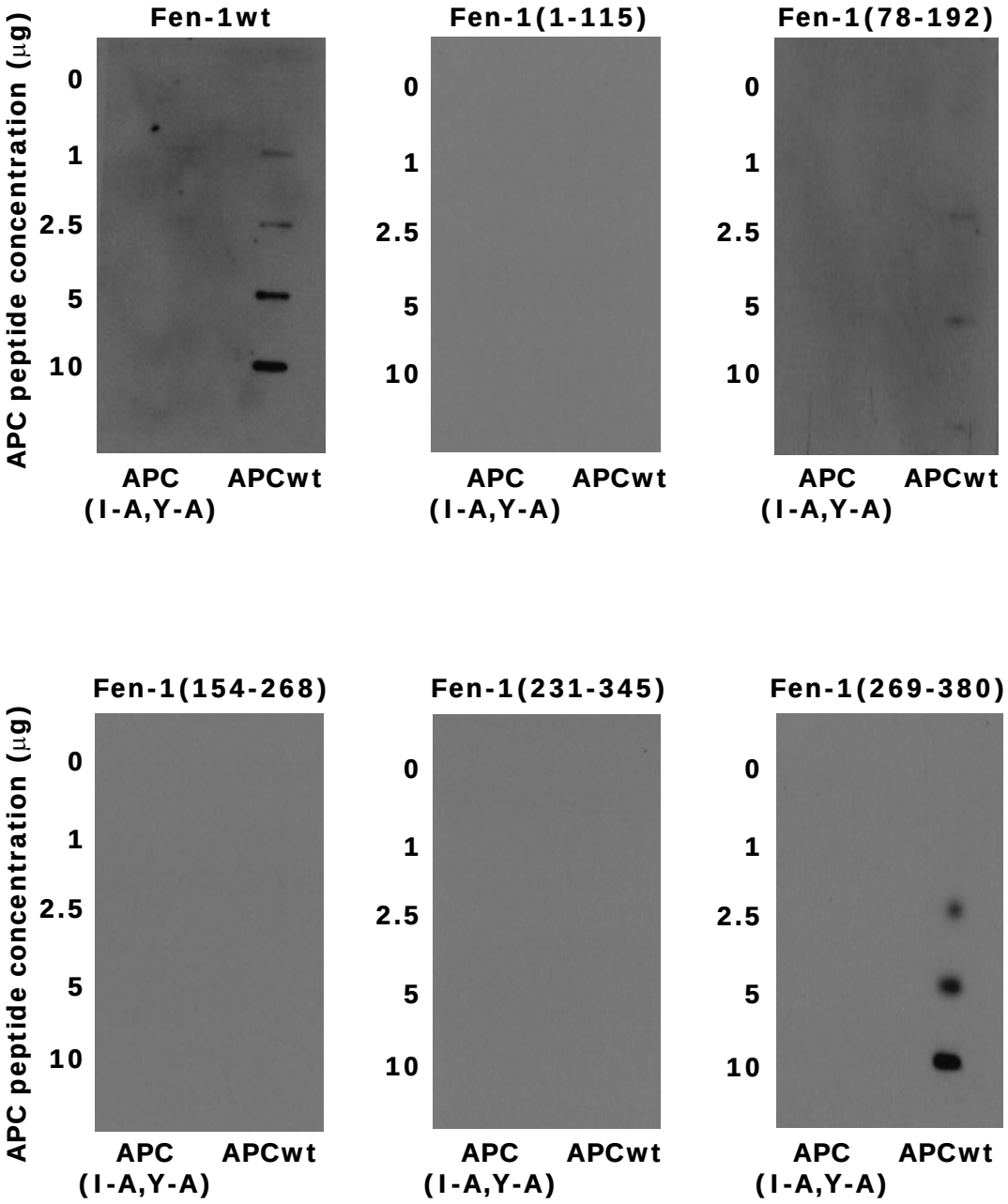


Figure 4-7. Far-western analysis of the interaction of APCwt and APC(I-A,Y-A) peptides and Fen-1 protein fragments. APC peptide was immobilized onto a PVDF membrane and incubated with either Fen-1 wild type or the indicated Fen-1 protein fragment. Binding was detected through the use of a mouse anti-human Fen-1 antibody followed by chemiluminescence analysis. Fen-1 protein fragment 269-380 was the only fragment able to interact with APCwt.

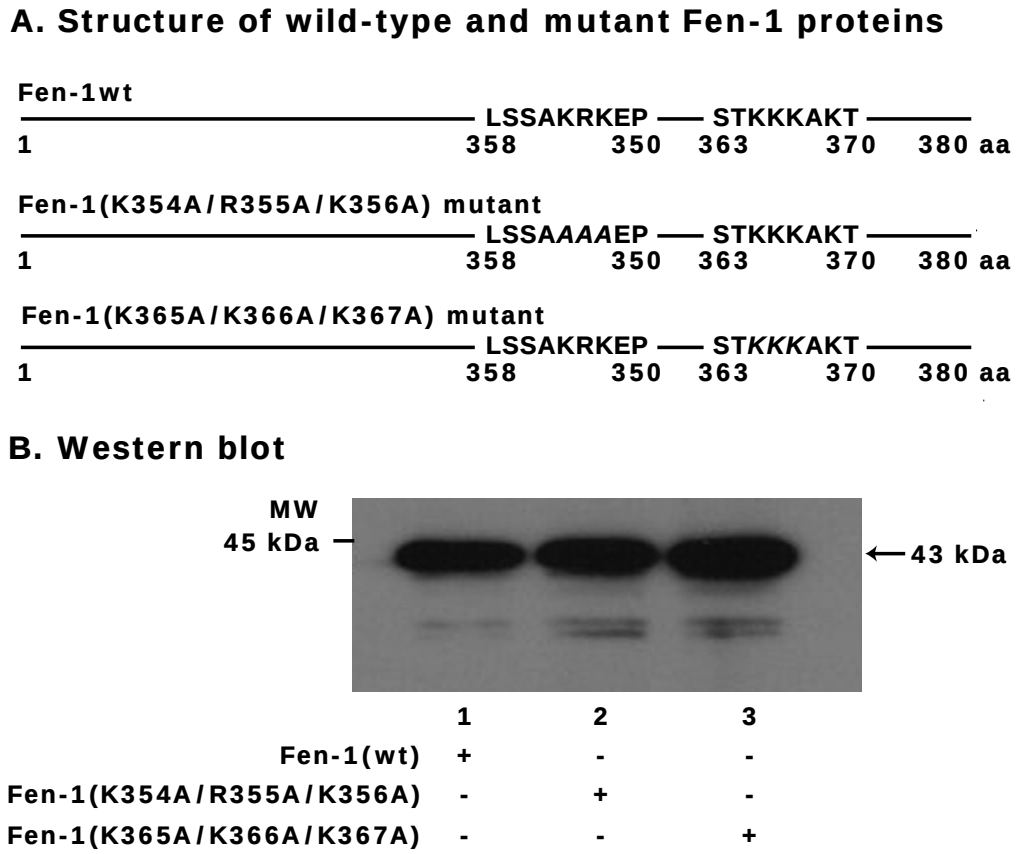


Figure 4-8. Protein levels of *in vitro* transcription/translation of wild-type and mutant Fen-1. A) Depiction of Fen-1 mutant proteins. B) Western blot Analysis Depicting Result of TNT Synthesis of Fen-1 wild-type and mutant Proteins. Fen-1 wild-type and mutant proteins were synthesized using the TNT PCR Quick Kit (Promega, Madison, WI). Synthesized proteins were run on a 10% SDS-PAGE and then transferred onto a PVDF membrane. Membrane was probed for Fen-1 wild-type and mutant proteins using a mouse anti-human Fen-1 antibody and the signal was detected using chemiluminescence analysis.

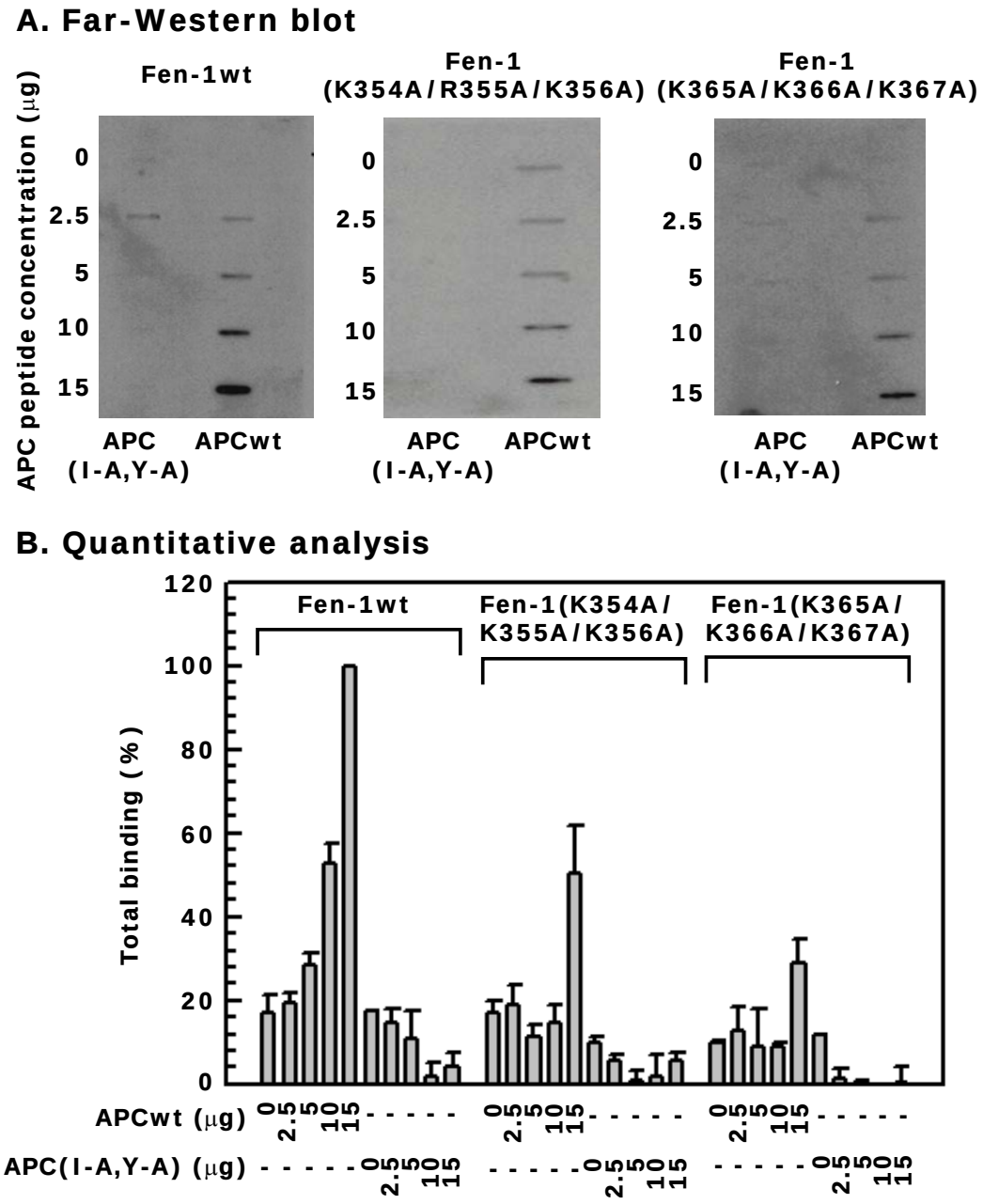


Figure 4-9. Far-western analysis of the interaction of Fen-1 wild-type and mutant proteins with APC. A) APCwt and mut peptides were immobilized on a PVDF membrane and incubated with Fen-1 wild-type and mutant proteins. Binding was detected using a mouse anti-human Fen-1 antibody followed by chemiluminescence. B) Percent binding of APCwt and Fen-1 wild-type and mutant proteins. Percent binding was determined by using the density of APC and Fen-1 wild-type binding band as 100 % binding and comparing Fen-1 mutant proteins and APC-binding to the density of the APC and Fen-1 wild-type band [(Density of APCwt and Fen-1 mutant binding/Density of APCwt and Fen-1 wild-type) x 100 = Percent bound)]. Experiment was repeated twice.

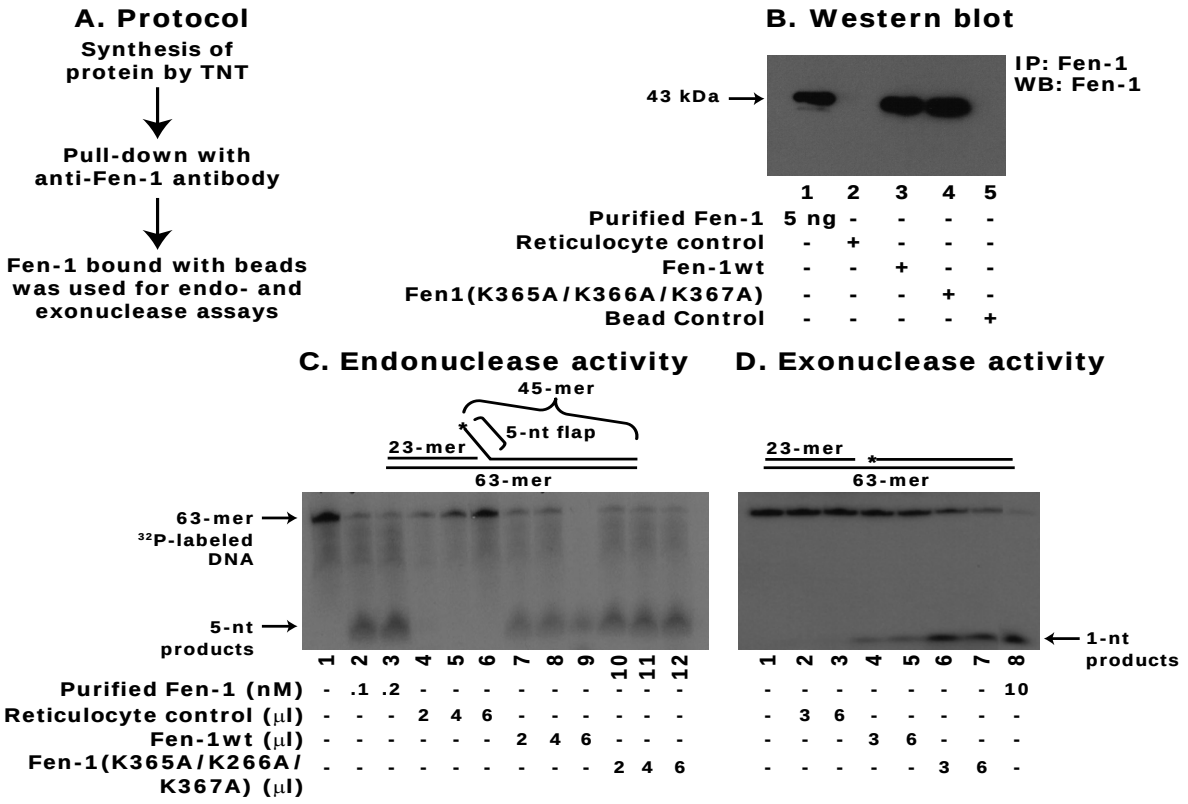


Figure 4-10. Fen-1(K365A/K366A/K367A) mutant retains 5'-flap endonuclease and 5'-3' exonuclease activity. A) Pull-down assay of wild-type and mutant Fen-1 proteins. Fen-1 wild type and Fen-1 (K365A/K366A/K367A) mutant protein were synthesized using the TNT PCR Quick Kit (Promega, Madison, WI) and then pulled down from the TNT reaction mixture using protein A sepharose beads and rabbit anti human Fen-1 antibody (Bethyl laboratories, Montgomery, TX). Beads were used in western blot analysis to detect presence of pulled-down Fen-1(wt) and Fen-1(K365A/K366A/K367A) mutant protein. Proteins were detected using anti-Fen-1 antibody (Novus Biologicals, Littleton, Co) and the chemiluminesence technique (GE Healthcare, Picastaway, NJ) B) Schematic of protocol used for endonuclease and exonuclease assay. C) Autoradiogram depicting endonuclease activity. Reaction mixtures in a 25 μL final volume contained 2, 4, or 6 μl of beads with Fen-1 wild-type or Fen-1(K365A/K366A/K367A) mutant protein. Purified Fen-1 wild-type protein at a concentration of 0.1 or 0.2 nM was used as a positive control. The mixture was incubated for 5 min on ice, and then 2.5 nM of the ^{32}P-labeled flapped-DNA substrate was added. The cleavage reaction was carried out at 37^{o}C for 30 min. X-ray film autoradiography determined the 5-nt cleaved product, and the electronic autoradiography measured the radioactivity (InstantImager; Packard Instrument Co., Meriden, CT). D) Autoradiogram depicting exonuclease activity. Reaction was carried out as described in B with the following changes: 1. 3 or 6 μl of beads with Fen-1 wild-type or Fen-1(K365A/K366A/K367A) was used in the reaction mixture; 2. 10 nm of purified Fen-1 wild-type protein was used as a postitive control; 3. A ^{32}P-labeled nicked-DNA substrate was used; 4. X-ray film autoradiography determined the 1-nt cleaved product and electronic autoradiography measured the radioactivity.

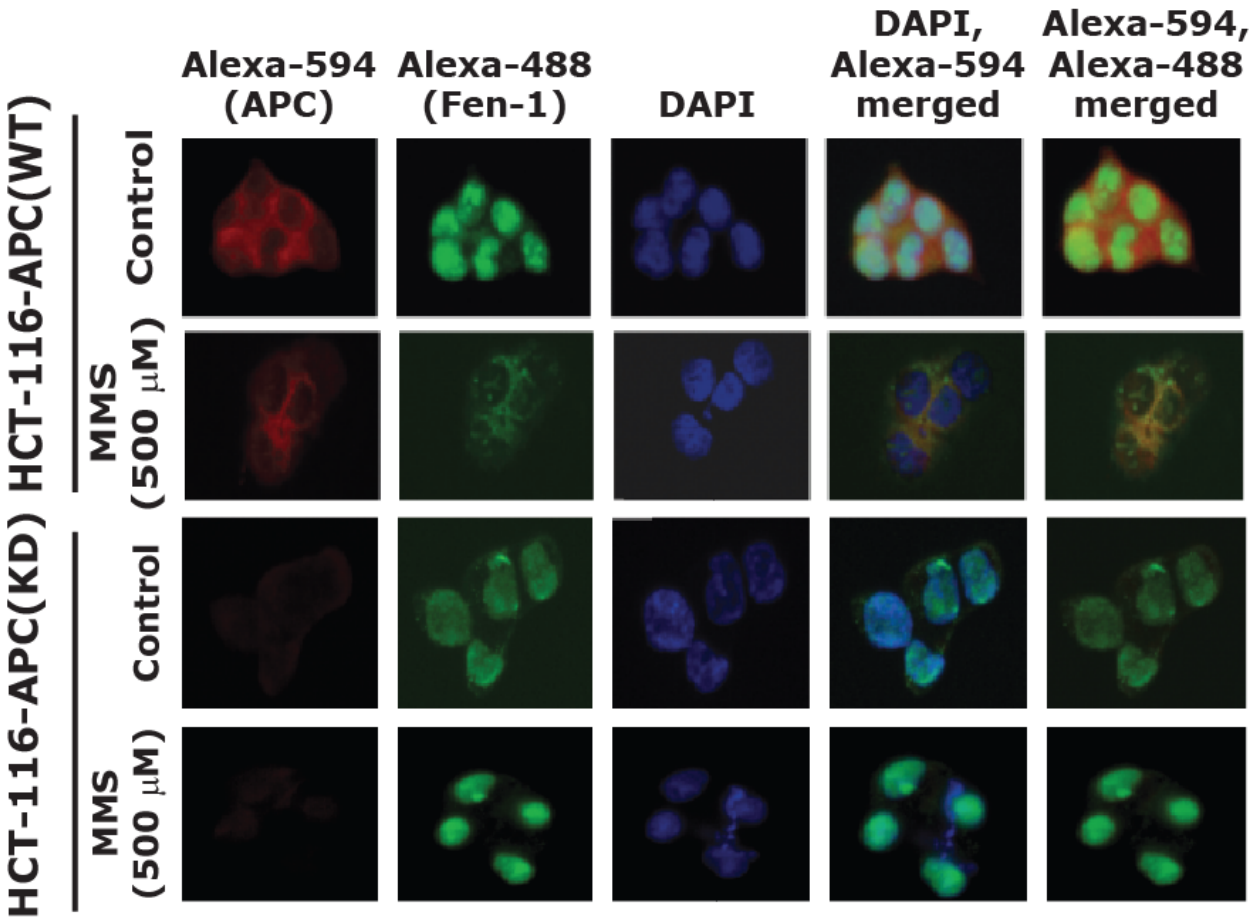


Figure 4-11. Imunohistochemical analysis of APC and Fen-1 co-localization in HCT-116-APC(WT) and HCT-116-APC(KD) cells. Cells were treated with the indicated amount of MMS for 24 h and then stained for Fen-1 (green), APC (red), and nuclei (blue). Images were merged to determine co-localization.

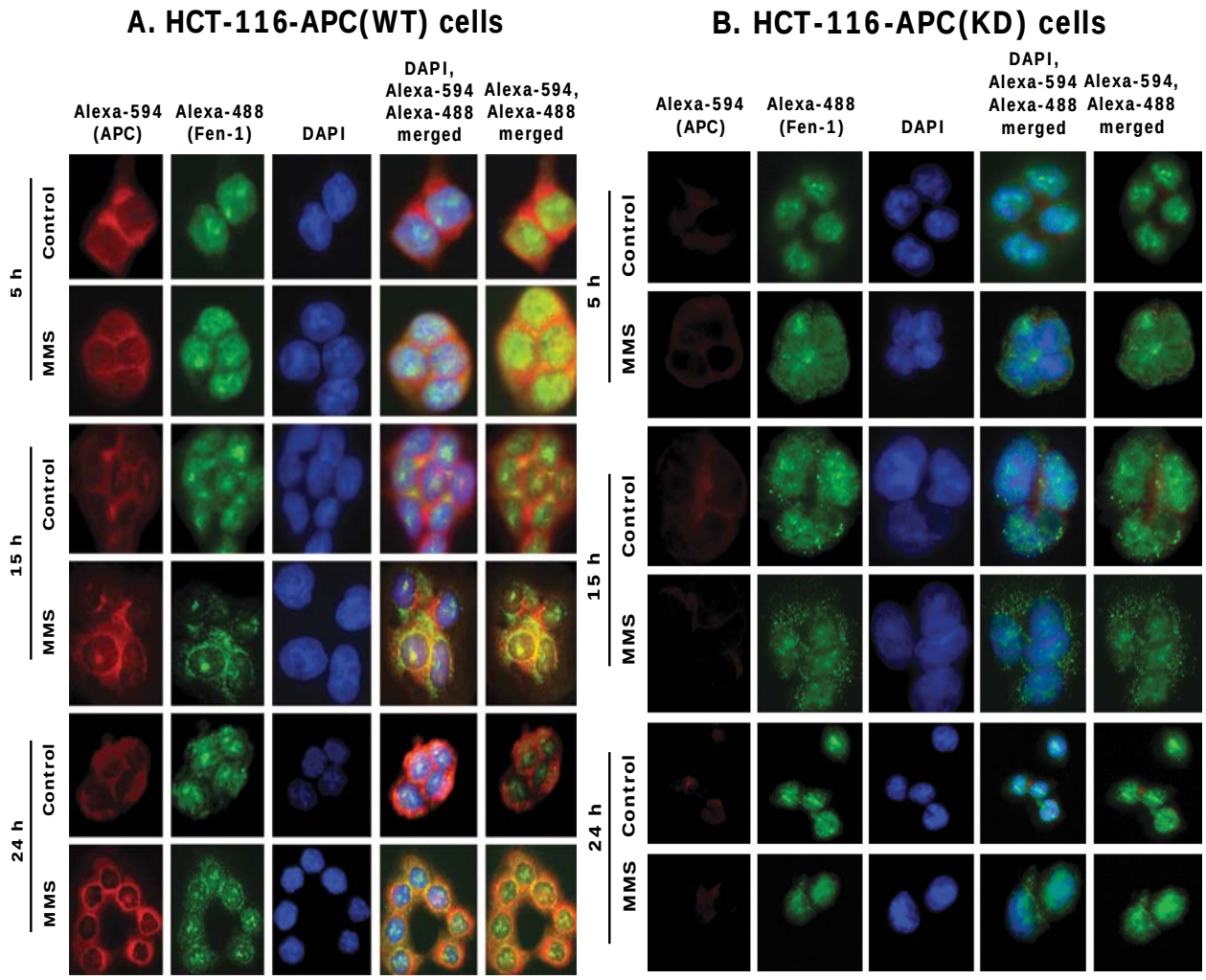


Figure 4-12. A time-course analysis of co-localization of APC and Fen-1 in colon cancer cells treated with MMS. A) HCT-116-APC(WT) cells were treated with 500 μM for the indicated amount of time. Cells were stained for Fen-1 (green), APC (red), and nuclei (blue). Pictures were then merged for co-localization. B) HCT-116-APC(KD) cells. Cells were treated the same as HCT-116-APC(WT).

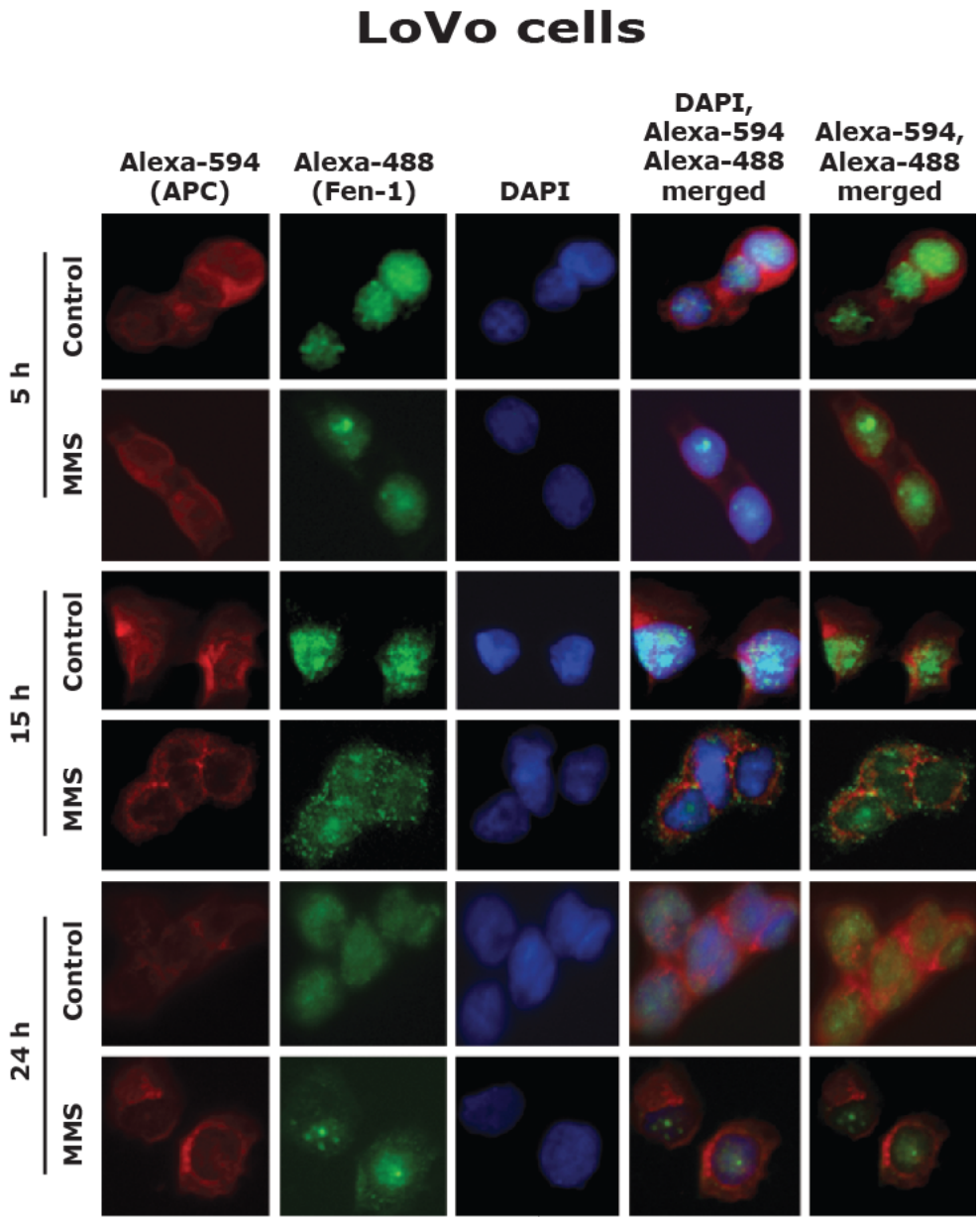


Figure 4-13. A time-course analysis of co-localization of APC and Fen-1 in LoVo cells treated with MMS. LoVo cells (truncated APC that lacks the DRI-domain) were treated with 500 μM MMS for the indicated amount of time. Cells were stained for Fen-1 (green), APC (red), and nuclei (blue). Pictures were then merged for co-localization.

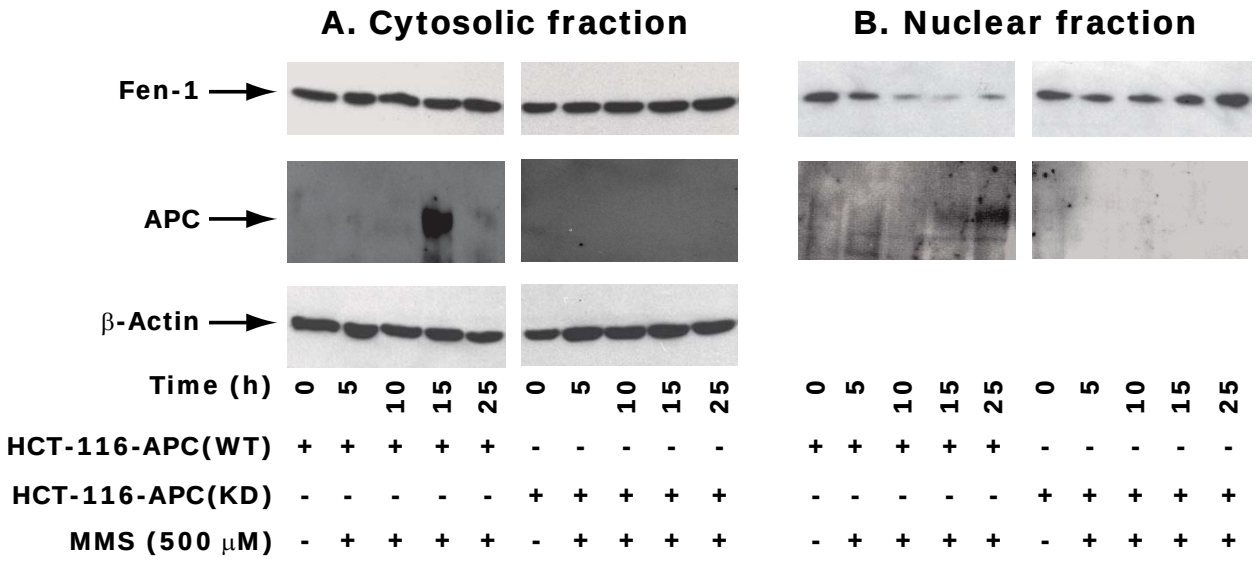


Figure 4-14.A time-course analysis of nuclear and cytosolic protein levels of APC and Fen-1 in MMS-treated colon cancer cells. HCT-116-APC(WT) and HCT-116-APC(KD) cells were treated with 500 μM MMS for the indicated amount of time, Nuclear and cyosolic fractions were prepared and analyzed using western blot analysis. Fen-1 was detected using mouse anti-Fen-1 antibody (Novus Biologicals, Littleton, Co) while APC was detected using mouse anti-APC antibody (Millipore, , CA). Signals were detected using the chemiluminescence technique. A) Western blot analysis of APC and Fen-1 levels in the cytosolic fractions of MMS treated HCT-116-APC(WT) and HCT-116-APC(KD) cells. β-actin is shown as a loading control. B) Western blot analysis of APC and Fen-1 levels in the nuclear fractions of MMS treated HCT-116-APC(WT) and HCT-116-APC(KD) cells.

CHAPTER 5
DISCUSSION

The Interaction of APC and DNA Polymerase β and its Implications in BER

In the present study the interaction sites of pol-β and APC involved in the APC and pol-β interaction were determined. Using Y2H analysis with single amino acid mutants of APC, it was found that only I1259 or Y1262 were necessary for the interaction of APC with pol-β. Furthermore, the mutation of either amino acid to alanine was enough to disrupt the interaction of APC with pol-β suggesting that the interaction of pol-β may be at the surface instead of in the groove of the APC protein. Additionally, the present study indicates that APC interacts with a specific region of pol-β. Based on the Y2H analysis with pol-β-deletion constructs and APC, it was determined that amino acids 80-120 of pol-β were critical for the interaction of pol-β with APC. Upon analysis of the crystal structure of pol-β and examining the solvent accessibility of amino acids 80-120, possible amino acids involved in the interaction with APC were determined. Mutagenesis of Thr79, Lys81, and Arg83 to alanine abolished the interaction between pol-β and APC. Thus, amino acids Thr79, Lys81, and Arg83 were identified as critical residues of pol-β which were important for the interaction with APC. Notably, these amino acids are located in the linker region of pol-β which connects its lyase and polymerase domains (Beard and Wilson, 2006).

The active site of the lyase domain of pol-β is Lys72 which is closely located to the amino acids Thr79, Lys81, and Arg83 (Srivastava et al., 1998). Due to this close proximity, it would be logical to hypothesize that the binding of APC can interfere with pol-β's deoxyribose phosphate (dRP)-lyase activity, and thus SP-BER. In fact, studies conducted indicate that this hypothesis is correct (Balusu et al., 2007). By reconstituting the SP-BER system *in vitro*, these authors showed

that SP-BER was blocked by APC in a dose-dependent manner. Furthermore, they determined the mechanism by which APC is blocking SP-BER by testing whether APC blocked pol-β dRP-lyase activity. The dRP-lyase activity of pol-β has been determined as the rate-limiting step in SP-BER. Removal of the 5'dRP moiety is crucial for the generation of the 5' phosphate required by DNA ligase to seal the nick and complete repair of the DNA (Beard and Wilson, 2006; Srivastava et al., 1998). Through the use of an *in vitro* dRP-lyase activity assay Balusu and colleagues were able to examine the effect of APC on pol-β's dRP-lyase activity (Balusu et al., 2007). They found that an APC wt peptide was able to block pol-β's dRP-lyase activity in a dose-dependent manner indicating that APC blocks SP-BER by modifying pol-β's dRP-lyase activity. Moreover, they were also able to show that the strand-displacement synthesis of LP-BER, which is blocked by APCwt, was unaffected with the pol-β mutant (T79/K81/R83) protein. These results indicated that the physical interaction of APC with pol-β is necessary to block its strand-displacement synthesis. Thus, it would appear that APC is a novel accessory protein in both SP and LP-BER.

The Interaction of APC with Fen-1 and its Implications in BER

Previous studies have shown that APC can block pol-β strand-displacement synthesis in LP-BER and interact with Fen-1 *in vitro* (Jaiswal et al., 2006; Narayan et al., 2005). In the current study these findings have been extended to examine whether APC and Fen-1 interact *in vivo*, and if this interaction can block Fen-1 5'enduonuclease and 5'-3' exonuclease activities providing another method of inhibiting LP-BER. Furthermore, critical amino acid residues on Fen-1 and APC that participate in the Fen-1 and APC interaction have been determined. Lastly, immunohistochemical analysis and Western blot analysis of MMS-treated cells have suggested a

novel role for APC whereby it prevents Fen-1 from entering the nucleus after exposure to DNA-alkylating agents.

Through Y2H analysis it was determined that APCwt and Fen-1 could interact *in vivo*. In light of this fact, it was decided to narrow down the interaction site on APC for Fen-1. Since the interaction site on APC for pol-β had already been determined, it was thought that APC may interact with Fen-1 at the same site. Using Y2H analysis of single amino acid mutants of APC, it was found that the interaction site of APC for Fen-1 and pol-β was in the same domain. Only amino acids I1259 or Y1262 were necessary for the interaction of APC with Fen-1. Furthermore, the mutation of either amino acid to alanine was enough to disrupt APC's interaction with Fen-1 suggesting that the interaction of Fen-1 may be at the surface instead of in the groove of the APC protein. Taken together, the fact that APC interacts with pol-β and Fen-1 at the same site indicates that APC cannot interact with both proteins at the same time. This suggests that APC may have a preference for either protein or that pol-β and Fen-1 may compete to bind with APC. Although how the differential interaction of these proteins occurs *in vivo* is not clear.

Fen-1 is the major protein involved in LP-BER. LP-BER can occur through two mechanisms: strand-displacement and "Hit-and-Run" synthesis. Fen-1 plays unique roles in each. In strand-displacement, Fen-1 cleaves the flap generated by pol-β, pol-δ, or pol-ε mediated strand-displacement synthesis. In hit-and-run synthesis, Fen-1 excises a nucleotide at the 3'-side of a nick to create a gap to be filled by pol-β (Klungland and Lindahl, 1997; Prasad et al., 2001; Qiu et al., 2001; Shibata and Nakamura, 2002) . More specifically, the two mechanisms proceed in the following manner. Once pol-β arrives at an oxidized or reduced abasic site after APE incision, it proceeds to perform strand-displacement synthesis until Fen-1 binds and cleaves the displaced flap leaving a nick which is sealed by DNA ligase. Alternatively, a hit and run

mechanism of LP-BER can occur in which pol-β incorporates 1-nt and the resulting flap is removed by Fen-1 5'endonuclease activity. Then a series of sequential events occurs in which pol-β binds to the nick left behind by Fen-1 endonuclease activity, extends the 3′-OH displacing the dRP moiety, fills the 1-nt gap, leaves a nick, and dissociates from the DNA. Fen-1 then binds to the nicked DNA, removes 1-nt by its 5′-3′exonuclease activity, creates a gap, and dissociates from the DNA. This cycle continues until DNA ligase seals the nick (Liu et al., 2005).

As APC can interact with Fen-1 and has already been shown to be involved in LP-BER, it was hypothesized that the interaction of APC with Fen-1 may cause a blockage in Fen-1 catalytic activity, thus blocking LP-BER. Through the use of *in vitro* Fen-1 activity assays it was found that an APCwt peptide could block both Fen-1 5'endonuclease and 5'-3' exonuclease cleavage abilities in a dose-dependent manner. Thus, APC is able to block both mechanisms of LP-BER. When APC blocks the 5'endonuclease cleavage activity of Fen-1, it effectively blocks pol-β directed strand-displacement synthesis of LP-BER. However, when APC blocks the 5'-3' exonuclease activity of Fen-1 it blocks hit and run synthesis of LP-BER. These results combined with previous data show that APC can block LP-BER in three ways: 1) By blocking pol-β directed strand-displacement synthesis through APC's interaction with pol-β; 2) By blocking pol-β directed strand-displacement through APC's blockage of the 5' endonuclease activity of Fen-1; and 3) By blocking hit and run synthesis of LP-BER through APC's blockage of the 5'-3' exonuclease activity of Fen-1.

Once it was found that APC's interaction with Fen-1 caused a block in its 5'endonuclease and 5'-3'exonuclease activities, the next logical step was to identify the amino acids of Fen-1 necessary for its interaction with APC. Protein fragments of Fen-1 were synthesized and used in a Far-western analysis with APC. Amino acids 346-380 of Fen-1 were found to interact with

APC. Most of these amino acids lie within the nuclear localization signal of Fen-1 suggesting that APC is interfering with nuclear localization of Fen-1. Indeed, two groups of amino acids essential for nuclear localization (K354, R355, K356 and K365, K366, K367) lie within the region of amino acids 346-380 (Shen et al., 2005). Furthermore, three proteins which interact with Fen-1 have been found to interact at Fen-1's C-terminus between amino acids 353-380 (Guo et al., 2008; Sharma et al., 2005). Based on this knowledge two sets of amino acids Fen-1(K354/R355/K356) and Fen-1(K365/K366/K367) were chosen for further studies. These amino acids of Fen-1 are important in nuclear localization, and due to the fact that they fell between amino acids 353-380 which have been shown to be the interaction site of Fen-1 with other proteins. Each amino acid was mutated to alanine and the mutant proteins were synthesized and used in a Far-western analysis with the APCwt peptide. Both mutants were shown to reduce the interaction between APCwt peptide and Fen-1. The Fen-1(K365A/K366A/K367A) mutant was able to reduce the interaction with APC to a greater extent. These results suggested that APC does interact with the nuclear localization signal of Fen-1. More specifically, it suggests that APC interacts with the amino acids that have been shown to be essential for nuclear localization.

To further characterize the interaction of APC with Fen-1, the synthesized Fen-1 (K365A/K366A/K367A) mutant was used in *in vitro* Fen-1 activity assays to determine if the Fen-1(K365A/K366A/K367A) mutant possessed 5'endonuclease and 5'-3'exonuclease activities. Surprisingly, the Fen-1(K365A/K366A/K367A) mutant retained both 5'endonuclease activity and 5'-3'exonuclease activity. It was expected that the Fen-1(K365A/K366A/K367A) mutant protein would lose its 5'endonuclease and 5'-3'exonuclease activities due to the fact that the interaction of APC blocks these activities. Since the Fen-1(K365A/K366A/K367A) mutant retains its endonuclease and exonuclease activities, the physical interaction between Fen-1 and

APC must be necessary for Fen-1 cleavage activity to be blocked. APC may cause a conformational change in Fen-1 upon binding that causes the active site of the endonuclease and exonuclease activities to become misaligned causing a blockage of Fen-1 endonuclease and exonuclease activities.

Due to the fact that the interaction of Fen-1 with APC appears to occur at the nuclear localization signal of Fen-1, it became necessary to determine the physiological significance of this interaction within the cell. Immunohistochemical analysis of HCT-116-APC(WT) cells treated with 500 μM MMS for 24 h showed that most cells had an increased co-localization of APC and Fen-1 in the cytoplasm as well as a reduction in nuclear Fen-1 as compared to control cells. This phenomenon was only seen in HCT-116-APC(WT) cells which contained wild-type APC. HCT-116-APC(KD) cells and LoVo cells were also treated with 500 μM MMS, but failed to show a reduction in nuclear Fen-1 after 24 h of treatment. Since HCT-116-APC(KD) cells contain stably knocked down levels of APC and LoVo cells contain truncated APC which lacks the DRI-domain and therefore does not interact with Fen-1, these results indicate that wild-type APC must be present for the reduction in nuclear Fen-1 to occur. Furthermore, this reduction occurs in response to DNA-alkylation damage. To further understand how this reduction in nuclear Fen-1 occurs after treatment with MMS for 24 h, a time course experiment was conducted where the aforementioned cell lines were treated with MMS for 5, 15, or 24 h. Once again the reduction in levels of nuclear Fen-1 and increased co-localization between APC and Fen-1 were only seen in HCT-116-APC(WT) cells. After 15 h of treatment with 500 μM MMS about half of the HCT-116-APC(WT) cells exhibited a reduction in nuclear Fen-1 and by 24 h most of the HCT-116-APC(WT) cells exhibited a loss of nuclear Fen-1. Cells treated with MMS for 5 h did not exhibit a loss of nuclear Fen-1, but they did exhibit increased co-localization of

APC and Fen-1 in both the cytoplasm and the nucleus. In contrast these observations were not seen in HCT-116(KD) cells or LoVo cells that received the same treatment. Furthermore, the increased co-localization of APC and Fen-1 was not apparent in HCT-116-APC(WT) cells which did not receive the MMS treatment. These results were further confirmed through Western blot analysis. HCT-116-APC(WT) cells and HCT-116-APC(KD) cells were treated with MMS for 5, 10, 15, and 25 h and the nuclear and cytosolic fractions were collected. These fractions were then used in Western blot analysis to determine the levels of APC and Fen-1 in the nucleus and the cytoplasm in MMS-treated HCT-116-APC(WT) and HCT-116-APC(KD) cells. An increase in cytoplasmic APC was seen in HCT-116-APC(WT) cells with the maximum increase being at 15 h after MMS treatment as compared to control HCT-116-APC(WT) cells. Interestingly, levels of nuclear Fen-1 were decreased in HCT-116-APC(WT) cells in response to MMS treatment with the maximum decrease seen at 15 h suggesting that an increase in cytoplasmic APC may lead to a reduction in nuclear Fen-1. As expected, this phenomenon was not seen in the cytoplasmic or nuclear fractions of MMS-treated HCT-116-APC(KD) cells. Levels of APC were not detected in the cytosolic or nuclear fractions of MMS treated HCT-116-APC(KD) cells, while Fen-1 levels showed no significant change in both the cytosolic and nuclear fractions. These results support the hypothesis that APC interacts with Fen-1 at its nuclear localization signal and blocks the translocation of Fen-1 to the nucleus. Interestingly, the highest level of nuclear APC in HCT-116-APC(WT) cells was seen at 25 h after MMS treatment. This correlated with a modest increase in the levels of nuclear Fen-1 providing further support for the hypothesis that APC can interact with Fen-1 and block its catalytic activity.

Combined, these results suggest another mechanism of APC-mediated block of BER by retaining Fen-1 in the cytoplasm in response to DNA alkylation damage. The data suggests that

APC becomes upregulated in response to MMS treatment. Over the course of treatment, APC interacts with Fen-1 at its nuclear localization signal in the cytoplasm and prevents it from entering the nucleus causing a loss of Fen-1 in the nucleus due to the fact that the Fen-1 protein cannot be replenished. In summation, APC can block LP-BER through its interaction with Fen-1 via two mechanisms. First, APC can block LP-BER by binding to Fen-1 and causing a block in both Fen-1 5'endonuclease and 5'-3'exonuclease cleavage activities. Secondly, in response to DNA damage, APC can block LP-BER by binding to Fen-1 at its nuclear localization signal and prevent it from replenishing nuclear Fen-1. Whether APC interacts with newly synthesized Fen-1 and whether post-translational modifications in APC and/or Fen-1 after DNA-alkylation damage causes cytoplasmic co-localization of APC and Fen-1 is not yet known.

Conclusions and Future Implications

Mutations in the *APC* gene are among the earliest events in colorectal carcinogenesis. The blockage of BER by APC may be viewed as a paradox as APC is generally viewed as a tumor suppressor. The blockage of BER by APC could serve as a tumor suppressor if the blockage of BER leads to apoptosis as a result of the accumulation of DNA damage. Indeed, studies have found that treatment of human colon cancer cells and mouse embryonic fibroblast cells with MMS enhanced the levels of APC and blocked BER, resulting in increased sensitivity and apoptosis of cells harboring damaged DNA (Kundu et al., 2007b; Narayan et al., 2005). Furthermore, it has been shown that HCT-116-APC(WT) cells treated with MMS exhibited decreased colony formation in a clonogenic assay when treated with MMS compared to HCT-116-APC(KD) cells treated with MMS indicating that increased levels of APC after MMS treatment blocks BER and decreases cell growth (Kundu et al., 2007b). These results indicate that APC plays a role as a tumor suppressor by removing cells containing damaged DNA. Alternatively, studies have shown that cigarette smoke condensate induces APC levels in

spontaneously immortalized normal human breast epithelial cells, blocks BER, and causes transformation of these cells (Kundu et al., 2007a). These findings point to a role of APC in carcinogenesis.

In conclusion, the findings of the present study indicate that APC can block LP-BER by interacting with Fen-1 through two different mechanisms: direct interaction with Fen-1 causing blockage of its endonuclease and exonuclease activities and by binding to Fen-1 in response to DNA damage and preventing Fen-1 from entering the nucleus to replenish nuclear Fen-1. Furthermore, it was discovered that the linker region of pol-β is responsible for the interaction with APC indicating that this region of pol-β may play a role in the complex regulation of the BER process *in vivo*. These proteins, including APC, may be involved in specific subpathways of BER that could be triggered by unknown signaling. Studies characterizing these subpathways and the signaling processes that control them will serve to shed some light on the regulation of BER *in vivo* and the role of APC in this regulation.

The role of APC in the blockage of LP-BER may provide clinical implications in the future. Since the DRI-domain is located toward the N-terminal region of the APC protein, which is spared by the MCR region, both wild-type and mutant APC proteins (containing the DRI-domain) may block DNA damage-induced LP-BER. Therefore, if the chemopreventative agents that produce DNA damage can increase APC levels in the target cell, then the decreased LP-BER and increased DNA damage may induce cell death. Thus, the link between APC and DNA repair may prove important in the development of chemotherapeutic drugs in the future.

LIST OF REFERENCES

Almeida, K.H., and Sobol, R.W. (2007). A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification. DNA Repair (Amst) *6*, 695-711.

American Cancer Society (2008). Cancer Facts and Figures (Atlanta, GA: American Cancer Society).

Aoki, K., and Taketo, M.M. (2007). Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. J Cell Sci *120*, 3327-3335.

Arezi, B., and Kuchta, R.D. (2000). Eukaryotic DNA primase. Trends Biochem Sci *25*, 572-576.

Baeg, G.H., Matsumine, A., Kuroda, T., Bhattacharjee, R.N., Miyashiro, I., Toyoshima, K., and Akiyama, T. (1995). The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase. EMBO J *14*, 5618-5625.

Baglioni, S., and Genuardi, M. (2004). Simple and complex genetics of colorectal cancer susceptibility. Am J Med Genet C Semin Med Genet *129C*, 35-43.

Balusu, R., Jaiswal, A.S., Armas, M.L., Kundu, C.N., Bloom, L.B., and Narayan, S. (2007). Structure/function analysis of the interaction of adenomatous polyposis coli with DNA polymerase beta and its implications for base excision repair. Biochemistry *46*, 13961-13974.

Bambara, R.A., Murante, R.S., and Henricksen, L.A. (1997). Enzymes and reactions at the eukaryotic DNA replication fork. J Biol Chem *272*, 4647-4650.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat Rev Mol Cell Biol *5*, 792-804.

Batra, V.K., Beard, W.A., Shock, D.D., Pedersen, L.C., and Wilson, S.H. (2005). Nucleotide-induced DNA polymerase active site motions accommodating a mutagenic DNA intermediate. Structure *13*, 1225-1233.

Beard, W.A., Shock, D.D., Yang, X.P., DeLauder, S.F., and Wilson, S.H. (2002). Loss of DNA polymerase beta stacking interactions with templating purines, but not pyrimidines, alters catalytic efficiency and fidelity. J Biol Chem *277*, 8235-8242.

Beard, W.A., and Wilson, S.H. (2006). Structure and mechanism of DNA polymerase Beta. Chem Rev *106*, 361-382.

Ben Yehuda, A., Globerson, A., Krichevsky, S., Bar On, H., Kidron, M., Friedlander, Y., Friedman, G., and Ben Yehuda, D. (2000). Ageing and the mismatch repair system. Mech Ageing Dev *121*, 173-179.

Beroud, C., and Soussi, T. (1996). APC gene: database of germline and somatic mutations in human tumors and cell lines. Nucleic Acids Res *24*, 121-124.

Bienz, M. (2002). The subcellular destinations of APC proteins. Nat Rev Mol Cell Biol *3*, 328-338.

Brancolini, C., Sgorbissa, A., and Schneider, C. (1998). Proteolytic processing of the adherens junctions components beta-catenin and gamma-catenin/plakoglobin during apoptosis. Cell Death Differ *5*, 1042-1050.

Briggs, M.W., and Sacks, D.B. (2003). IQGAP1 as signal integrator: Ca2+, calmodulin, Cdc42 and the cytoskeleton. FEBS Lett *542*, 7-11.

Browne, S.J., MacFarlane, M., Cohen, G.M., and Paraskeva, C. (1998). The adenomatous polyposis coli protein and retinoblastoma protein are cleaved early in apoptosis and are potential substrates for caspases. Cell Death Differ *5*, 206-213.

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

Caldecott, K.W. (2002). Polynucleotide kinase: a versatile molecule makes a clean break. Structure *10*, 1151-1152.

Campbell, P.M., and Szyf, M. (2003). Human DNA methyltransferase gene DNMT1 is regulated by the APC pathway. Carcinogenesis *24*, 17-24.

Chen, T., Yang, I., Irby, R., Shain, K.H., Wang, H.G., Quackenbush, J., Coppola, D., Cheng, J.Q., and Yeatman, T.J. (2003). Regulation of caspase expression and apoptosis by adenomatous polyposis coli. Cancer Res *63*, 4368-4374.

Clairmont, C.A., Narayanan, L., Sun, K.W., Glazer, P.M., and Sweasy, J.B. (1999). The Tyr-265-to-Cys mutator mutant of DNA polymerase beta induces a mutator phenotype in mouse LN12 cells. Proc Natl Acad Sci U S A *96*, 9580-9585.

Clement, G., Bosman, F.T., Fontolliet, C., and Benhattar, J. (2004). Monoallelic methylation of the APC promoter is altered in normal gastric mucosa associated with neoplastic lesions. Cancer Res *64*, 6867-6873.

Crawford, H.C., Fingleton, B.M., Rudolph-Owen, L.A., Goss, K.J., Rubinfeld, B., Polakis, P., and Matrisian, L.M. (1999). The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. Oncogene *18*, 2883-2891.

D'Incalci, M., Citti, L., Taverna, P., and Catapano, C.V. (1988). Importance of the DNA repair enzyme O6-alkyl guanine alkyltransferase (AT) in cancer chemotherapy. Cancer Treat Rev *15*, 279-292.

Dalal, S., Kosa, J.L., and Sweasy, J.B. (2004). The D246V mutant of DNA polymerase beta misincorporates nucleotides: evidence for a role for the flexible loop in DNA positioning within the active site. J Biol Chem *279*, 577-584.

Date, T., Yamamoto, S., Tanihara, K., Nishimoto, Y., Liu, N., and Matsukage, A. (1990). Site-directed mutagenesis of recombinant rat DNA polymerase beta: involvement of arginine-183 in primer recognition. Biochemistry *29*, 5027-5034.

Date, T., Yamamoto, S., Tanihara, K., Nishimoto, Y., and Matsukage, A. (1991). Aspartic acid residues at positions 190 and 192 of rat DNA polymerase beta are involved in primer binding. Biochemistry *30*, 5286-5292.

Deng, G., Song, G.A., Pong, E., Sleisenger, M., and Kim, Y.S. (2004). Promoter methylation inhibits APC gene expression by causing changes in chromatin conformation and interfering with the binding of transcription factor CCAAT-binding factor. Cancer Res *64*, 2692-2698.

Dianov, G.L., Prasad, R., Wilson, S.H., and Bohr, V.A. (1999). Role of DNA polymerase beta in the excision step of long patch mammalian base excision repair. J Biol Chem *274*, 13741-13743.

Dianova, II, Bohr, V.A., and Dianov, G.L. (2001). Interaction of human AP endonuclease 1 with flap endonuclease 1 and proliferating cell nuclear antigen involved in long-patch base excision repair. Biochemistry *40*, 12639-12644.

Dihlmann, S., Gebert, J., Siermann, A., Herfarth, C., and von Knebel Doeberitz, M. (1999). Dominant negative effect of the APC1309 mutation: a possible explanation for genotype-phenotype correlations in familial adenomatous polyposis. Cancer Res *59*, 1857-1860.

Dikovskaya, D., Newton, I.P., and Nathke, I.S. (2004). The adenomatous polyposis coli protein is required for the formation of robust spindles formed in CSF Xenopus extracts. Mol Biol Cell *15*, 2978-2991.

Draviam, V.M., Shapiro, I., Aldridge, B., and Sorger, P.K. (2006). Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. EMBO J *25*, 2814-2827.

El-Andaloussi, N., Valovka, T., Toueille, M., Hassa, P.O., Gehrig, P., Covic, M., Hubscher, U., and Hottiger, M.O. (2007). Methylation of DNA polymerase beta by protein arginine methyltransferase 1 regulates its binding to proliferating cell nuclear antigen. FASEB J *21*, 26-34.

El-Andaloussi, N., Valovka, T., Toueille, M., Steinacher, R., Focke, F., Gehrig, P., Covic, M., Hassa, P.O., Schar, P., Hubscher, U., and Hottiger, M.O. (2006). Arginine methylation regulates DNA polymerase beta. Mol Cell *22*, 51-62.

Erdmann, K.S., Kuhlmann, J., Lessmann, V., Herrmann, L., Eulenburg, V., Muller, O., and Heumann, R. (2000). The Adenomatous Polyposis Coli-protein (APC) interacts with the protein tyrosine phosphatase PTP-BL via an alternatively spliced PDZ domain. Oncogene *19*, 3894-3901.

Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S.J., *et al.* (2000). Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res *60*, 4366-4371.

Faux, M.C., Ross, J.L., Meeker, C., Johns, T., Ji, H., Simpson, R.J., Layton, M.J., and Burgess, A.W. (2004). Restoration of full-length adenomatous polyposis coli (APC) protein in a colon cancer cell line enhances cell adhesion. J Cell Sci *117*, 427-439.

Fodde, R., Kuipers, J., Rosenberg, C., Smits, R., Kielman, M., Gaspar, C., van Es, J.H., Breukel, C., Wiegant, J., Giles, R.H., and Clevers, H. (2001). Mutations in the APC tumour suppressor gene cause chromosomal instability. Nat Cell Biol *3*, 433-438.

Frank, G., Qiu, J., Zheng, L., and Shen, B. (2001). Stimulation of eukaryotic flap endonuclease-1 activities by proliferating cell nuclear antigen (PCNA) is independent of its in vitro interaction via a consensus PCNA binding region. J Biol Chem *276*, 36295-36302.

Gary, R., Park, M.S., Nolan, J.P., Cornelius, H.L., Kozyreva, O.G., Tran, H.T., Lobachev, K.S., Resnick, M.A., and Gordenin, D.A. (1999). A novel role in DNA metabolism for the binding of Fen1/Rad27 to PCNA and implications for genetic risk. Mol Cell Biol *19*, 5373-5382.

Gerson, S.L. (2004). MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer *4*, 296-307.

Gorbunova, V., and Levy, A.A. (1997). Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. Nucleic Acids Res *25*, 4650-4657.

Green, R.A., and Kaplan, K.B. (2003). Chromosome instability in colorectal tumor cells is associated with defects in microtubule plus-end attachments caused by a dominant mutation in APC. J Cell Biol *163*, 949-961.

Green, R.A., Wollman, R., and Kaplan, K.B. (2005). APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. Mol Biol Cell *16*, 4609-4622.

Grote, H.J., Schmiemann, V., Kiel, S., Bocking, A., Kappes, R., Gabbert, H.E., and Sarbia, M. (2004). Aberrant methylation of the adenomatous polyposis coli promoter 1A in bronchial aspirates from patients with suspected lung cancer. Int J Cancer *110*, 751-755.

Guo, Z., Chavez, V., Singh, P., Finger, L.D., Hang, H., Hegde, M.L., and Shen, B. (2008). Comprehensive mapping of the C-terminus of flap endonuclease-1 reveals distinct interaction sites for five proteins that represent different DNA replication and repair pathways. J Mol Biol *377*, 679-690.

Hamada, F., and Bienz, M. (2002). A Drosophila APC tumour suppressor homologue functions in cellular adhesion. Nat Cell Biol *4*, 208-213.

Hamada, F., and Bienz, M. (2004). The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. Dev Cell *7*, 677-685.

Hamid, S., and Eckert, K.A. (2005). Effect of DNA polymerase beta loop variants on discrimination of O6-methyldeoxyguanosine modification present in the nucleotide versus template substrate. Biochemistry *44*, 10378-10387.

Hanawalt, P.C. (2002). Subpathways of nucleotide excision repair and their regulation. Oncogene *21*, 8949-8956.

Hanson, C.A., and Miller, J.R. (2005). Non-traditional roles for the Adenomatous Polyposis Coli (APC) tumor suppressor protein. Gene *361*, 1-12.

Harrington, J.J., and Lieber, M.R. (1994). The characterization of a mammalian DNA structure-specific endonuclease. EMBO J *13*, 1235-1246.

Hasan, S., El-Andaloussi, N., Hardeland, U., Hassa, P.O., Burki, C., Imhof, R., Schar, P., and Hottiger, M.O. (2002). Acetylation regulates the DNA end-trimming activity of DNA polymerase beta. Mol Cell *10*, 1213-1222.

Hasan, S., Stucki, M., Hassa, P.O., Imhof, R., Gehrig, P., Hunziker, P., Hubscher, U., and Hottiger, M.O. (2001). Regulation of human flap endonuclease-1 activity by acetylation through the transcriptional coactivator p300. Mol Cell *7*, 1221-1231.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509-1512.

Hefferin, M.L., and Tomkinson, A.E. (2005). Mechanism of DNA double-strand break repair by non-homologous end joining. DNA Repair (Amst) *4*, 639-648.

Heinen, C.D., Goss, K.H., Cornelius, J.R., Babcock, G.F., Knudsen, E.S., Kowalik, T., and Groden, J. (2002). The APC tumor suppressor controls entry into S-phase through its ability to regulate the cyclin D/RB pathway. Gastroenterology *123*, 751-763.

Helleday, T. (2003). Pathways for mitotic homologous recombination in mammalian cells. Mutat Res *532*, 103-115.

Henderson, B.R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat Cell Biol *2*, 653-660.

Henderson, B.R., and Fagotto, F. (2002). The ins and outs of APC and beta-catenin nuclear transport. EMBO Rep *3*, 834-839.

Henneke, G., Koundrioukoff, S., and Hubscher, U. (2003). Phosphorylation of human Fen1 by cyclin-dependent kinase modulates its role in replication fork regulation. Oncogene *22*, 4301-4313.

Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F., *et al.* (2000). Identification of CDK4 as a target of c-MYC. Proc Natl Acad Sci U S A *97*, 2229-2234.

Hosfield, D.J., Frank, G., Weng, Y., Tainer, J.A., and Shen, B. (1998). Newly discovered archaebacterial flap endonucleases show a structure-specific mechanism for DNA substrate binding and catalysis resembling human flap endonuclease-1. J Biol Chem *273*, 27154-27161.

Houtgraaf, J.H., Versmissen, J., and van der Giessen, W.J. (2006). A concise review of DNA damage checkpoints and repair in mammalian cells. Cardiovasc Revasc Med *7*, 165-172.

Hughes, S.A., Carothers, A.M., Hunt, D.H., Moran, A.E., Mueller, J.D., and Bertagnolli, M.M. (2002). Adenomatous polyposis coli truncation alters cytoskeletal structure and microtubule stability in early intestinal tumorigenesis. J Gastrointest Surg *6*, 868-874; discussion 875.

Ischenko, A.A., and Saparbaev, M.K. (2002). Alternative nucleotide incision repair pathway for oxidative DNA damage. Nature *415*, 183-187.

Ishidate, T., Matsumine, A., Toyoshima, K., and Akiyama, T. (2000). The APC-hDLG complex negatively regulates cell cycle progression from the G0/G1 to S phase. Oncogene *19*, 365-372.

Ishikawa, K., Ishii, H., and Saito, T. (2006). DNA damage-dependent cell cycle checkpoints and genomic stability. DNA Cell Biol *25*, 406-411.

Jackson, S.P. (2002). Sensing and repairing DNA double-strand breaks. Carcinogenesis *23*, 687-696.

Jaiswal, A.S., Balusu, R., Armas, M.L., Kundu, C.N., and Narayan, S. (2006). Mechanism of adenomatous polyposis coli (APC)-mediated blockage of long-patch base excision repair. Biochemistry *45*, 15903-15914.

Jaiswal, A.S., Marlow, B.P., Gupta, N., and Narayan, S. (2002). Beta-catenin-mediated transactivation and cell-cell adhesion pathways are important in curcumin (diferuylmethane)-induced growth arrest and apoptosis in colon cancer cells. Oncogene *21*, 8414-8427.

Jaiswal, A.S., and Narayan, S. (2001). p53-dependent transcriptional regulation of the APC promoter in colon cancer cells treated with DNA alkylating agents. J Biol Chem *276*, 18193-18199.

Jaiswal, A.S., and Narayan, S. (2004). Reduced levels of the adenomatous polyposis coli (APC) protein are associated with ceramide-induced apoptosis of colon cancer cells. J Cancer Res Clin Oncol *130*, 695-703.

Jimbo, T., Kawasaki, Y., Koyama, R., Sato, R., Takada, S., Haraguchi, K., and Akiyama, T. (2002). Identification of a link between the tumour suppressor APC and the kinesin superfamily. Nat Cell Biol *4*, 323-327.

Jiricny, J. (2006). The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol *7*, 335-346.

Johnson, K.A. (1993). Conformational coupling in DNA polymerase fidelity. Annu Rev Biochem *62*, 685-713.

Kang, H.Y., Choi, E., Bae, S.H., Lee, K.H., Gim, B.S., Kim, H.D., Park, C., MacNeill, S.A., and Seo, Y.S. (2000). Genetic analyses of Schizosaccharomyces pombe dna2(+) reveal that dna2 plays an essential role in Okazaki fragment metabolism. Genetics *155*, 1055-1067.

Kaplan, K.B., Burds, A.A., Swedlow, J.R., Bekir, S.S., Sorger, P.K., and Nathke, I.S. (2001). A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nat Cell Biol *3*, 429-432.

Karran, P. (1996). Microsatellite instability and DNA mismatch repair in human cancer. Semin Cancer Biol *7*, 15-24.

Kastan, M.B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Kawasaki, Y., Sato, R., and Akiyama, T. (2003). Mutated APC and Asef are involved in the migration of colorectal tumour cells. Nat Cell Biol *5*, 211-215.

Kawasaki, Y., Senda, T., Ishidate, T., Koyama, R., Morishita, T., Iwayama, Y., Higuchi, O., and Akiyama, T. (2000). Asef, a link between the tumor suppressor APC and G-protein signaling. Science *289*, 1194-1197.

Kay, A.J., and Hunter, C.P. (2001). CDC-42 regulates PAR protein localization and function to control cellular and embryonic polarity in C. elegans. Curr Biol *11*, 474-481.

Khanna, K.K., and Jackson, S.P. (2001). DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet *27*, 247-254.

Klungland, A., and Lindahl, T. (1997). Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). EMBO J *16*, 3341-3348.

Kosa, J.L., and Sweasy, J.B. (1999). The E249K mutator mutant of DNA polymerase beta extends mispaired termini. J Biol Chem *274*, 35866-35872.

Krahn, J.M., Beard, W.A., and Wilson, S.H. (2004). Structural insights into DNA polymerase beta deterrents for misincorporation support an induced-fit mechanism for fidelity. Structure *12*, 1823-1832.

Kraynov, V.S., Showalter, A.K., Liu, J., Zhong, X., and Tsai, M.D. (2000). DNA polymerase beta: contributions of template-positioning and dNTP triphosphate-binding residues to catalysis and fidelity. Biochemistry *39*, 16008-16015.

Kroboth, K., Newton, I.P., Kita, K., Dikovskaya, D., Zumbrunn, J., Waterman-Storer, C.M., and Nathke, I.S. (2007). Lack of adenomatous polyposis coli protein correlates with a decrease in cell migration and overall changes in microtubule stability. Mol Biol Cell *18*, 910-918.

Kundu, C.N., Balusu, R., Jaiswal, A.S., Gairola, C.G., and Narayan, S. (2007a). Cigarette smoke condensate-induced level of adenomatous polyposis coli blocks long-patch base excision repair in breast epithelial cells. Oncogene *26*, 1428-1438.

Kundu, C.N., Balusu, R., Jaiswal, A.S., and Narayan, S. (2007b). Adenomatous polyposis coli-mediated hypersensitivity of mouse embryonic fibroblast cell lines to methylmethane sulfonate treatment: implication of base excision repair pathways. Carcinogenesis *28*, 2089-2095.

Lieber, M.R. (1997). The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. Bioessays *19*, 233-240.

Lieber, M.R. (1999). The biochemistry and biological significance of nonhomologous DNA end joining: an essential repair process in multicellular eukaryotes. Genes Cells *4*, 77-85.

Lieber, M.R., Ma, Y., Pannicke, U., and Schwarz, K. (2003). Mechanism and regulation of human non-homologous DNA end-joining. Nat Rev Mol Cell Biol *4*, 712-720.

Lindahl, T. (1971). The action pattern of mammalian deoxyribonuclease IV. Eur J Biochem *18*, 415-421.

Liu, Y., Beard, W.A., Shock, D.D., Prasad, R., Hou, E.W., and Wilson, S.H. (2005). DNA polymerase beta and flap endonuclease 1 enzymatic specificities sustain DNA synthesis for long patch base excision repair. J Biol Chem *280*, 3665-3674.

Liu, Y., Kao, H.I., and Bambara, R.A. (2004). Flap endonuclease 1: a central component of DNA metabolism. Annu Rev Biochem *73*, 589-615.

Louie, R.K., Bahmanyar, S., Siemers, K.A., Votin, V., Chang, P., Stearns, T., Nelson, W.J., and Barth, A.I. (2004). Adenomatous polyposis coli and EB1 localize in close proximity of the mother centriole and EB1 is a functional component of centrosomes. J Cell Sci *117*, 1117-1128.

Matsumine, A., Ogai, A., Senda, T., Okumura, N., Satoh, K., Baeg, G.H., Kawahara, T., Kobayashi, S., Okada, M., Toyoshima, K., and Akiyama, T. (1996). Binding of APC to the human homolog of the Drosophila discs large tumor suppressor protein. Science *272*, 1020-1023.

Matsumoto, Y., and Kim, K. (1995). Excision of deoxyribose phosphate residues by DNA polymerase beta during DNA repair. Science *269*, 699-702.

Menge, K.L., Hostomsky, Z., Nodes, B.R., Hudson, G.O., Rahmati, S., Moomaw, E.W., Almassy, R.J., and Hostomska, Z. (1995). Structure-function analysis of the mammalian DNA polymerase beta active site: role of aspartic acid 256, arginine 254, and arginine 258 in nucleotidyl transfer. Biochemistry *34*, 15934-15942.

Middleton, M.R., and Margison, G.P. (2003). Improvement of chemotherapy efficacy by inactivation of a DNA-repair pathway. Lancet Oncol *4*, 37-44.

Mitra, S., Boldogh, I., Izumi, T., and Hazra, T.K. (2001). Complexities of the DNA base excision repair pathway for repair of oxidative DNA damage. Environ Mol Mutagen *38*, 180-190.

Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T., and Nakamura, Y. (1992). Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum Mol Genet *1*, 229-233.

Mordes, D.A., Glick, G.G., Zhao, R., and Cortez, D. (2008). TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. Genes Dev *22*, 1478-1489.

Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1996). Apoptosis and APC in colorectal tumorigenesis. Proc Natl Acad Sci U S A *93*, 7950-7954.

Morrison, E.E., Wardleworth, B.N., Askham, J.M., Markham, A.F., and Meredith, D.M. (1998). EB1, a protein which interacts with the APC tumour suppressor, is associated with the microtubule cytoskeleton throughout the cell cycle. Oncogene *17*, 3471-3477.

Moss, S.F., Liu, T.C., Petrotos, A., Hsu, T.M., Gold, L.I., and Holt, P.R. (1996). Inward growth of colonic adenomatous polyps. Gastroenterology *111*, 1425-1432.

Mullen, G.P., and Wilson, S.H. (1997). DNA polymerase beta in abasic site repair: a structurally conserved helix-hairpin-helix motif in lesion detection by base excision repair enzymes. Biochemistry *36*, 4713-4717.

Murante, R.S., Huang, L., Turchi, J.J., and Bambara, R.A. (1994). The calf 5'- to 3'-exonuclease is also an endonuclease with both activities dependent on primers annealed upstream of the point of cleavage. J Biol Chem *269*, 1191-1196.

Murante, R.S., Rust, L., and Bambara, R.A. (1995). Calf 5' to 3' exo/endonuclease must slide from a 5' end of the substrate to perform structure-specific cleavage. J Biol Chem *270*, 30377-30383.

Nakamura, M., Zhou, X.Z., and Lu, K.P. (2001). Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. Curr Biol *11*, 1062-1067.

Narayan, S., and Jaiswal, A.S. (1997). Activation of adenomatous polyposis coli (APC) gene expression by the DNA-alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine requires p53. J Biol Chem *272*, 30619-30622.

Narayan, S., Jaiswal, A.S., and Balusu, R. (2005). Tumor suppressor APC blocks DNA polymerase beta-dependent strand displacement synthesis during long patch but not short patch base excision repair and increases sensitivity to methylmethane sulfonate. J Biol Chem *280*, 6942-6949.

Narayan, S., and Roy, D. (2003). Role of APC and DNA mismatch repair genes in the development of colorectal cancers. Mol Cancer *2*, 41.

Nateri, A.S., Spencer-Dene, B., and Behrens, A. (2005). Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. Nature *437*, 281-285.

Nathke, I.S. (2004). The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. Annu Rev Cell Dev Biol *20*, 337-366.

Neri, S., Gardini, A., Facchini, A., Olivieri, F., Franceschi, C., Ravaglia, G., and Mariani, E. (2005). Mismatch repair system and aging: microsatellite instability in peripheral blood cells from differently aged participants. J Gerontol A Biol Sci Med Sci *60*, 285-292.

Niimi, N., Sugo, N., Aratani, Y., and Koyama, H. (2005). Genetic interaction between DNA polymerase beta and DNA-PKcs in embryogenesis and neurogenesis. Cell Death Differ *12*, 184-191.

Nyberg, K.A., Michelson, R.J., Putnam, C.W., and Weinert, T.A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet *36*, 617-656.

Opresko, P.L., Shiman, R., and Eckert, K.A. (2000). Hydrophobic interactions in the hinge domain of DNA polymerase beta are important but not sufficient for maintaining fidelity of DNA synthesis. Biochemistry *39*, 11399-11407.

Opresko, P.L., Sweasy, J.B., and Eckert, K.A. (1998). The mutator form of polymerase beta with amino acid substitution at tyrosine 265 in the hinge region displays an increase in both base substitution and frame shift errors. Biochemistry *37*, 2111-2119.

Orford, K., Crockett, C., Jensen, J.P., Weissman, A.M., and Byers, S.W. (1997). Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem *272*, 24735-24738.

Parrish, J.Z., Yang, C., Shen, B., and Xue, D. (2003). CRN-1, a Caenorhabditis elegans FEN-1 homologue, cooperates with CPS-6/EndoG to promote apoptotic DNA degradation. EMBO J *22*, 3451-3460.

Peifer, M., and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis--a look outside the nucleus. Science *287*, 1606-1609.

Pelletier, H., Sawaya, M.R., Kumar, A., Wilson, S.H., and Kraut, J. (1994). Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP. Science *264*, 1891-1903.

Pelletier, H., Sawaya, M.R., Wolfle, W., Wilson, S.H., and Kraut, J. (1996). Crystal structures of human DNA polymerase beta complexed with DNA: implications for catalytic mechanism, processivity, and fidelity. Biochemistry *35*, 12742-12761.

Plo, I., Liao, Z.Y., Barcelo, J.M., Kohlhagen, G., Caldecott, K.W., Weinfeld, M., and Pommier, Y. (2003). Association of XRCC1 and tyrosyl DNA phosphodiesterase (Tdp1) for the repair of topoisomerase I-mediated DNA lesions. DNA Repair (Amst) *2*, 1087-1100.

Plug, A.W., Clairmont, C.A., Sapi, E., Ashley, T., and Sweasy, J.B. (1997). Evidence for a role for DNA polymerase beta in mammalian meiosis. Proc Natl Acad Sci U S A *94*, 1327-1331.

Podlutsky, A.J., Dianova, II, Podust, V.N., Bohr, V.A., and Dianov, G.L. (2001). Human DNA polymerase beta initiates DNA synthesis during long-patch repair of reduced AP sites in DNA. EMBO J *20*, 1477-1482.

Polakis, P. (1997). The adenomatous polyposis coli (APC) tumor suppressor. Biochim Biophys Acta *1332*, F127-147.

Prasad, R., Batra, V.K., Yang, X.P., Krahn, J.M., Pedersen, L.C., Beard, W.A., and Wilson, S.H. (2005). Structural insight into the DNA polymerase beta deoxyribose phosphate lyase mechanism. DNA Repair (Amst) *4*, 1347-1357.

Prasad, R., Beard, W.A., Chyan, J.Y., Maciejewski, M.W., Mullen, G.P., and Wilson, S.H. (1998a). Functional analysis of the amino-terminal 8-kDa domain of DNA polymerase beta as revealed by site-directed mutagenesis. DNA binding and 5'-deoxyribose phosphate lyase activities. J Biol Chem *273*, 11121-11126.

Prasad, R., Beard, W.A., Strauss, P.R., and Wilson, S.H. (1998b). Human DNA polymerase beta deoxyribose phosphate lyase. Substrate specificity and catalytic mechanism. J Biol Chem *273*, 15263-15270.

Prasad, R., Dianov, G.L., Bohr, V.A., and Wilson, S.H. (2000). FEN1 stimulation of DNA polymerase beta mediates an excision step in mammalian long patch base excision repair. J Biol Chem *275*, 4460-4466.

Prasad, R., Kumar, A., Widen, S.G., Casas-Finet, J.R., and Wilson, S.H. (1993). Identification of residues in the single-stranded DNA-binding site of the 8-kDa domain of rat DNA polymerase beta by UV cross-linking. J Biol Chem *268*, 22746-22755.

Prasad, R., Lavrik, O.I., Kim, S.J., Kedar, P., Yang, X.P., Vande Berg, B.J., and Wilson, S.H. (2001). DNA polymerase beta -mediated long patch base excision repair. Poly(ADP-ribose)polymerase-1 stimulates strand displacement DNA synthesis. J Biol Chem *276*, 32411-32414.

Qiu, J., Li, X., Frank, G., and Shen, B. (2001). Cell cycle-dependent and DNA damage-inducible nuclear localization of FEN-1 nuclease is consistent with its dual functions in DNA replication and repair. J Biol Chem *276*, 4901-4908.

Qiu, J., Liu, R., Chapados, B.R., Sherman, M., Tainer, J.A., and Shen, B. (2004). Interaction interface of human flap endonuclease-1 with its DNA substrates. J Biol Chem *279*, 24394-24402.

Qiu, J., Qian, Y., Frank, P., Wintersberger, U., and Shen, B. (1999). Saccharomyces cerevisiae RNase H(35) functions in RNA primer removal during lagging-strand DNA synthesis, most efficiently in cooperation with Rad27 nuclease. Mol Cell Biol *19*, 8361-8371.

Ranalli, T.A., Tom, S., and Bambara, R.A. (2002). AP endonuclease 1 coordinates flap endonuclease 1 and DNA ligase I activity in long patch base excision repair. J Biol Chem *277*, 41715-41724.

Sabharwal, A., and Middleton, M.R. (2006). Exploiting the role of O6-methylguanine-DNA-methyltransferase (MGMT) in cancer therapy. Curr Opin Pharmacol *6*, 355-363.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S.*, et al.* (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev *18*, 1385-1390.

Sargent, R.G., Brenneman, M.A., and Wilson, J.H. (1997). Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. Mol Cell Biol *17*, 267-277.

Sawaya, M.R., Prasad, R., Wilson, S.H., Kraut, J., and Pelletier, H. (1997). Crystal structures of human DNA polymerase beta complexed with gapped and nicked DNA: evidence for an induced fit mechanism. Biochemistry *36*, 11205-11215.

Schmeiser, K., and Grand, R.J. (1999). The fate of E- and P-cadherin during the early stages of apoptosis. Cell Death Differ *6*, 377-386.

Schweitzer, J.K., and Livingston, D.M. (1998). Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation. Hum Mol Genet *7*, 69-74.

Scott, S.P., and Pandita, T.K. (2006). The cellular control of DNA double-strand breaks. J Cell Biochem *99*, 1463-1475.

Seeling, J.M., Miller, J.R., Gil, R., Moon, R.T., White, R., and Virshup, D.M. (1999). Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science *283*, 2089-2091.

Seluanov, A., Mittelman, D., Pereira-Smith, O.M., Wilson, J.H., and Gorbunova, V. (2004). DNA end joining becomes less efficient and more error-prone during cellular senescence. Proc Natl Acad Sci U S A *101*, 7624-7629.

Seo, E., and Jho, E.H. (2007). Axin-independent phosphorylation of APC controls beta-catenin signaling via cytoplasmic retention of beta-catenin. Biochem Biophys Res Commun *357*, 81-86.

Shah, A.M., Li, S.X., Anderson, K.S., and Sweasy, J.B. (2001). Y265H mutator mutant of DNA polymerase beta. Proper teometric alignment is critical for fidelity. J Biol Chem *276*, 10824-10831.

Shah, A.M., Maitra, M., and Sweasy, J.B. (2003). Variants of DNA polymerase Beta extend mispaired DNA due to increased affinity for nucleotide substrate. Biochemistry *42*, 10709-10717.

Shapiro, D.J., Sharp, P.A., Wahli, W.W., and Keller, M.J. (1988). A high-efficiency HeLa cell nuclear transcription extract. DNA *7*, 47-55.

Sharma, S., Sommers, J.A., Gary, R.K., Friedrich-Heineken, E., Hubscher, U., and Brosh, R.M., Jr. (2005). The interaction site of Flap Endonuclease-1 with WRN helicase suggests a coordination of WRN and PCNA. Nucleic Acids Res *33*, 6769-6781.

Shen, B., Nolan, J.P., Sklar, L.A., and Park, M.S. (1997). Functional analysis of point mutations in human flap endonuclease-1 active site. Nucleic Acids Res *25*, 3332-3338.

Shen, B., Qiu, J., Hosfield, D., and Tainer, J.A. (1998). Flap endonuclease homologs in archaebacteria exist as independent proteins. Trends Biochem Sci *23*, 171-173.

Shen, B., Singh, P., Liu, R., Qiu, J., Zheng, L., Finger, L.D., and Alas, S. (2005). Multiple but dissectible functions of FEN-1 nucleases in nucleic acid processing, genome stability and diseases. Bioessays *27*, 717-729.

Shibata, Y., and Nakamura, T. (2002). Defective flap endonuclease 1 activity in mammalian cells is associated with impaired DNA repair and prolonged S phase delay. J Biol Chem *277*, 746-754.

Showalter, A.K., and Tsai, M.D. (2002). A reexamination of the nucleotide incorporation fidelity of DNA polymerases. Biochemistry *41*, 10571-10576.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A *96*, 5522-5527.

Siegal, G., Turchi, J.J., Myers, T.W., and Bambara, R.A. (1992). A 5' to 3' exonuclease functionally interacts with calf DNA polymerase epsilon. Proc Natl Acad Sci U S A *89*, 9377-9381.

Skinner, A.M., and Turker, M.S. (2005). Oxidative mutagenesis, mismatch repair, and aging. Sci Aging Knowledge Environ *2005*, re3.

Smits, R., Kielman, M.F., Breukel, C., Zurcher, C., Neufeld, K., Jagmohan-Changur, S., Hofland, N., van Dijk, J., White, R., Edelmann, W.*, et al.* (1999). Apc1638T: a mouse model delineating critical domains of the adenomatous polyposis coli protein involved in tumorigenesis and development. Genes Dev *13*, 1309-1321.

Sobol, R.W., Prasad, R., Evenski, A., Baker, A., Yang, X.P., Horton, J.K., and Wilson, S.H. (2000). The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity. Nature *405*, 807-810.

Sobol, R.W., and Wilson, S.H. (2001). Mammalian DNA beta-polymerase in base excision repair of alkylation damage. Prog Nucleic Acid Res Mol Biol *68*, 57-74.

Srivastava, D.K., Berg, B.J., Prasad, R., Molina, J.T., Beard, W.A., Tomkinson, A.E., and Wilson, S.H. (1998). Mammalian abasic site base excision repair. Identification of the reaction sequence and rate-determining steps. J Biol Chem *273*, 21203-21209.

Starcevic, D., Dalal, S., and Sweasy, J. (2005). Hinge residue Ile260 of DNA polymerase beta is important for enzyme activity and fidelity. Biochemistry *44*, 3775-3784.

Steigerwald, K., Behbehani, G.K., Combs, K.A., Barton, M.C., and Groden, J. (2005). The APC tumor suppressor promotes transcription-independent apoptosis in vitro. Mol Cancer Res *3*, 78-89.

Su, L.K., Burrell, M., Hill, D.E., Gyuris, J., Brent, R., Wiltshire, R., Trent, J., Vogelstein, B., and Kinzler, K.W. (1995). APC binds to the novel protein EB1. Cancer Res *55*, 2972-2977.

Su, L.K., Johnson, K.A., Smith, K.J., Hill, D.E., Vogelstein, B., and Kinzler, K.W. (1993). Association between wild type and mutant APC gene products. Cancer Res *53*, 2728-2731.

Su, L.K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., and Dove, W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256*, 668-670.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422-426.

Tighe, A., Johnson, V.L., and Taylor, S.S. (2004). Truncating APC mutations have dominant effects on proliferation, spindle checkpoint control, survival and chromosome stability. J Cell Sci *117*, 6339-6353.

Tseng, H.M., and Tomkinson, A.E. (2004). Processing and joining of DNA ends coordinated by interactions among Dnl4/Lif1, Pol4, and FEN-1. J Biol Chem *279*, 47580-47588.

Tsuchiya, T., Tamura, G., Sato, K., Endoh, Y., Sakata, K., Jin, Z., Motoyama, T., Usuba, O., Kimura, W., Nishizuka, S.*, et al.* (2000). Distinct methylation patterns of two APC gene promoters in normal and cancerous gastric epithelia. Oncogene *19*, 3642-3646.

Turchi, J.J., and Bambara, R.A. (1993). Completion of mammalian lagging strand DNA replication using purified proteins. J Biol Chem *268*, 15136-15141.

Turchi, J.J., Huang, L., Murante, R.S., Kim, Y., and Bambara, R.A. (1994). Enzymatic completion of mammalian lagging-strand DNA replication. Proc Natl Acad Sci U S A *91*, 9803-9807.

Wahl, G.M., and Carr, A.M. (2001). The evolution of diverse biological responses to DNA damage: insights from yeast and p53. Nat Cell Biol *3*, E277-286.

Watanabe, T., Wang, S., Noritake, J., Sato, K., Fukata, M., Takefuji, M., Nakagawa, M., Izumi, N., Akiyama, T., and Kaibuchi, K. (2004). Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. Dev Cell *7*, 871-883.

Wen, Y., Eng, C.H., Schmoranzer, J., Cabrera-Poch, N., Morris, E.J., Chen, M., Wallar, B.J., Alberts, A.S., and Gundersen, G.G. (2004). EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. Nat Cell Biol *6*, 820-830.

White, R.N. (1999). Management of a proximal pelvic limb skin laceration in a dog using a skin flap and an external fixator. J Small Anim Pract *40*, 84-87.

Wiederhold, L., Leppard, J.B., Kedar, P., Karimi-Busheri, F., Rasouli-Nia, A., Weinfeld, M., Tomkinson, A.E., Izumi, T., Prasad, R., Wilson, S.H.*, et al.* (2004). AP endonuclease-independent DNA base excision repair in human cells. Mol Cell *15*, 209-220.

Williams, A.C., Hague, A., Elder, D.J., and Paraskeva, C. (1996). In vitro models for studying colorectal carcinogenesis: cellular and molecular events including APC and Rb cleavage in the control of proliferation, differentiation and apoptosis. Biochim Biophys Acta *1288*, F9-19.

Wilson, T.E., and Lieber, M.R. (1999). Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway. J Biol Chem *274*, 23599-23609.

Yang, J., Zhang, W., Evans, P.M., Chen, X., He, X., and Liu, C. (2006). Adenomatous polyposis coli (APC) differentially regulates beta-catenin phosphorylation and ubiquitination in colon cancer cells. J Biol Chem *281*, 17751-17757.

Zhang, T., Otevrel, T., Gao, Z., Ehrlich, S.M., Fields, J.Z., and Boman, B.M. (2001). Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res *61*, 8664-8667.

Zheng, L., Li, M., Shan, J., Krishnamoorthi, R., and Shen, B. (2002). Distinct roles of two Mg2+ binding sites in regulation of murine flap endonuclease-1 activities. Biochemistry *41*, 10323-10331.

Zheng, L., Zhou, M., Chai, Q., Parrish, J., Xue, D., Patrick, S.M., Turchi, J.J., Yannone, S.M., Chen, D., and Shen, B. (2005). Novel function of the flap endonuclease 1 complex in processing stalled DNA replication forks. EMBO Rep *6*, 83-89.

Zou, L., Cortez, D., and Elledge, S.J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev *16*, 198-208.

BIOGRAPHICAL SKETCH

Melissa Liz Armas was born in Miami, Florida, to Cuban parents. She is the only daughter of Mayra and Eser Armas. Since an early age, she was interested in Science. Melissa attended Southwest Miami Senior High School, where she was an honors student and was active in the Phi Beta Chi Science club. She was also active in the Foreign Language club and the Future Business Leaders of America.

After graduating high school, Melissa attended the University of South Florida, where she received a B.S. in biology. She was also part of the Honors Program. Melissa was accepted into the Ronald E. McNair Postbacculaurete Achievement Program. As a McNair Scholar, Melissa received her first research experience. Her research project was on Brain cancer (Glioma) cells. She was able to present her research at McNair conferences and the Honors research symposia.

After graduating from the University of South Florida, Melissa enrolled into the IDP program at the University of Florida to pursue her doctorate. She pursued her doctoral research on Adenomatous Polyposis Coli (APC) and its interaction with Base Excision Repair proteins: Its role in colorectal carcinogenesis in the laboratory of Satya Narayan. Melissa has presented her research in departmental seminars and has had two abstracts presented at national meetings.

Melissa plans to pursue a master's in public health. By combining the knowledge from her doctorate and her master's, Melissa plans to focus on Health Policy and its relation to minority populations.