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Regulation of BACH1/FANCJ Function in DNA Damage Repair: A Dissertation

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REGULATION OF BACH1/FANCJ FUNCTION IN DNA DAMAGE REPAIR

A Dissertation Presented

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By
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Statement of Contribution

The work presented in Chapter 2 was collaboration with research associate Ming Peng and previous graduate student Rachel Flynn. The publication that resulted from this collaboration was a second author paper with Min and Rachel as co-first authors. The work presented in Chapter 4 was collaboration with Rachel Flynn and Shu Wang, a previous post-doc in Dr. Cantor's lab. The potential publication will be a co-first author paper between Rachel, Shu and myself. Min also contributed to the chromatin immunoprecipitation experiment in Chapter 3 and the sensitivity assay in Chapter 4. Our collaborator Dr. Robert Brosh preformed the *in vitro* ELISA and helicase assays in Chapter 2. In addition, another collaborator Dr. Jianyuan Luo performed the *in vitro* acetylation assay in Chapter 5.

Regulation of BACH1/FANCJ function in DNA damage repair

ABSTRACT

The DNA damage response (DDR) pathway is a complicated network of interacting proteins that function to sense and remove DNA damage. Upon exposure to DNA damage, a signaling cascade is generated. The damage is either removed, restoring the original genetic sequence, or apoptosis is activated. In the absence of DDR, cells are unable to effectively process DNA damage. Unprocessed DNA damage can lead to chromosomal changes, gene mutations, and malignant transformation. Thus, the proteins involved in DDR are critical for maintaining genomic stability.

One essential DDR protein is the BRCA1 Associated C-terminal Helicase, BACH1. BACH1 was initially identified through its direct association with the BRCT domain of the Breast Cancer Associated Gene, BRCA1. Similar to BRCA1, germline mutations in BACH1were identified in patients with early onset breast cancer. Interestingly, the disease-associated mutations in BACH1 were shown to have altered helicase activity *in vitro*, providing a direct link between BACH1 helicase activity and disease development. The correlation between BACH1 and cancer predisposition was further confirmed by the identification of BACH1 as the cancer syndrome Fanconi anemia (FA) gene product, FANCJ. Similar to other FA proteins, suppression of FANCJ leads to decreased

homologous recombination, enhanced sensitivity to DNA interstrand crosslinking (ICL) agents, and chromosomal instability.

In an effort to further understand the function of FANCJ in DDR, FANCJ was shown to directly associate with the mismatch repair (MMR) protein MLH1. This interaction is facilitated by lysines 141 and 142 within the helicase domain of FANCJ. Importantly, the FANCJ/MLH1 interaction is critical for ICL repair. Furthermore, in an attempt to dissect the binding site of FANCJ on MLH1, we discovered an HNPCC associated MLH1 mutation (L607H) that has intact mismatch repair, but lacks FANCJ interaction. In contrast to the MLH1 interaction, the FANCJ/BRCA1 interaction was not required for correcting the cellular defects in FANCJ null cells. Thus, in an effort to understand the functional significance of the FANCJ/BRCA1 interaction, we discovered that FANCJ promotes Pol η dependent translesion synthesis (TLS) bypass when uncoupled from BRCA1. In this thesis, we provide evidence suggesting that FANCJ and MLH1 are functionally linked and that the interaction of these proteins is critical for repair choice.

CHAPTER I

INTRODUCTION

DNA Damage Response

Approximately 30,000 DNA lesions are generated spontaneously in a mammalian cell per day (Lindahl and Barnes, 2000). These lesions can interfere with normal DNA replication and transcription, thus detecting and processing such lesions is of critical importance to maintain genomic stability. Mammalian cells have evolved a complex DNA damage response (DDR) network for detecting DNA lesions, initiating cell cycle checkpoints, repair pathways, and/or apoptosis. Unprocessed DNA damage can lead to chromosomal changes, gene mutations, and malignant transformation. Not surprisingly, cancer susceptibility is intimately linked to mutations in the DDR genes which can either be inherited or acquired during an organism's lifetime.

The DDR is a network of interacting pathways that are activated by DNA damage. DNA damage stems from endogenous sources, such as cellular metabolic byproducts including reactive oxygen species, base hydrolysis, and cytosine deamination (Lindahl and Barnes, 2000). Exogenous sources of DNA damage arise from environmental factors including ionizing radiation (IR) and ultraviolet (UV) radiation (Friedberg E, 2006). Damaging agents give rise to a wide range of DNA adducts including oxidation, alkylation, and hydrolysis of bases (Friedberg E, 2006). These types of DNA adducts when processed can

generate DNA lesions including base-base mismatches, single stranded breaks (SSB), double stranded breaks (DSBs), and covalent linkages of opposite strands of DNA called interstrand cross links (ICLs) (Friedberg E, 2006).

The DDR network is responsible for recognizing and processing DNA lesions. Proteins involved in DDR are categorized as sensors, mediators, transducers, and effectors (Niida and Nakanishi, 2006). Sensor proteins are responsible for recognizing DNA damage. Although the identities of sensor proteins are not well characterized there are several possible candidates including proteins of the 9-1-1 complex (Rad9, Rad1, and Hus1), Rad17, and the mismatch repair proteins (Duckett et al., 1996; Longhese et al., 1998; Melo and Toczyski, 2002; Paulovich et al., 1998; Yamada et al., 1997; Zhang et al., 2002). Once DNA lesions are recognized, mediator and transducer proteins are recruited to facilitate downstream repair signaling. Mediator proteins facilitate DNA damage signaling by promoting interactions between components of the DDR pathway. Most mediator proteins contain BRCA1 carboxyl terminal (BRCT) domains, which function as protein-phosphoprotein interaction modules (Manke et al., 2003; Yu et al., 2003). Transducer proteins affect the activity of downstream proteins through regulation of phosphorylation and de-phosphorylation. Kinases including Ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad-3-related (ATR), Chk1, and Chk2 are well characterized transducer proteins. These transducer proteins phosphorylate many effectors in the sequence context of Serine-Glutamine (S/Q) or Threonine- Glutamine (TQ)

(Niida and Nakanishi, 2006). Once activated, effector proteins initiate the activation of cell cycle checkpoints.

In response to DNA damage, DDR network can arrest cells in the G1/S, intra-S, or G2/M phases to allow time for lesion repair. The G1/S checkpoint can occur at the end of G1 phase, just before the entry into S-phase. This checkpoint is a decision step for determining whether a cell should duplicate its DNA, arrest, or enter apoptosis. Once cells have passed the G1/S checkpoint they enter a synthesis (S) phase, where replication occurs. There is an intra-S phase checkpoint, which is activated by stalled replication forks. This checkpoint is important for preventing damaged DNA from going through replication. The G2/M checkpoint is at the end of G2 phase, just before entry into mitosis. At this checkpoint, the cell has to ensure it is ready for mitosis by confirming there is no unrepaired damage that escaped the previous two cell cycle checkpoints (Kaufmann and Paules, 1996). These three checkpoints are critical for efficient repair and for maintaining the fidelity of the genome.

Mammalian cells have evolved multiple pathways to repair a wide range of DNA lesions including base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end-joining (NHEJ) (Hakem, 2008). Activation and recruitment of these repair pathways is dependent on the type of damage. BER functions to repair small base distortions in the genome. MMR functions to repair base-base mismatches and small insertion or deletion loops. NER functions to repair bulky

adducts generated by, for example, UV damage. HR functions to repair DSBs by promoting strand exchange using a homologous template. When DSBs occur during the G1 phase, cells can also utilize the error prone pathway, NHEJ, which directly ligates broken DNA ends together.

Replication blocking lesions can also recruit the post replication repair (PRR) pathway to bypass lesions. DNA lesions, such as ICLs that cannot be accommodated into the active sites of the replicative polymerases in S phase are extremely dangerous due to their ability to block the replication fork. Prolonged stalling can lead to collapsed replication forks resulting in the formation of DSBs and/or chromosomal rearrangements, which activate apoptosis(Lee and Myung, 2008). To prevent prolonged DNA replication fork stalling, the PRR pathway is used to bypass the lesion. PRR is generally error prone; it allows the bypass of DNA lesions to prevent cell death at the expense of increased mutation frequency. Mutations are avoided in normal cells since the bypassed lesions can be repaired later in the G2 phase using an error free repair mechanism (Lee and Myung, 2008).

In mammalian cells, the most well characterized PRR lesion bypass pathway is the translesion synthesis (TLS) pathway. The TLS pathway utilizes error prone polymerases to bypass DNA lesions. These TLS polymerases are characterized by low fidelity, low processivity, lack of proofreading activity, lack of exonuclease activity, and non-restrictive active sites. Compared with normal replication polymerases, these TLS polymerases can accommodate bulky

lesions (Lehmann et al., 2007; Prakash et al., 2005; Waters et al., 2009). Due to the mutagenic ability of the TLS polymerases, the activation of the TLS pathway is highly regulated. Studies from mammalian cells have revealed a link between the recruitment of TLS polymerases and the ubiqutin modification of proliferating cell nuclear antigen (PCNA). PCNA exists as a homotrimeric doughnut-shaped clamp structure that encircles the DNA and functions as a processivity factor for DNA polymerases. PCNA can be modified by monoubiquitination and polyubiqutination. These modifications facilitate the switch between error-prone and error-free repair pathways. PCNA monoubiqutination is carried out by the E2 Ub conjugating enzyme Rad6 and the E3 Ub ligase Rad18 at the Lysine (K) 164 residue of PCNA (Hoege et al., 2002). PCNA polyubiquitination is carried out by E2 Ub conjugating enzymes Ubc13 and Mms2 along with the E3 Ub ligases, SHPRH and HLTF (Frampton et al., 2006; Hofmann and Pickart, 1999; Motegi et al., 2006; Ulrich and Jentsch, 2000; Unk et al., 2006). Polyubiquitination occurs at K164 via a non-canonical K63 Ub linkage (Frampton et al., 2006; Hofmann and Pickart, 1999; Ulrich and Jentsch, 2000). Ubiquitination modifications of PCNA are critical for switching repair pathways depending on the cell cycle and the type of lesion to avoid activation of apoptosis and generation of mutations.

In this dissertation, I dissected the role of key DDR proteins including Breast Cancer Susceptibility Gene 1 (BRCA1), its direct partner the BRCA1 Associated C-terminal Helicase (BACH1), and the mismatch repair (MMR)

protein, MLH1. These proteins function in complexes and loss of their function may have drastic consequences for DDR and genomic integrity. Moreover BACH1 interacts with both BRCA1 and MLH1 (Cantor et al., 2001; Peng et al., 2007). Thus, understanding the function of BACH1/BRCA1 and BACH1/MLH1 interactions in DDR has been the goal of my work.

Breast Cancer Susceptibility Gene 1 (BRCA1) and Cancer

BRCA1 was the first gene identified to associate with hereditary breast, ovarian and fallopian tube cancers. BRCA1 was mapped to the long arm of chromosome 17, in the interval of 17q12-21 (Hall et al., 1990). It contains 24 exons that encode a protein of 1,863 amino acids. Mutations in BRCA1 and the other hereditary breast cancer associating gene, BRCA2 account for 45% of all families with multiple cases of breast cancer, and up to 90% of all families with both breast and ovarian cancers. Mutation in a single copy of BRCA1 confers breast cancer susceptibility with a 90% life-time risk of developing cancer (Couch and Weber, 1996). Notably, development of cancer requires the inactivation of the second copy of BRCA1. Although mutations in BRCA1 are not commonly found in sporadic breast and ovarian cancers, the BRCA1 promoter is often methylated in the tumors. Thus, BRCA1 may also contribute to the prevention of sporadic breast cancer (Esteller et al., 2000; Wiley et al., 2006; Wilson et al., 1999).

The function of BRCA1 in DDR

BRCA1 is a critical DNA damage repair protein that functions in DSB repair. The first indication that BRCA1 functioned in DSB repair came from the finding that BRCA1 deficient cells were highly sensitive to DSBs and exhibited chromosomal instability (Shen et al., 1998; Xu et al., 1999). In addition, BRCA1 was shown to interact and colocalize with the critical HR protein Rad51 in nuclear foci (Scully et al., 1997). Unlike BRCA2, which has a direct role with Rad51 in HR, BRCA1 functions upstream of the pathway at the branch point of gene conversion (GC) and single-strand annealing (SSA), which are two mechanisms of DSB repair (Stark et al., 2004). Thus in BRCA1 deficient cells, both GC and SSA are reduced (Moynahan et al., 1999; Moynahan et al., 2001a; Stark et al., 2002).

In addition to its repair function, BRCA1 also functions in DNA damage induced G2/M and intra-S phase checkpoint activation. G2/M checkpoint activation requires the phosphorylation of CDC2 kinase and its dephosphorylation by the phosphatase CDC25C, initiates mitosis. BRCA1 regulates this checkpoint at multiple levels. First, BRCA1 is required for the activation of Chk1 kinase which functions to inhibit the CDC25C activity (Yarden et al., 2002). Second, BRCA1 is required for the expression of 14-3-3 proteins which form a complex with CDC25C and exclude it from the nucleus (Yarden et al., 2002). Third, BRCA1 is also required for the expression of WEE1 and GADD45 (Mullan et al., 2001; Xu et al., 2001; Yarden et al., 2002). Both function

to prevent CDC2-CylinB complex formation, thus, preventing the initiation of mitosis (Mullan et al., 2001; Xu et al., 2001; Yarden et al., 2002). The function of BRCA1 in intra-S checkpoint requires its phosphorylation on serine (S) 1387 by ATM, but is less well characterized compared to the G2/M checkpoint (Tibbetts et al., 2000; Xu et al., 2001). In addition to the well defined functions of BRCA1 in DSB repair and cell cycle checkpoint regulation, it may also function in global genomic repair (GGR), chromatin remodeling, and transcription-coupled repair (Bochar et al., 2000; Gowen et al., 1998; Harkin et al., 1999; Hartman and Ford, 2002).

BRCA1 domains and interacting proteins

Elucidation of BRCA1 function has also come from identification and characterization of BRCA1-associated proteins. In fact, BRCA1 has been reported to interact directly or indirectly with nearly one hundred proteins. Many of BRCA1-interacting proteins have been reported to bind to the BRCA1 RING domains, these include the BRCA1 associated RING domain 1 (BARD1), the BRCA1 associated protein 1 (BAP1), and UbcH5c (Brzovic et al., 2003; Jensen et al., 1998; Wu et al., 1996). These proteins function to facilitate the BRCA1 E3 ubiquitin ligase activity. On the other hand, CtBP-interacting protein (CtIP), BACH1, and Abraxas interact with BRCA1 through the BRCT domains (Cantor et al., 2001; Wang et al., 2007; Wong et al., 1998; Yu et al., 1998). It follows that a mediator protein, such as BRCA1 binds and recruits DDR proteins in response to

DNA damage. Most likely these proteins are bridged together by BRCA1 to facilitate the DNA damage induced cell cycle checkpoint activation and repair.

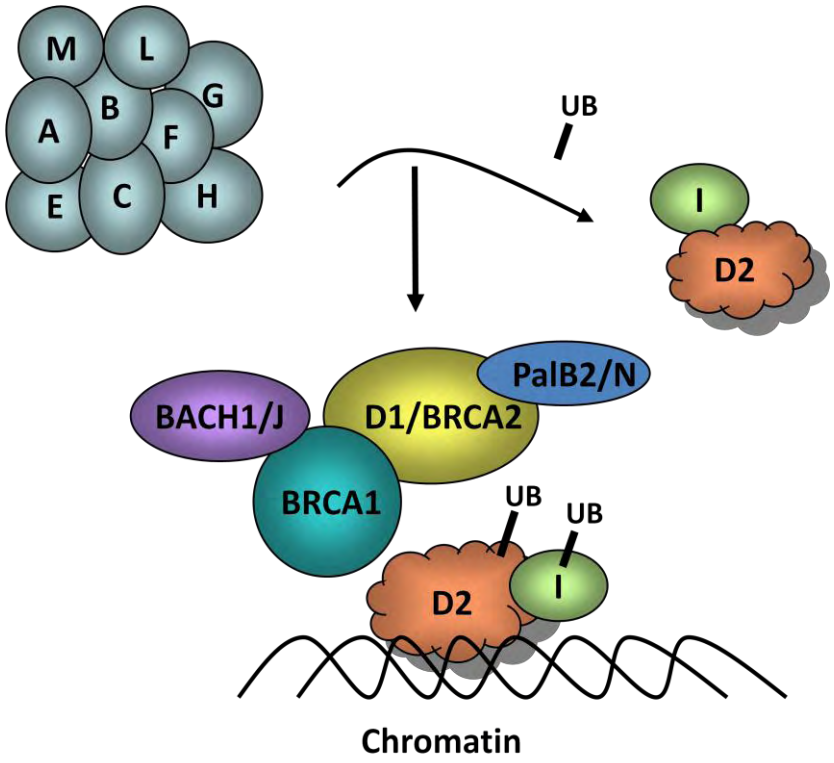
In addition to its interaction with individual DDR proteins, BRCA1 was also linked to the Fanconi anemia (FA) pathway. The FA pathway consists of 13 complementation groups (FA-A, FA-B, FA-C, FA-D1, FA-D2, FA-E, FA-F, FA-G, FA-I, FA-J, FA-L, FA-M, and FA-N) and is divided into upstream proteins, FANCD2/FANCI, and downstream proteins (Figure 1-1). The upstream proteins include FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM. These eight proteins form the FA core complex, which is phosphorylated and activated by ATR in response to DNA damage. Once activated, the FA core complex translocates to chromatin and is recruited to sites of break. The E3 ligase, FANCL, of the core complex works in concert with the E2 subunit UBE2T and facilitates the monoubiquitination of FANCD2 on lysine 561 and 523, respectively (Longerich et al., 2009; Machida et al., 2006; Meetei et al., 2003; Smogorzewska et al., 2007). Once monoubiquitinated, FANCD2 and FANCI form a heterodimer and translocate to chromatin, where they are recruited to nuclear foci containing BRCA1 and downstream FA proteins (Taniguchi and D'Andrea, 2006). Mutations in BRCA1 have not been linked to FA, thus it is not an official FA protein. However, BRCA1 is indirectly linked to the FA pathway on multiple levels. First, BRCA1 not only co-localizes with FANCD2 and FANCI in nuclear foci, but also regulates their ability to form damage induced foci (Garcia-Higuera et al., 2001). Second, BRCA1 facilitates the relocalization of FANCD2 to

sites of stalled replication forks (Vandenberg et al., 2003). Third, BRCA1 interacting partners were identified as downstream FA proteins (Litman et al., 2005). Downstream FA proteins include FANCD1, FANCN, and FANCJ. All three groups were later identified as BRCA2, PALB2, and BACH1 respectively. The intimate connection between the FA and the BRCA pathway again demonstrated the overlapping nature and complexity of the DDR network.

Identification of BACH1 and its link to Cancer

BACH1 was identified through its direct association with BRCA1. In an effort to understand the contribution of BRCT sequences to BRCA1 function, GST fusion BRCT motifs (GST-BRCT) were generated (Figure 1-1) (Cantor et al., 2001). In a far western experiment, a protein of ~130 kDa was detected with the GST-BRCT probe that was labeled by *in vitro* phosphorylation with protein kinase A. Furthermore, GST-BRCT probes containing clinically relevant point mutants, P1749R and M1775R, reduced or failed to bind to the130 kDa protein respectively. Subsequently, the130 kDa protein was charaterized using microcapillary reverse phase HPLC and nanoelectrospray tandem mass spectrometry (MS/MS). The 130 kDa protein contained 1249 residues and its N-terminal 888 residues revealed strong homology to the DEAH helicase family. In addition, the 130 kDa protein also contains seven helicase motifs that are characteristic of the DEAH helicase family. Unlike DEAH helicases, the 130 kDa protein also contains a C-terminal region that shares 39% homology with the

Figure 1-1: **Cartoon of the FA pathway.** The FA pathway is composed of core complex proteins, FANCD2/FANCI, and downstream proteins. In response to damage, FANCD2 and FANCI are monoubiquitinated by the FA core complex and form a heterodimer. The FANCD2/FANCI heterodimer translocates to chromatin where it colocalizes with BRCA1 and the downstream proteins, FANCJ/BACH1, FANCD1/BRCA2, and FANCN/PalB2. The FA pathway is critical for interstrand crosslink repair (ICL) and maintaining genomic stability.

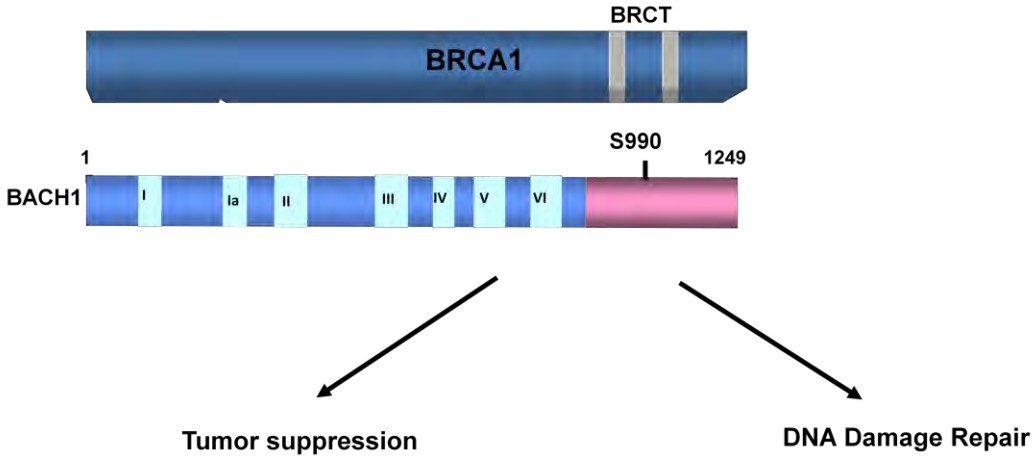


synaptonemal complex protein 1 (SCP1) (Schmekel et al., 1996). Given the interacting domain on BRCA1 and its helicase domains, the 130 kDa protein was named BACH1 for BRCA1 associated C-terminal helicase.

In addition to the interacting domain of BACH1 on BRCA1, the Serine 990 (S990) phosphorylation on BACH1 was shown to be critical for its interaction with BRCA1 (Yu et al., 2003). The residue following S990 is a Proline (P) and phosphorylation of sites containing SP is usually regulated by cyclin-dependent kinases. Although BACH1 expression is stable throughout the cell cycle, S990 is phosphorylated only from S to G2/M phase (Yu et al., 2003). Thus, the interaction of BACH1 and BRCA1 is cell cycle regulated.

The interaction with BRCA1 suggested that BACH1 could also be linked to hereditary breast cancer. Consistent with this idea, germ-line mutations in BACH1were identified in patients with early onset breast cancer (Cantor et al., 2001). Also, mutations in BACH1 enhanced breast cancer risk two fold (Walsh and King, 2007). Interestingly, the disease-associated mutations in BACH1 were shown to alter its helicase activity *in vitro*, providing a direct link between BACH1 helicase activity and disease development (Cantor et al., 2004). In addition to hereditary breast cancer, BACH1 is also associated with the FA syndrome (Levitus et al., 2005b; Litman et al., 2005).

Figure 1-2**: Established BRCA1/BACH1 interaction and functions.** BACH1 is a 5' to 3' DNA helicase with seven helicase motifs (I to VI), and it interacts with BRCA1 through the BRCT domain. In addition, the BRCA1 and BACH1 interaction is also dependent on the S990 phosphorylation on BACH1located in its C-terminal domain (red region). Both BRCA1 and BACH1 are critical DNA damage response proteins and are both associated with hereditary breast cancer. Furthermore, the interaction of BRCA1 and BACH1 is also critical for DNA damage repair and tumor suppression.



Function of BACH1 in DDR

The most obvious clue to BACH1 function was that its amino acid sequence was homologous to DNA helicases. Furthermore, *in vitro* helicaes assay demonstrated that BACH1 unwound DNA in the 5' to 3' direction. BACH1 requires the presence of at least 15 nucleotides at the 5' ssDNA tail to initiate DNA unwinding (Gupta et al., 2005). In addition, BACH1 also preferentially binds and unwinds forked duplex DNA substrates (Gupta et al., 2005). Furthermore, BACH1 also unwinds recombination intermediates, the three-stranded D-loop structure (Gupta et al., 2005). These *in vitro* results suggest that BACH1 functions at the DNA replication fork and in DSB repair.

Given the direct interaction of BACH1 with BRCA1 experiments were designed to assess whether BACH1 functioned in DDR akin to BRCA1. In response to DNA damage, BACH1 displayed a BRCA1 like nuclear foci pattern, colocalized with γ-H2AX, and was modified by phosphorylation (Peng et al., 2006). What's more, BACH1 functioned similar to BRCA1, in response to DSBs (Litman et al., 2005). Specifically, overexpression of a helicase inactive BACH1 mutant ($BACH1^{K52R}$), disrupted DSB repair (Cantor et al., 2001). In addition, BACH1 deficient cells were sensitive to DSBs. Furthermore, BACH1 depleted cells had reduced DSB repair by HR induced by the site-specific I-*SceI* endonuclease (Litman et al., 2005). However, unlike BRCA1, BACH1 deficient cells had normal Rad51 foci formation (Litman et al., 2005). BACH1 was recently shown to destabilize Rad51 nucleoprotein filaments and inhibit the DNA strand

exchange activity of the Rad51 recombinase (Sommers et al., 2009). The mechanistic function of BACH1 in DSB repair remains elusive, but due its ability to unwind Rad51nucleoprotein filaments, BACH1 may either function to exit the repair process or monitor homologous strand exchange.

In addition to DSB repair, BACH1 also functions in ICL repair. ICLs can be introduced into DNA either endogenously during cellular metabolism through the acidification of nitrites, or exogenously by agents such as, melphalan, cisplatin, and mitomycin C (MMC). ICLs are extremely toxic due to their ability to inhibit DNA replication, transcription, and segregation resulting from the impediment of DNA strand separation. The first indication that BACH1 functioned in ICL repair came from the finding that BACH1 deficient cells were extremely sensitive to MMC (Litman et al., 2005). In addition, BACH1 deficient cells underwent MMC-induced chromosome instability resulting in chromosome breaks, quadriradial, and tri-radial chromosomes (Litman et al., 2005). These phenotypes provided the first evidence for a potential link between BACH1 and the FA pathway. Subsequent experiments demonstrated that BACH1 did not affect the monoubiquitination status of FANCD2 and BACH1 is the FA gene product, FANCJ (Levitus et al., 2005b; Litman et al., 2005). The function of FANCJ in ICL repair required its helicase and MLH1 binding activities, but not BRCA1 binding (Peng et al., 2007). Thus, the contribution of BRCA1 binding to the function of FANCJ in ICL repair remains elusive. Further complicating our understanding of FANCJ's function is that the function of FANCJ in double

stranded break repair (DSBR) is potentially not conserved between species. For example, in mammalian cells, FANCJ was shown to be critical for DSB repair. However, in chicken DT40 cells, FANCJ was shown to function independent of BRCA1and HR (Bridge et al., 2005). FANCJ in chicken DT40 cells lacks the binding domain required for BRCA1 interaction, thus FANCJ may still function in mammalian HR, but requires the interaction of BRCA1. Whether FANCJ functions independent of BRCA1 remains to be determined in mammalian cells.

Besides its repair function, BACH1 is also involved in the activation of intra-S phase checkpoint. In S phase, FANCJ increased its association with chromatin and the interaction with BRCA1 was enhanced (Kumaraswamy and Shiekhattar, 2007). In addition, the DNA dependent ATPase activity of FANCJ was also activated (Kumaraswamy and Shiekhattar, 2007). All together, these data suggest that FANCJ functions in S-phase. The function of FANCJ in intra-S-phase checkpoint came from the finding that depletion of FANCJ exhibited an elevated S-phase accumulation in response to aphidicolin treatment, which triggers an intra-S-phase response in a phosphoinositide-3-kinase-related protein kinase (PIKK) dependent manner (Greenberg et al., 2006). In response to IR, normal cells have a 50-60% reduction in deoxynucleotide uptake, whereas, FANCJ suppressed cells have a less extensive diminution in DNA synthesis (Greenberg et al., 2006). The IR induced intra-S-phase checkpoint requires the inhibition of DNA synthesis at late firing sites of replication initiation, which are partly controlled by blocking the loading of Cdc45. Furthermore, FANCJ interacts

with a critical intra-S phase checkpoint protein TopBP1, which plays an essential role in the loading of Cdc45. FANCJ forms a DNA damage induced complex with TopBP1, BRCA1, and BARD1 (Greenberg et al., 2006). This super-complex formation was impaired in the absence of FANCJ or when FANCJ cannot interact with BRCA1. The function of this super-complex was suggested to prevent TopBP1 dependent Cdc45 loading in response to DNA damage. Thus, in FANCJ depleted cells, Cdc45 remains at the replication origin and cells enter mitosis. Altogether the data implicate that FANCJ functions in the DDR. How DDR is affected by the contribution of FANCJ binding to BRCA1 or MLH1 is less clear.

MMR pathway and Cancer

MLH1 is a MMR protein that functions in DNA mismatch repair. Mutations in MMR genes are associated with hereditary nonpolyposis colon cancer (HNPCC). HNPCC accounts for 5% of all colorectal cancer cases. It is an autosomal-dominant inherited cancer predisposition syndrome caused by mutations in MMR genes, mutS homolog 2 (MSH2), mutS homolog 6 (MSH6), mutL homolog 1 (MLH1), postmeiotic segregation increased 2 (PMS2), and postmeiotic segregation increased 1(PMS1). Mutations in MSH2 and MLH1 account for 90% of all HNPCC cases. In addition to HNPCC, somatic mutations of MMR genes and epigenetic silencing of MLH1 expression have been observed in many sporadic cancers.

Function of MMR pathway in DDR

The MMR pathway is best characterized for its ability to recognize and repair DNA mismatches (Figure 1-3). DNA mismatches can occur during DNA replication and recombination due to erroneous insertions, deletions, and mis-incorporation of bases. Conversely, DNA mismatches can also occur upon exposure to DNA methylating agents such as, N-methyl-N-nitrosourea (MNU) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG). MNU and MNNG are SN1 type (unimolecular nucleophilic substitution) agents, they generate a variety of DNA adducts. However, the most mutagenic adduct is O6-methylguanine (O6-MeG). O6-MeG is initially repaired by a direct reversal process using the methylguanine methyltransferase (MGMT) enzyme. During replication, unrepaired O6-MeG is perceived as an adenine (A) thus, it is paired with thymine (T), resulting in O6-MeG:T mismatch. The DNA mismatch is sensed and processed by the MMR pathway. Sensing of DNA mismatches is mediated by one of the two heterodimers of the *E. coli* homologs, MutSα, and MutSβ. MutSα is composed of MSH2 and MSH6, whereas MutSβ is composed of MSH2 and MSH3 (Jiricny, 2006). Once a lesion is sensed, MutS heterodimers recruit the MutL homolog MutLα, which is composed of MLH1 and PMS2. MutSα and MutLα form a stable complex to initiate cell signaling and repair.

MMR dependent checkpoint activation has been controversial. There are two contradicting models for MMR dependent checkpoint activation. First, the futile DNA repair cycle model suggests that MMR proteins only have one function,

DNA repair (Karran, 2001; Wang and Edelmann, 2006). Thus activation of the G2/M checkpoint is a consequence of DSBs generated by multiple attempts to repair O6-MeG:T mismatches. Since MMR pathway only repairs the mismatch on the newly synthesized strand, thus MGMT would be required to remove the mismatch completely. In the absence of MGMT, MMR processing of O6-MeG:T mismatches results in the excision and re-synthesis of the strand containing T. Since O6-MeG lesions would remain on the template strand, a T would be reinserted opposite the O6-MeG during the next round of replication, thus, this would result in a futile cycle of repair. This futile cycle of repair could eventually lead to replication fork arrest and DSB formation (Wang and Edelmann, 2006).

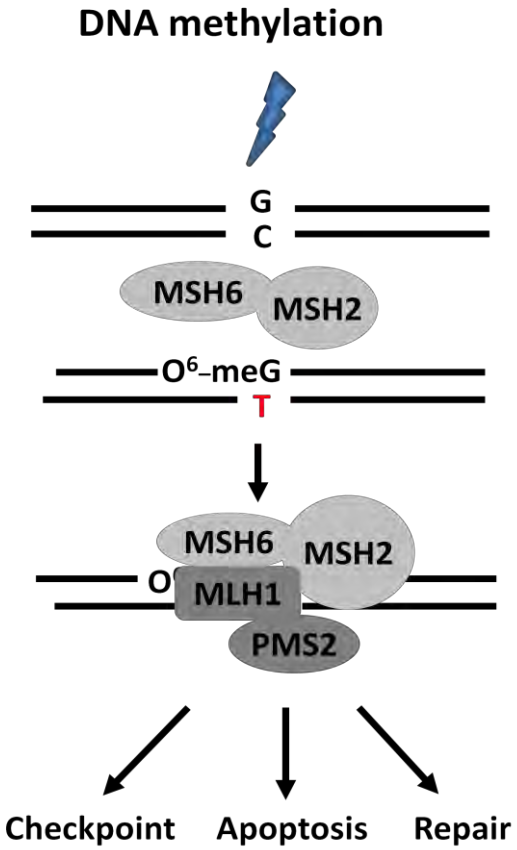
The direct signaling model suggests that MMR proteins have two distinct functions, repair and signaling (Fishel, 2001; Kat et al., 1993). This model suggests that the MutSα complex functions as a DNA damage sensor to activate a downstream signaling cascade involving ATR and Chk1. There is multiple evidence supporting the direct signaling model. First, MutSα binds to the O6-MeG:T mismatch with higher affinity compared to O6-MeG:C or the G:T mismatch (Yoshioka et al., 2006). In addition, ATR/ATRIP also preferentially interacted with the O6-MeG:T mismatch, but not with the G:T mismatch. (Yoshioka et al., 2006). The DNA-protein complex assembled with the O6-MeG:T mismatch, but not the G:T mismatch, result in the phosphorylation of Chk1 (Yoshioka et al., 2006). Second, genetic evidence for the direct signaling model was provided with the discovery of “separation of function” mutants.

Missense mutations in the ATPase domain of MSH2 and MSH6 ablated their ability to perform mismatch repair, but did not affect their ability to activate cell cycle checkpoint and apoptosis in response to O6-MeG lesions (Lin et al., 2004; Yang et al., 2004).

Similar to other DDR pathways, MMR also functions with additional repair proteins such as, PCNA and exonuclease-1 (EXO1), for efficient repair and signaling. MSH2, MLH1, MSH6, and MSH3 all interact with PCNA (Bowers et al., 2001; Clark et al., 2000; Flores-Rozas et al., 2000; Gu et al., 1998; Kleczkowska et al., 2001; Lee and Alani, 2006; Umar et al., 1996). These interactions are critical for the DNA mismatch induced MutSα and MutLα complex formation. In addition, PCNA was suggested to localize with MMR proteins to the newly synthesized DNA. Recently, MutLα was identified to contain PCNA/replication factor C (RFC) dependent endonuclease activity (Kadyrov et al., 2006). This endonuclease activity is located in the PMS2 subunit and it is critical for 3' nick-directed MMR because no helicase has yet been identified and EXO1 is a 5'-3' exonuclease (Erdeniz et al., 2007; Li, 2008).

MMR proteins can also signal for apoptosis when the lesion is too severe to repair. MMR proteins are linked to the activation of apoptosis through p53 dependent and independent mechanisms. In response to methylating damage, activation of p53 and its phosphorylation on S15 is MMR dependent (Duckett et al., 1999). The stabilization of the functional homologue of p53, p73 may involve

Figure 1-3: **Cartoon illustration of the Mismatch Repair Pathway.** The mismatch repair pathway is responsible for recognizing and repairing DNA mismatches, which could be generated by exogenous DNA methylating agents. In response to methylation damage, adducts such as O6-MeG are generated and through processing these adducts results in O6-MeG:T mismatch. The MSH2/MSH6 heterodimer recognizes the mismatch and recruits the MLH1/PMS2 heterodimer to initiate checkpoint, repair, and/or apoptosis.



MMR proteins. Specifically, PMS2 directly associates and stabilizes p73 (Shimodaira et al., 2003). Overall, MMR proteins are critical for mutation avoidance and for tumor suppression. Although the mammalian MMR pathway is well characterized, there could still be additional proteins required. The interaction between FANCJ and MLH1 predicts a potential role for FANCJ in the MMR pathway.

Concluding remarks

DDR involves many repair proteins and is a complex network of pathways that function together to coordinate and repair DNA damage to maintain genomic stability and suppress tumor formation. My focus was to understand the function of critical DDR proteins including BRCA1, FANCJ, and MLH1. FANCJ was originally discovered as a BRCA1 interacting protein, the subsequent novel connection to MLH1 links FANCJ to the MMR pathway. Thus, the goal of this thesis was to understand the significance of BRCA1/ FANCJ /MLH1 interactions in DDR. First, what is the functional relevance of FANCJ /MLH1 interaction in the ICL response? Second, what is the functional relevance of FANCJ /MLH1 interaction in MMR? Third, does BACH1 function independent of BRCA1?

CHAPTER II

The FANCJ/MutLα interaction is required for correction of the ICL-response in FA-J cells

Abstract

FANCJ was first linked to hereditary breast cancer through its direct interaction with BRCA1. FANCJ was also recently identified as a Fanconi anemia (FA) gene product, establishing FANCJ as an essential tumor suppressor. Similar to other FA cells, FANCJ-null (FA-J) cells accumulate 4N DNA content in response to DNA interstrand crosslinks (ICLs). This accumulation is corrected by re-introduction of wild-type FANCJ. Here, we show that FANCJ interacts with the mismatch repair complex MutLα, composed of PMS2 and MLH1. Specifically, FANCJ directly interacts with MLH1 independent of BRCA1 through its helicase domain. Genetic studies reveal that FANCJ helicase activity and MLH1 binding, but not BRCA1 binding, are essential to correct the FA-J cells ICL-induced 4N DNA accumulation and sensitivity to ICLs. These results suggest that the FANCJ/MutLα interaction, but not the FANCJ/BRCA1 interaction, is essential for establishment of a normal ICL-induced response. The functional role of the FANCJ/MutLα complex demonstrates a novel link between FA and MMR and predicts a broader role for FANCJ in DNA damage signaling independent of BRCA1.

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I contributed to Figure 2-3C and Figure 2-4.

Introduction

In the absence of DNA repair proteins, cell cycle checkpoints and/or DNA damage repair pathways are not properly activated. This inability to actively respond to DNA damage can lead to massive chromosomal damage and even cell death. In some cases, mutations in DNA repair proteins can contribute to multiple cancer syndromes. Studies on the genetic causes of the cancer-prone syndrome Fanconi anemia (FA) revealed that gene mutations associated with hereditary breast cancer were also associated with FA. For example, the hereditary breast cancer gene, *BRCA2* was shown to be the gene defect in the FA-D1 patient complementation group, revealing that BRCA2 was FANCD1 (Howlett et al., 2002). Likewise, *FANCJ* (formally called BACH1/BRIP1) was identified as the gene defective in the FA-J patient complementation group (Levitus et al., 2005a; Levran et al., 2005; Litman et al., 2005), and was initially linked to hereditary breast cancer. This link was based on its direct binding to BRCA1 and through the identification of two breast cancer patients with mutations in FANCJ, which also altered its helicase activity in vitro (Cantor et al., 2004; Cantor et al., 2001). This connection was furthered by the finding that

FANCJ (BRIP1) mutations confer a two-fold increase in the risk of developing breast cancer (Seal et al., 2006).

While other FA genes have not been linked to breast cancer, the network of at least 13 genes (designated *FANCA* to *FANCN*) are critical for maintaining chromosomal integrity (Thompson, 2005). Although the molecular function of these proteins is not clear, several gene products, including FANCA, B, C, E, F, G, H, L, and M, form a nuclear core complex (the FA core complex), that is required for monoubiquitination of FANCD2. The FA proteins BRCA2/FANCD1, PALB2/FANCN and FANCJ are not required for this event and are considered downstream of FANCD2 monoubiquitination. Nevertheless, all FA proteins contribute to processing interstrand-cross-links (ICLs) (Thompson, 2005). Consequently, in the absence of FA proteins, ICL-treatment leads to reduced cell viability and an accumulation of cells with a 4N DNA content representing cells in either late S or G2/M, and has been referred to as a G2/M arrest (Akkari et al., 2001). This ICL-induced cell cycle progression defect and sensitivity to ICLs is restored upon re-introduction of the missing FA gene (Chandra et al., 2005; Dutrillaux et al., 1982; Heinrich et al., 1998; Kaiser et al., 1982; Kupfer and D'Andrea, 1996; Kupfer et al., 1997; Sala-Trepat et al., 2000). However, the FA-related function or associated partners required for a proper ICL-response is not known**.**

Consistent with other FA cells, FANCJ-null (FA-J) cells have an ICL-induced cell cycle progression defect that can be corrected upon re-introduction

of wild-type (WT) FANCJ cDNA (Litman et al., 2005). This cell cycle progression defect has also been described as a prolonged G2/M arrest (Miglierina et al., 1991) or 4N DNA content accumulation (Akkari et al., 2001). The cause of this ICL-response in FA cells is not presently understood, but thought to involve delayed repair and/or failure to restart replication (Thompson et al., 2005). Unlike the majority of FA proteins, FANCJ has defined domains. Specifically, FANCJ binds directly to BRCA1 (Cantor et al., 2001) and FANCJ is a DNA helicase (Cantor et al., 2004). Dissecting the importance of these domains could further our understanding of how FA proteins function in an ICL-induced response. Attempts to define the functions of FANCJ domains in the ICL-response have been limited to chicken DT40 cells where the FANCJ /BRCA1 interaction is not conserved (Bridge et al., 2005). If FANCJ operates independent of BRCA1 for a particular ICL-response function, a remaining question will be whether FANCJ forms a complex with other proteins independent of BRCA1 to perform that function.

Here, we investigated whether FANCJ helicase activity or the FANCJ interaction with two distinct proteins was required for restoring FANCJ's ICL-response. Specifically, we identified that FANCJ interacts with the MutLα mismatch repair complex, independent of BRCA1. Our findings demonstrate for the first time that the FANCJ /MLH1 interaction is as critical as FANCJ helicase activity for restoring a normal cell cycle progression and resistance of FA-J cells to ICLs. In contrast, the FANCJ /BRCA1 interaction is dispensable for

normalizing the response of FA-J cells to ICLs, suggesting that FANCJ functions in distinct complexes to facilitate multifaceted DNA repair functions.

Results

FANCJ functions independently of BRCA1 to correct FA-J null cells

We had previously shown that introduction of WT FANCJ cDNA into FA-J cells corrects the ICL-induced cell cycle progresssion defect (Litman et al., 2005). However, it is unclear how FANCJ contributes to the ICL-response to restore the FA pathway especially given that FANCJ's role in the FA pathway appears to be independent of BRCA1, at least in chicken cells (Bridge et al., 2005). To verify and extend this finding, we addressed whether FANCJ binding to BRCA1 was required to correct the ICL-induced cell cycle progression defect in FA-J cells. We reconstituted FA-J cells with vector, WT, or the S990A FANCJ construct that is ablated for BRCA1 binding (Yu et al., 2003) (Figure 2-1C). Both WT and S990A versions of FANCJ corrected the ICL-induced cell cycle progression defect observed in FA-J cells compared to vector alone (Figure 2-1A and B). These data support the finding that FANCJ operates independent of BRCA1 to correct FA-J cells.

FANCJ is physically linked to the MutLα complex

Since FANCJ binding with BRCA1 was not required to correct the ICL-induced cell cycle progression defect in FA-J cells, we set out to identify

additional FANCJ interacting partners that may function with FANCJ in this ICL-induced response. A WT double-tagged FANCJ construct was used to create a stable line of HeLa S3 cells. Using a two-step immunoaffinity strategy, the double-tagged FANCJ was sequentially immunopurified (Nakatani and Ogryzko, 2003). Interacting proteins co-purifying with the double-tagged WT FANCJ were eluted and visualized by silver stain. FANCJ migrated at the expected ~140KD size as well as a single larger species that appears to derive from the vector (Figure 2-2A). Individual bands were excised from the gel and analyzed by mass spectrometry (LC-MS/MS). As expected, FANCJ co-purified with BRCA1 that was identified as the 250kD band. Unique partners were identified, including the MMR proteins, MLH1 and PMS2, which form the MutLα heterodimer (Schofield and Hsieh, 2003) (Figure 2-2A). Western blot analyses using specific antibodies confirmed the presence of these proteins (Figure 2-2B). To determine whether the MutLα complex associated with the native FANCJ protein, MCF7 cell extracts were immunoprecipitated (IP) with FANCJ antibodies (Abs) E67 and E47, and the presence of coprecipitating MLH1, PMS2, and BRCA1 proteins were evaluated by Western blot (Figure 2-2C). While FANCJ Ab precipitated the MutLα complex in the MCF7 cells, a MutLα complex was not precipitated with preimmune Abs (PI) or with FANCJ Abs in 293T cells, which lack expression of the MutLα complex (Trojan et al., 2002). Moreover, FANCJ was not precipitated with the MLH1 Ab in FA-J cells, which lack expression of FANCJ, unless FANCJ was re-introduced (Figure 2-2D). In contrast, a FANCJ/MLH1 interaction was

readily detected in other FA cell lines irrespective of gene correction, such as FA-A, FA-D1 and FA-D2 (Supplemental Figure S2-8). Furthermore, the interaction between FANCJ and the MutLα complex was stable in HeLa cells in the presence or absence of DNA damage (Figure 2-2E).

The helicase domain of FANCJ binds directly to MLH1 independent of BRCA1

MLH1 was previously reported to be part of a BRCA1 complex (Greenberg et al., 2006; Wang et al., 2000), therefore, we examined whether BRCA1 mediated the interaction between FANCJ and the MutLα complex. First, we noted that unlike FANCJ, BRCA1 was not readily detected in an MLH1 precipitation (Figure 2-2C). Next, we addressed whether FANCJ precipitated with the MutLα complex in BRCA1-deficient cells. Expression of BRCA1 was stably suppressed in MCF7 cells by a shRNA vector, as previously demonstrated (Litman et al., 2005). In cells expressing both a control shRNA specific to eGFP or an shRNA specific to BRCA1, FANCJ antibodies efficiently co-precipitated the two components of the MutLα complex (Figure 2-3A), suggesting that FANCJ binds the MutLα complex independent of BRCA1. In support of this finding, the helicase domain of FANCJ was required for MLH1 binding while a C-terminal region of FANCJ was required for BRCA1 binding (Figure 2-3B). To further assess the nature of the FANCJ/MLH1 interaction, we incubated recombinant FANCJ or BRCA1 with MLH1 that had been translated *in vitro*. MLH1 and

FANCJ were precipitated by their corresponding Abs, and their interactions were analyzed by Western blot. FANCJ and MLH1 proteins were co-precipitated with both FANCJ and MLH1 IPs, whereas BRCA1 was robustly precipitated only in the FANCJ IP (Figure 2-3C). A direct interaction between FANCJ and MLH1 was confirmed by ELISA assay using purified recombinant proteins. FANCJ bound MLH1 in a protein concentration dependent manner (Figure 2-3D). Furthermore, the interaction of FANCJ and MLH1 was demonstrated to be DNA-independent as evidenced by the similar colorimetric signal observed for FANCJ/MLH1 interaction in the presence of ethidium bromide (EtBr) or DNaseI (Figure 2-3E). These results suggest that FANCJ makes direct contacts with MLH1, independent of BRCA1 or PMS2.

PMS2 contributes to the FANCJ/MLH1 interaction in vivo

Given that MLH1 forms a heterodimer with PMS2, we next assessed whether PMS2 binding to MLH1 contributed to the MLH1/FANCJ interaction in vivo. To address this possibility, we tested the ability of different MLH1 constructs to precipitate FANCJ in the absence or presence of PMS2. WT full-length MLH1 and several MLH1-myc fusion proteins of varying length were generated and transiently transfected into MutLα-null 293T cells. To determine which of these MLH1 fragments were expressed and/or co-IPed FANCJ, MLH1 was precipitated from cell lysates with either myc or MLH1 Abs. While co-

transfecting PMS2 with MLH1 did not alter the expression of MLH1, the ability of FANCJ to form a complex with MLH1 was enhanced. With the addition of PMS2, FANCJ precipitated with the MLH1 constructs N2, C2, and C3, which in the absence of PMS2 had failed to precipitate FANCJ (Figure 2-4A). Thus, in the presence of PMS2, only one of the two MLH1-FANCJ interacting domains (478-508)(D1) or (736-744)(D2) was required (see Figure 2-4C) suggesting that PMS2 facilitates the MLH1/FANCJ interaction. PMS2 stability is dependent on the MLH1 C-terminus (Mohd et al., 2006); not surprisingly, we found that ablation of a C-terminal region of MLH1 (703-725) reduced both PMS2 expression and FANCJ binding (Figure 2-4B).

MutLα functions downstream of FANCD2 monoubiquitination

To appreciate the physiological significance of a FANCJ/MutLα interaction, we next, addressed whether the MutLα complex functioned with FANCJ in the FA pathway. We had previously shown that in FANCJ deficient cells, DNA damage induced FANCD2 monubuiquitination was intact (Litman et al., 2005). Similarly, we found that incubation of MutLα deficient cells (HCT116 and HEC-1A) with hydroxyurea (HU), lead to efficient FANCD2 monoubiquitination (Supplemental Figure S2-9A), suggesting that similar to FANCJ, MutLα functions downstream of FANCD2.

Given that suppression of MMR proteins has been reported to reduce the survival of cells upon ICL-treatment, (Aquilina et al., 1998; Fiumicino et al., 2000), we next asked whether similar to FANCJ deficiency, MutLα deficiency also sensitizes cells to ICLs. First, we suppressed MutLα using siRNA reagents in MCF7 cells vs. a luciferase control. Second, we reconstituted HCT116 cells null for MutLα with vector or MutLα expressing cDNAs. In both experiments, there was no measurable change in ICL sensitivity in the presence or absence of MutLα expression (Supplemental Figure S2-9B and data not shown). Given that MMR proteins bind and process ICLs (Duckett et al., 1996; Yamada et al., 1997; Zhang et al., 2002), activate multiple DNA damage-induced checkpoints, such as intra S and G2/M (4N) arrest (Brown et al., 2003; Cejka et al., 2003), and participate in the repair of ICLs by promoting recombination (Zheng et al., 2006), we considered that MutLα-suppression could bypass ICL-sensitivity through loss checkpoint (Cejka et al., 2003) and/or by activating default non-recombination based repair pathways as reported (Zheng et al., 2006). Thus, we considered that to unmask function of MutLα in the ICL-response with FANCJ, it would be necessary to selectively ablate the MLH1/FANCJ interaction, while maintaining other MLH1 functional interactions (i.e. PMS2 binding).

Disruption of the native MLH1/FANCJ interaction generates ICL-sensitivity

To define the domain on FANCJ required for MLH1 binding, we generated several FANCJ-myc fusion proteins of varying length and expressed them in MCF7 cells (Figure 2-5A and E). To determine which of these FANCJ fragments were expressed and/or co-IPed MLH1, FANCJ was precipitated from cell lysates with myc antibodies (Abs). Full-length FANCJ and FANCJ expression constructs including the FANCJ N-terminal amino acid residues 1-145 precipitated MLH1 (Figure 2-5A and C). These results suggested that FANCJ N-terminal residues 1-145 were required for binding to MLH1. To assess whether residues in this region were sufficient for MLH1 binding, we inserted FANCJ residues 128-158 within the eGFP gene sequence to create an eGFP-fusion protein. In contrast to eGFP alone, the eGFP-FANCJ fusion protein readily co-precipitated MLH1 (Figure 5B), suggesting that FANCJ 128-158 was sufficient for MLH1 binding. Furthermore, expression of the eGFP-FANCJ fusion protein in cells perturbed the formation of the native FANCJ/MLH1 interaction as determined by both FANCJ and MLH1 IP and Western blot experiments (Figure 2-5D) confirming that this region of FANCJ was essential for mediating the MLH1 interaction.

Next, we addressed whether expression of the 128-158 FANCJ-eGFP fusion protein and the resulting perturbation of the native FANCJ/MLH1 interaction would render cells sensitive to ICLs. MCF7 cells were transfected with vectors expressing either the 128-158 FANCJ-eGFP fusion protein or eGFP

alone, plated, and treated with increasing concentrations of Mitomycin C (MMC). The overall trend upon expression of the 128-158 FANCJ-eGFP fusion protein was reduced cellular survival compared to expression of the eGFP control, despite some variability between experiments (Figure 2-5E). While the enhanced sensitivity was consistent with the possibility that a FANCJ/MLH1 interaction was required for ICL-repair, we considered that binding of the fusion protein to MLH1 might have altered additional MLH1 functions not specific to FANCJ. Thus, we sought to identify a method to ablate the FANCJ/MLH1 interaction without altering native MLH1 protein or being reliant on transfection efficiency to disrupt the native FANCJ/MLH1 interaction.

Lysines 141 and 142 of FANCJ are required for the FANCJ/MLH1 interaction

Given that mutational analysis revealed that FANCJ co-precipitated with MLH1 except when FANCJ residues 140-145 were absent (Figure 2-5A and C), we assessed the importance of these residues for binding MLH1 within the context of the full-length FANCJ protein. Thus, we generated three independent FANCJ mutant constructs that converted lysine 141 and 142 to alanine (K141/142A), glutamine 143 to a glutamic acid (Q143E), or serine 145 to an alanine (S145A). While the WT FANCJ and all three mutant versions were expressed and efficiently co-precipitated BRCA1, the K141/142A version demonstrated a dramatic reduction in the co-precipitation of MLH1 (Figure 2-6A) suggesting that these two lysines were required for MLH1 binding.

We considered that the K141/142A mutation in FANCJ could have not only disrupted MLH1 binding, but also FANCJ helicase activity. Thus, we generated recombinant versions of WT (Cantor et al., 2004) and the K141/142A FANCJ proteins to assess whether this mutant version was enzymatically active. The recombinant K141/142 FANCJ protein was detected as a single Coomassie stained band analyzed by SDS-PAGE that co-migrated with the WT FANCJ recombinant protein (Supplemental Figure S2-10A and D). The DNA unwinding activity of K141/142A FANCJ on a forked duplex DNA substrate was compared to unwinding activity of WT FANCJ. Both K141/142A FANCJ and WT FANCJ were found to be proficient in unwinding whereas K52R FANCJ, as previously demonstrated, failed to unwind the forked duplex substrate (Gupta et al., 2005) (Supplemental Figure S2-10B). Furthermore, K141/142A FANCJ and WT FANCJ unwound the forked duplex substrate in a protein concentration dependent manner achieving 90% of unwound substrate at the highest helicase concentration tested (Supplemental Figure S2-10C). Thus, the K141/142 mutant only disrupts MLH1 binding, but not FANCJ helicase activity.

FANCJ function depends on MLH1 binding to correct FA-J cells

Next, we tested the ability of K141/142A FANCJ cDNA to correct the cell cycle progression defect in FA-J cells. We used retroviral infection to stably infect FA-J cells with cDNA encoding the vector, WT, K141/142A, or K52R Flag/HA-tagged FANCJ constructs, which expressed similarly (Figure 2-6A).

Moreover, an MLH1 IP demonstrated that the MLH1/PMS2 complex was intact in FA-J cells and was able to precipitate the reconstituted FANCJ (Figure 2-2D). As in MCF7 cells, in FA-J cells MLH1 co-IPed with WT FANCJ, but was dramatically reduced in the K141/142A mutant FANCJ (Figure 2-6A). FA-J cells containing vector, WT, K52R, or K141/142A FANCJ were treated with melphalan to induce ICLs as described (Litman et al., 2005). The proportion of vector-containing FA-J cells with 4N DNA content increased after melphalan treatment, similar to previous experiments. As before the proportion of WT FANCJ-containing FA-J cells with 4N DNA content (~30%) was about half that of vector-containing cells (~70%). We found that cells containing the catalytically inactive FANCJ helicase (K52R) failed to correct the 4N accumulation defect (~66%). Likewise, the proportion of K141/142A FANCJ-containing FA-J cells with 4N DNA content (~68%) resembled that of vector-containing FA-J cells, suggesting that introduction of K141/142A FANCJ did not correct the cell cycle progression defect in FA-J cells (Figure 2-6B). These data suggest that the FANCJ/MLH1 interaction is essential for restoration of the FA pathway in FA-J cells.

To further confirm and examine the importance of FANCJ binding to MLH1 for the ICL-induced response, we assessed whether FANCJ binding to MLH1 was required for FANCJ to correct the ICL-induced sensitivity of FA-J cells. However, the ability of WT FANCJ to correct the ICL sensitivity of FA-J cells had not been previously reported. Thus, we first tested and confirmed that re-introduction of WT FANCJ corrected the ICL-sensitivity of FA-J cells treated with

MMC. The vector reconstituted FA-J cells were more sensitive to MMC than the WT FANCJ reconstituted cells with an IC_{50} of 250mM and ~ 900mM MMC, respectively (Figure 2-6C). In contrast to WT, both the K141/142A, and K52R FANCJ reconstituted FA-J cells were sensitive to ICLs with an IC_{50} of less than 250mM MMC. Moreover, the K141/142A FANCJ reconstituted FA-J cells were more sensitive than the vector or K52R FANCJ reconstituted FA-J cells ($P< 0.01$). As before, the S990A FANCJ corrected the ICL-response similar to WT. Although the correction of the ICL sensitivity was greater with the S990A FANCJ than with the WT FANCJ, at 250mM MMC the values were not significantly different (with P-values between 0.0027-0.22) (Figure 2-6C). These findings clearly demonstrate that both FANCJ helicase activity and FANCJ binding to MLH1 are required for FANCJ to functionally correct the ICL sensitivity of FA-J cells.

Discussion

In this study, we addressed whether FANCJ helicase activity or different FANCJ complexes are essential for FANCJ's function in the ICL response. Specifically, we have shown that FANCJ forms a complex with the MutLα heterodimer, which is composed of the mismatch repair proteins MLH1 and PMS2. FANCJ directly interacts with MLH1 independent of BRCA1, and this DNA-independent interaction is within the FANCJ helicase domain, C-terminal to nucleotide binding box 1, and includes lysines 141 and 142. This is the first

report that demonstrates that a direct interaction between FANCJ and MLH1 is as essential for the ICL-induced response as the FANCJ helicase activity. Furthermore, our data suggest that formation of a FANCJ/BRCA1 complex is not required for normalization of the ICL-induced response in FA-J cells.

The question remains as to how FANCJ or mismatch repair proteins function in the ICL-response. Following exposure to ICLs, ATR is activated and initiates a signal cascade through the phosphorylation of downstream substrates ultimately leading to checkpoint activation, DNA damage repair, and/or apoptosis. Intriguingly, MMR proteins were recently proposed to act as direct sensors of DNA methylation and initiate the intra S-phase checkpoint by helping to recruit ATR-ATRIP to sites of DNA damage (Yoshioka et al., 2006). Furthermore, mismatch repair proteins have been implicated in sensing and processing ICLs. In particular, the MutSβ complex was shown to bind to intrastrand crosslinks produced by cisplatin (Duckett et al., 1996; Yamada et al., 1997), and the MutSβ complex was shown to be involved with the removal of ICLs produced by psoralen (Zhang et al., 2002). Furthermore, in the absence of MMR signaling ICL-repair proceeded in an alternate pathway promoting a non-recombination dependent mechanism (Zheng et al., 2006). Our study now suggests that MLH1 binding to FANCJ is functionally important for the ICL-induced response, as disruption of the native FANCJ/MLH1 interaction reduced cell survival following ICL treatment. Moreover, FANCJ K141/142A mutant, defective in MLH1 binding, fails to correct FA-J cells. The finding that MLH1-deficiency did not generate

gross changes in the cellular survival following ICLs may stem from the loss of both MMR DNA damage repair and signaling. In fact, the loss of DNA damage signaling and the subsequent repair is essential in establishing the resistance of MMR-deficient cells to DNA methylation (O'Brien and Brown, 2006). The multiplicity of MMR functions in the DNA damage response has been recently uncovered through separation-of-function mutations (O'Brien and Brown, 2006).

Here, we attempted a similar approach to dissect the role of MLH1 and FANCJ in the ICL-response. Three approaches were attempted to selectively ablate the FANCJ/MLH1 interaction in vivo. In one of these approaches, we attempted to generate an MLH1 mutant that lacked FANCJ binding. While MLH1 and FANCJ bind directly in vitro, the interaction is facilitated by PMS2 in vivo. In the absence of PMS2, FANCJ binding to MLH1 requires two MLH1 C-terminal domains D1 (478-508) and D2 (736-744). However, in the presence of PMS2, only one of these domains was required. Moreover, both the region on MLH1 (703-725) required for FANCJ binding in the presence of PMS2 is also essential for PMS2 stabilization. The complexity of the MLH1/PMS2/FANCJ interaction confounded attempts to selectively ablate the FANCJ/MLH1 interaction through MLH1 mutagenesis. Fortunately, the binding of MLH1 to FANCJ was less complex and therefore, the FANCJ/MLH1 interaction could be selectively disrupted by both mutagenesis and peptide disruption. We found that disruption of this complex caused defects in the ICL-response, consistent with the MLH1/FANCJ interaction being required for ICL-repair.

Conceivably, for ICL repair, mismatch repair complexes including MutLα, mobilize or regulate FANCJ helicase activity to unwind DNA in the vicinity of the DNA damage to facilitate repair processes. Thus, disruption of FANCJ helicase activity or MLH1 binding could interfere with ICL-response. In support of this possibility, the MLH1 homologue in *E. coli*, MutL binds the DNA helicase UvrD gene product Helicase II (Hall et al., 1998; Spampinato and Modrich, 2000) and stimulates its helicase activity (Dao and Modrich, 1998; Yamaguchi et al., 1998). However, we did not detect an effect of the MutLα complex, inhibitory or stimulatory, on FANCJ catalyzed unwinding of a forked duplex, 5' flap, or Holliday Junction substrate (data not shown). It is possible that regulation of FANCJ helicase activity by MLH1 may require additional MMR proteins and/or that the physical interaction between FANCJ and MLH1 serves a non-catalytic role in mediating the ICL-response.

Alternatively, the catalytic activity of FANCJ may serve an entirely different purpose (see Figure 2-7). For example the FANCJ helicase activity could serve to displace MutLα from DNA. This type of model has also been proposed for other helicases. For example, the Srs2 helicase is proposed to displace a checkpoint protein to facilitate checkpoint exit (Vaze et al., 2002). If true, in the absence of FANCJ (FA-J cells), or in FA-J cells with a catalytically inactive FANCJ and/or a FANCJ mutant that lacks MLH1 binding, the MutLα complex would fail to be displaced from DNA. As such, the MutLα complex would be stuck or take longer to be displaced from DNA leading to a prolonged G2/M

arrest and/or delay in the completion of repair. In support of this model, ICL treatment of FA-J cells reconstituted with vector, K52R, or K141/142A FANCJ constructs demonstrate both hyper-G2/M arrest and -sensitivity to ICLs. Furthermore, peptide perturbation of the MLH1/FANCJ interaction in MCF7 cells lead to enhanced ICL-sensitivity.

This proposed role for FANCJ in displacing MutLα is also not at odds with the finding that suppression of the MutLα complex in MCF7 cells did not lead to MMC sensitivity (Supplemental Figure S2-9). It has been reported that in the absence of MMR signaling there is a reduced G2/M arrest following DNA damage (O'Brien and Brown, 2006) and compensating non-recombination repair pathways are engaged (Zheng et al., 2006). The expectation from these findings would be that MMR-deficient cells would have a normalized sensitivity to ICLs due to the defective checkpoint and compensation by alternative mechanisms of repair (see model in Figure 2-7). Ultimately, it will be critical to establish whether a FANCJ/MLH1 complex facilitates ICL-repair by promoting homologous recombination or other repair functions. While ablation of BRCA1 binding to FANCJ may not affect the ability of FANCJ to correct the defective response of FA-J cells, the timing or mechanism of repair may be altered. Thus, it is important to consider that the FA-J cells lacking the FANCJ/BRCA1 interaction resist the ICL-induced 4N arrest and sensitivity through an alternative mechanism perhaps by checkpoint avoidance. Experiments are currently underway to investigate this possibility.

While MMR proteins are essential to elicit a G2/M arrest, it is presently unclear as to how this arrest is overcome. In cells lacking the active FANCJ helicase and/or a FANCJ/MLH1 interaction, diffusion of the MutLα complex from DNA may lead to an eventual exit from this G2/M arrest. In contrast this exit may not be achieved in FA-J cells expressing the K52R FANCJ, if the MutLα complex is locked on the DNA by the inactive helicase. It follows that a complete failure to re-enter the cell cycle, as opposed to a slower entry, would be more toxic to cells. Consistent with this, we found that the FA-J K52R expressing cells are short lived in tissue culture and forced expression of K52R FANCJ in other cell lines is not stable (data not shown).

Given that the FANCJ/MutLα interaction is intact in other FA cells, including FA-A, FA-D2, and FA-D1 (Supplemental Figure S2-8), loss of this complex is not a general feature of FA cells. However, it remains to be determined whether additional FA-MMR interactions are altered. Moreover, similar to FANCJ deficiency, MLH1 deficiency does not affect the ATR-mediated FANCD2 monoubiquitination (Supplemental Figure S2-9) (Andreassen et al., 2004); (Bridge et al., 2005; Levitus et al., 2004; Litman et al., 2005), suggesting that the FANCJ/MLH1 interaction is not essential for FA-pathway activation. Interestingly, we find that deletions in the MLH1 C-terminus (703-725), which are important for maintaining the stability of PMS2 (Mohd et al., 2006) also disrupted the FANCJ/MLH1 interaction. This finding has implications for MLH1 clinical mutations identified in colon cancer patients, potentially linking MLH1 function not

only to PMS2, but also to FANCJ.

In conclusion, these studies have provided the first evidence for a role of the FANCJ/MutLα complex in the ICL induced response. This work extends the already implicated role of MMR proteins in the ICL-response. Further study of the role of FANCJ, BRCA1, and MMR proteins in this process should advance the understanding of how ICL-induced responses are regulated to preserve genomic integrity.

Figure 2-1: **The FANCJ/BRCA1 interaction is dispensible for correction of the 4N DNA accumulation defect in FA-J cells.** A) FA-J cells were reconstituted with vector, WT or S990A and were either left untreated or treated with melphalan and the percentage of cells with 4N DNA content was analyzed by FACS. B) The percent of cells with 4N DNA content after ICL-treatment was averaged for each cell line from four independent experiments with standard deviation (SD) indicated by error bars. C)FANCJ expression was analyzed in whole cell extracts (WCE) by immunoblot. β-actin serves as a loading control for the WCE samples. B) Immunoprecipitations with FANCJ (E67) were analyzed by Western blot with the noted Abs.

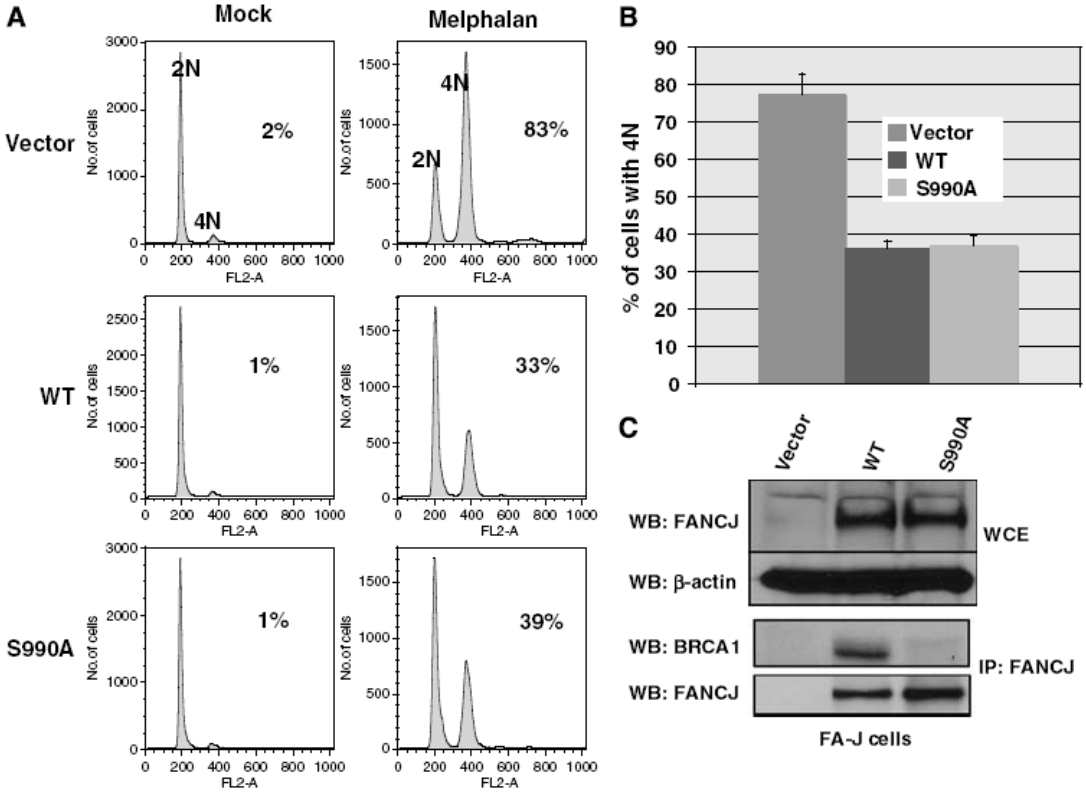


Figure 2-2: **FANCJ interacts with the MMR proteins MLH1 and PMS2.** A) Silver-stained gel of the WT FANCJ (F) compared to a Vector (V) purified complexes from HeLa S3 cells by consecutive Flag and HA purification steps (Flag/HA). Identified unique bands are indicated and FANCJ is observed as two species, the 140kD band is labeled. B) Western blot detection of Flag/HA purified FANCJ complexes. C) Immunoprecipitations with either FANCJ (E67 or E47) or MLH1 Abs from MCF7 or 293T cells were analyzed by Western blot with the noted Abs. D) Western blot shows the presence of the indicated proteins from MLH1 IPs from FA-J cells reconstituted with vector or WT FANCJ. E) HeLa cells were either left untreated or treated with 1mM hydroxyurea (HU) for 24hr or 2.4ug/mL MMC for 1hr. HeLa cell lysates were immunoprecipitated with preimmune (PI) or FANCJ Abs followed by Western blot analysis with the noted Abs.

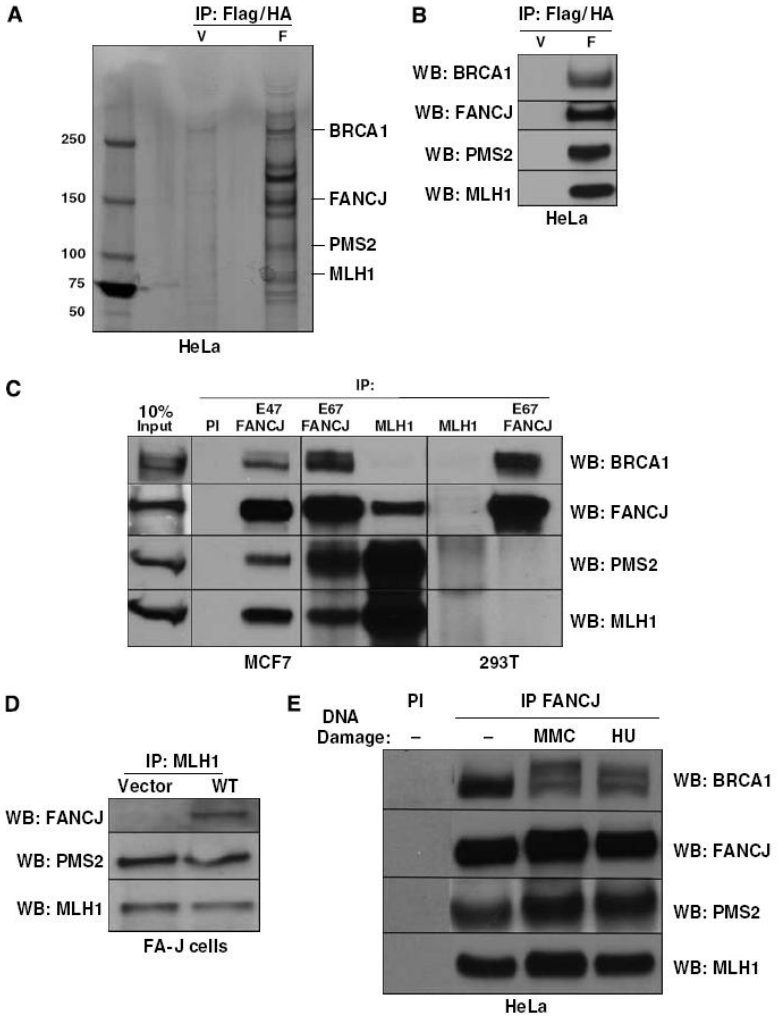


Figure 2-3: **FANCJ helicase domain associates with the MutLα complex independent of BRCA1 and through a direct interaction with MLH1.** A) MCF7 cells were stably infected with a lentivirus encoding shRNA for either eGFP or BRCA1. FANCJ IP was performed followed by Western blot for the indicated proteins. B) MCF7 cells were transiently transfected with pCDNA3 vectors containing no insert (-), full length FANCJ (FL), helicase domain including amino acid residues 1-882 (HD) or C-terminus including residues 882- 1249 (CT) of FANCJ, then immunoprecipitated with the Myc Ab (9E10). Arrows designate the respective FANCJ myc-tag species. Immunoglobulin (IgG) is shown. C) Western blot of the indicated IP experiments in which in vitro translated MLH1 was incubated with recombinant FANCJ or BRCA1 proteins. D) Purified recombinant MLH1 or BSA was coated onto ELISA plates. Following blocking with 3% BSA, the wells were incubated with increasing concentrations of purified recombinant FANCJ (0-40 nM) for 1 hr at 30 ^{o}C, and bound FANCJ was detected by ELISA using a rabbit polyclonal Ab against FANCJ followed by incubation with secondary horseradish peroxidase (HRP)-labeled antibodies and OPD substrate. Data points are the mean of three independent experiments performed in duplicate with SD indicated by error bars. E) ELISA was performed as described in Panel D using 4.9 nM FANCJ alone or in the presence of EtBr (50 ug/ml) or DNaseI (2 ug/ml). BSA (3%) was used as a control instead of MLH1 during the coating step.

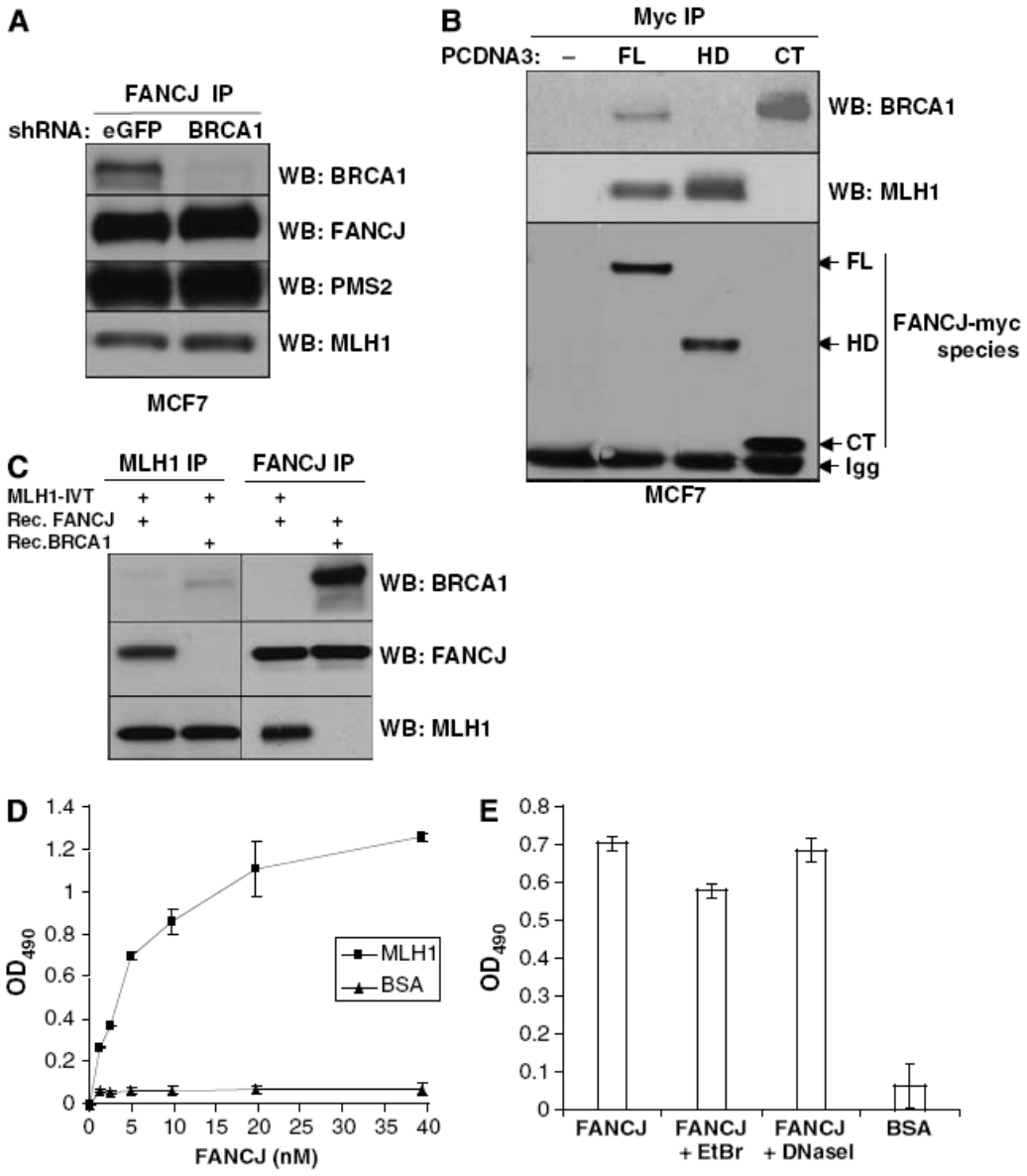

A
FANCJ IP
shRNA: eGFP BRCA1
WB: BRCA1
WB: FANCJ
WB: PMS2
WB: MLH1
MCF7
B
Myc IP
PCDNA3: – FL HD CT
WB: BRCA1
WB: MLH1
FL
HD
CT
Igg
FANCJ-myc species
MCF7
C
MLH1 IP
FANCJ IP
MLH1-IVT + + +
Rec. FANCJ + + +
Rec.BRCA1 + +
WB: BRCA1
WB: FANCJ
WB: MLH1
D
OD490
MLH1
BSA
FANCJ (nM)
E
OD490
FANCJ
FANCJ + EtBr
FANCJ + DNaseI
BSA

Figure 2-4: **PMS2 facilitates the FANCJ interaction with the MLH1 C-terminus.** A) MLH1 or Myc (9E10) IP experiment were performed from 293T cells that were transfected with vector alone (V), full-length MLH1 (WT), or MLH1 species alone or in combination with PMS2 (C1-C3, N1, N2). IP products were analyzed by Western blot with FANCJ, PMS2 and MLH1 Abs. B) MLH1 IP experiment were performed from 293T cells that were transfected with vector alone (V), full-length MLH1 (WT), or MLH1 species in combination with PMS2 (C4-C7). IP products were analyzed by Western blot with FANCJ, PMS2 and MLH1 Abs. C) Schematic representation of the MLH1/FANCJ dimer domains (D1, D2) and the region between 703-725 is highlighted as an essential element for maintaining the MLH1/PMS2/FANCJ complex.

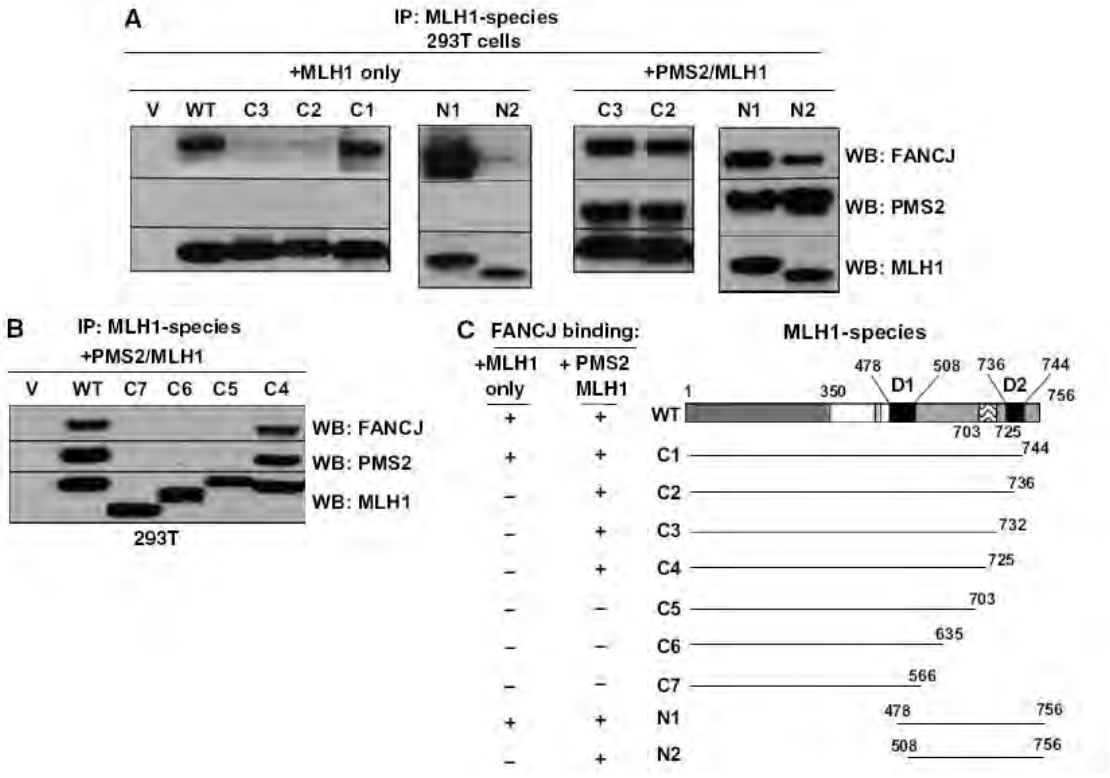


Figure 2-5: **Expression of FANCJ residues 128-158 disrupt the FANCJ/MLH1 interaction to generate ICL-sensitivity.** A) Myc (9E10) IP experiments were performed from MCF7 cells that were transfected with vector alone (-), full-length FANCJ (FL), and the different FANCJ constructs (A-G) shown in C, followed by Western blot with MLH1, and Myc Abs. The asterisk denotes the migration of the different myc-tagged FANCJ species. B) Myc IP experiments were performed from MCF7 cells that were transfected with either eGFP empty vector or the 128-158 FANCJ-eGFP constructs followed by Western blot with the indicated Abs. C) The different FANCJ constructs are shown with a positive (+) or negative (-) to indicate binding to MLH1. E) MCF7 cells transfected with vector alone or the 128-158 FANCJ-eGFP construct, treated with increasing concentrations of MMC and incubated for 4-5days. Cell growth was measured by ATP content. Three independent representative experiments are shown and depicted by red, yellow, and blue lines. Solid lines represent cells transfected with empty-eGFP vector and hatched lines represent cells transfected with 128-158-eGFP vector.

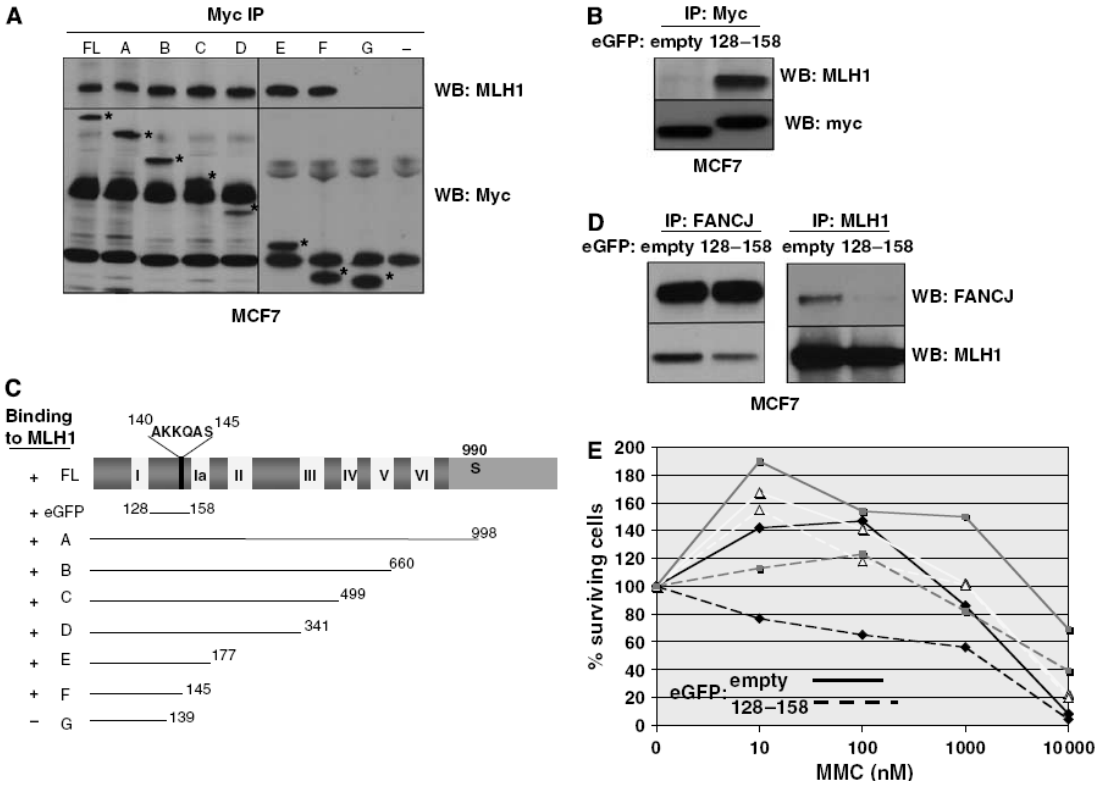


Figure 2-6: **MLH1 binding to FANCJ is essential to correct FA-J cells.** A) Myc IP experiments were performed from MCF7 cells that were transfected with vector alone (-), FL, V, Q143E, S145A, and K141/142A FANCJ constructs, followed by Western blot with the indicated Abs. FA-J cells were reconstituted with empty vector, WT, K141/142A, or K52R FANCJ vectors and FANCJ expression was analyzed by whole cell extracts, β-actin serves as a loading control for the WCE samples. Western blot shows the presence of the indicated proteins from FANCJ IPs from FA-J cells reconstituted with vector, WT, or K141/142A FANCJ. B) FA-J cell lines reconstituted with empty vector, WT, K141/142A, or K52R FANCJ were either left untreated or treated with melphalan. The percent of cells with 4N DNA content after ICL-treatment was averaged for each cell line from four independent experiments with standard deviation (SD) indicated by error bars. C, D) FA-J cells reconstituted with vector, WT, K141/142A, K52R, or S990A FANCJ were seeded on 24 well plates and incubated overnight under normal growth conditions. The cells were then treated with the indicated doses of MMC and incubated for eight days. On the final day, the cells were counted and the percentage of live cells was calculated. Experiments were performed in triplicate and a representative graph is shown. The IC_{50} dose for the FA-J vector (250nM) was compared for all mutants and error bars represent the standard deviation.MLH1 binding to FANCJ requires lysine residues 141 and 142.

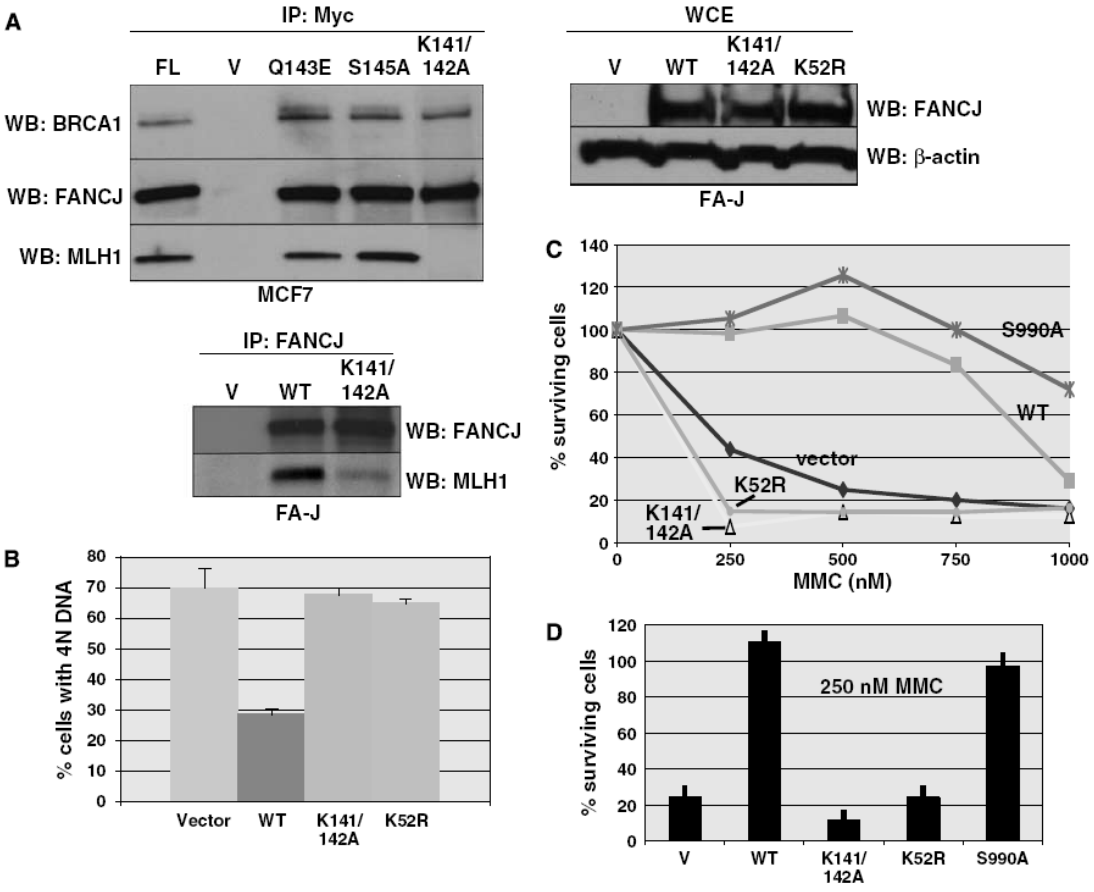
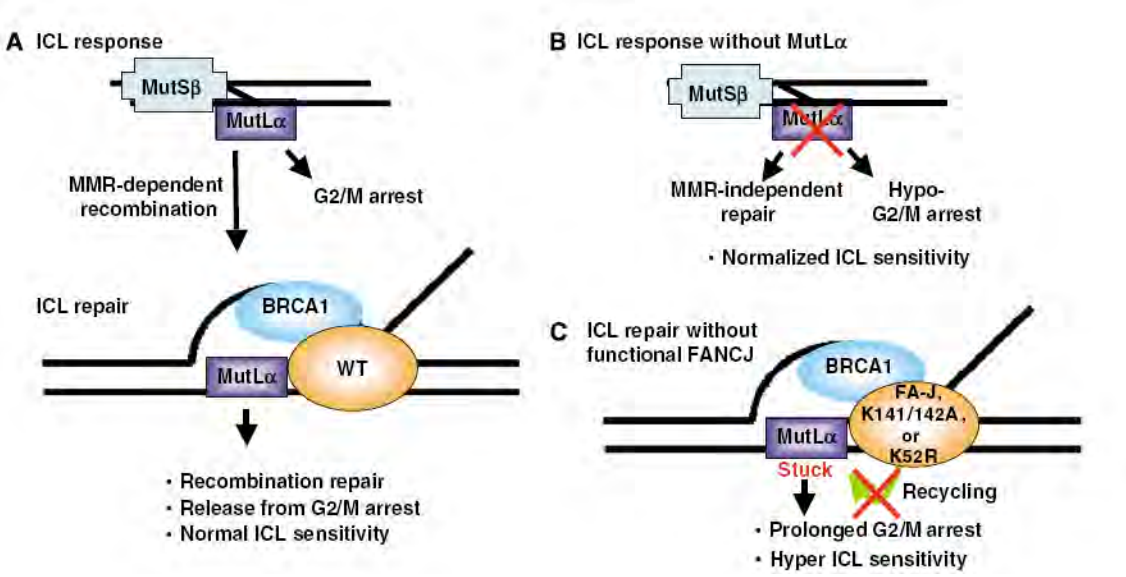
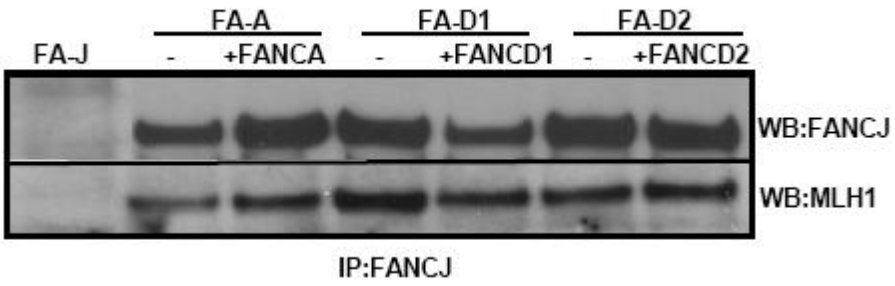

A
IP: Myc
FL
V
Q143E
S145A
K141/ 142A
WB: BRCA1
WB: FANCJ
WB: MLH1
MCF7
IP: FANCJ
V
WT
K141/ 142A
WB: FANCJ
WB: MLH1
FA-J
WCE
V
WT
K141/ 142A
K52R
WB: FANCJ
WB: β-actin
FA-J
B
% cells with 4N DNA
Vector
WT
K141/ 142A
K52R
C
% surviving cells
MMC (nM)
S990A
WT
vector
K52R
K141/ 142A
D
% surviving cells
250 nM MMC
V
WT
K141/ 142A
K52R
S990A

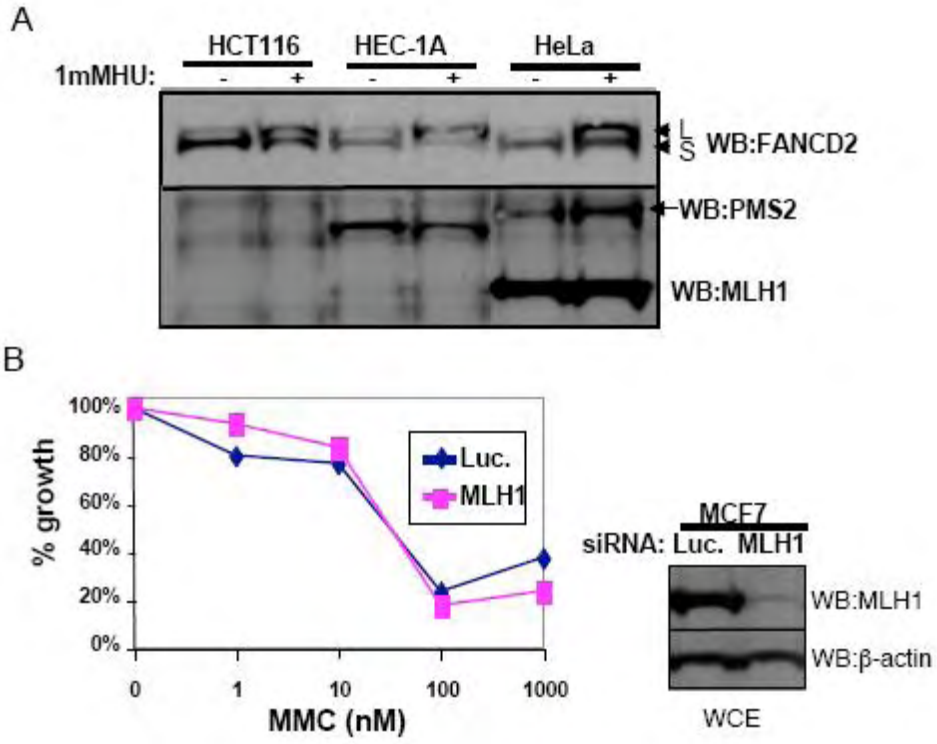
Figure 2-7: **Model depicting how FANCJ and MutLα proteins function to mediate the ICL response.** A) The normal ICL-response is proposed to include MMR proteins. This is supported by the findings that the MMR machinery has been shown to specifically bind crosslinked DNA adducts (Duckett et al., 1996; Yamada et al., 1997; Zhang et al., 2002), mediate ICL-recombination repair (Zheng et al., 2006), and induce a G2/M arrest (Cejka et al., 2003). Furthermore, MMR proteins including the MutLα complex, similar to BRCA1 and FANCJ are essential for recombination processes (de Wind et al., 1995; Jasin, 2002; Litman et al., 2005; Mohindra et al., 2002). B) The ICL-response without MutLα is predicted to lead to an ICL-response through a MMR-independent non-recombination based mechanism with a minimal G2/M arrest so that ICL-sensitivity is normalized. C) ICL-repair without functional FANCJ is predicted to be directed to recombination as in A, but generate a prolonged G2/M arrest due to absent or dysfunctional FANCJ protein. In the absence of FANCJ protein, helicase activity, or MLH1 binding, FANCJ is unable to displace MutLα from recombination intermediates and consequently, the MutLα complex remains stuck or tethered to DNA longer delaying the exit from the G2/M arrest and enhancing ICL-sensitivity.



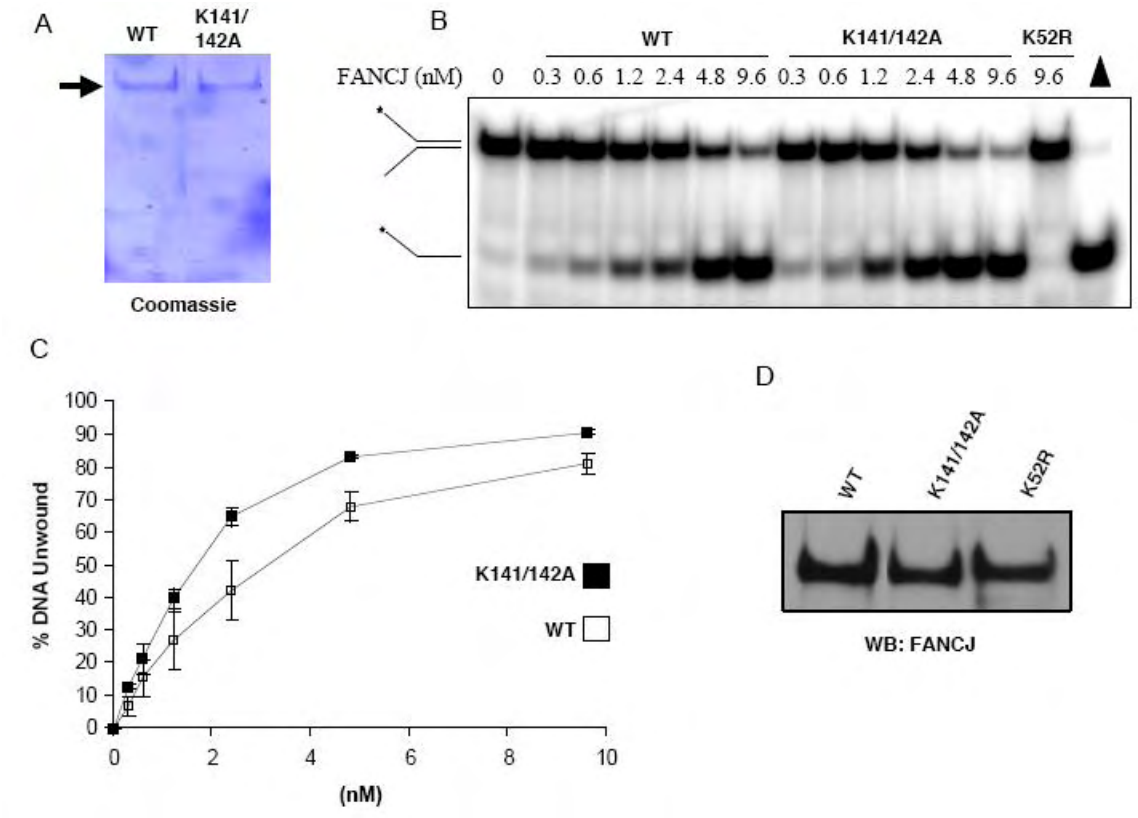
Supplemental Figure S2-8: **FANCJ/MLH1 interaction is intact in FA cells.** FA cells were lysed in 150mM NETN and lysates were normalized. Immunopreciptations were performed using FANCJ Ab (E47) and precipitates were analyzed by Western blot using FANCJ and MLH1 specific Abs.



Supplemental Figure S2-9: **MutLα complex is downstream of FANCD2 monoubiquitination and MLH1-deficient cells do not have enhanced ICL-sensitivity.** A) HCT116, HEC-1A, and HeLa cells were either left untreated or treated with 1 mM hydroxyurea (HU) for 24 hours, lysed with 600mM NETN, and analyzed by Western blot with the indicated Abs. Both long (L) and short (S) forms of FANCD2 are noted. B) MCF7 cells transfected with control siRNA (Luc) or MLH1 siRNA were seeded in a 96-well plate and incubated overnight. Transfected cells were treated with increase concentrations of MMC and incubated for 4 days. Cell survival was analyzed by ATP content. The graph on the left is a representative experiment from 3 independent experiments. Whole cell extracts from the MCF7 transfected cells were analyzed by Western blot using the specified antibodies.



Supplemental Figure S2-10: **The K141/142A FANCJ mutant maintains robust helicase activity.** A) Recombinant K141/142A FANCJ protein was immunoprecipitated with Flag antibodies and eluted using Flag peptide. The purity of the protein sample was visualized by Coomassie stain and arrow denotes both WT and K141/142A FANCJ species B) Helicase reactions (20 µl) were performed by incubating the indicated concentrations of WT, K141/142A, or K52R FANCJ as with 0.5 nM forked duplex DNA substrate at 30 ^{o}C for 15 min in the presence of ATP (2 mM) under standard helicase assay conditions as described under "Materials and Methods." The duplex substrate runs at the top of the gel and the unwound DNA fragment runs below as shown. Filled triangle, heat-denatured DNA substrate control. A phosphorimage of a typical gel is shown. C) Quantitative helicase data represent the mean of at least three independent experiments with standard deviation (SD) indicated by *error bars*. Open squares, FANCJ-WT; filled squares, FANCJ-K141/142A. D) Recombinant WT, K141/142A, and K52R proteins used in B were separated by SDS-PAGE and analyzed by Western blot using FANCJ specific antibodies.



Materials and methods

Cell lines

HeLa, MCF7, 293T and HeLa S3 cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL each). FA-J (EUFA30-F) cells were cultured as previously described (Litman et al., 2005). HCT116 cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/mL each). Hi5 insect cell were grown in Grace's Insect Media supplemented with 10% Fetal Bovine Serum and 1% genetemycin at 28°C without $C0_2$. FA-J cells were infected with the pOZ retroviral vector (Nakatani and Ogryzko, 2003) containing no insert, WT, K141/142A, or K52R FANCJ inserts, or with the lentiviral vector pLentiV5 (Invitrogen) vector, containing no insert, WT, or S990A FANCJ inserts. Stable FA-J pOZ cell lines were generated by sorting pOZ infected cells with anti-IL-2 magnetic beads (Dyna Beads) and expanding IL-2 positive cells. Stable FA-J pLenti cell lines were generated through blasticidin selection (7ug/mL).

Purification of a FANCJ complex

A FANCJ complex was purified from nuclear extracts (NE) derived from ~8 $X10^9$ HeLa cells stably expressing the double tagged-FANCJ by two step immunoaffinity chromatography according to the standard method (Nakatani and Ogryzko, 2003). Flag-HA double purified material was electrophoresed in 3-8%

Tris-Acetate Gel (Invitrogen). Individual Silver-stained bands were excised and subsequently analyzed by Mass spectrometry (Genomine Inc., South Korea).

Immunoprecipitations, immunoblotting and antibodies

Cells were harvested and lysed in 150mM NETN lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10μg/ml leupeptin, 10μg/ml aprotinin) for 30 min on ice. Cell extracts were clarified by centrifugation. The cell lysates were boiled in SDS loading buffer. For immunoprecipitation assays cells lysates were incubated with protein-A beads and either FANCJ (E67 or E47), MLH1 (BD Bioscience) or Myc (9e10) Abs at 4°C for 2 hr. Beads were subsequently washed and boiled in SDS loading buffer. Proteins were separated using SDS-PAGE and electrotransferred to nitrocellulose. Membranes were blocked in 5% milk PBS/tween and incubated with primary Ab for 1hr. Abs for Western blot analysis included anti-MLH1 (BD Bioscience,1:500), anti-PMS2 (BD Biosciences 1:200), anti-BRCA1(ms110, hybridoma cell,1:3), anti-FANCJ (Monoclonal pool 2G7, 2C10,1B4), anti-FANCD2 (Fanconi Anemia Research Foundation), and Myc (9E10, hybridoma cell, 1:3). Membranes were washed, then incubated with horseradish peroxidase-linked secondary antibodies (Amersham,1:5000), and detected by chemiluminescence (Amersham).

ICL-induced 4N accumulation assay and sensitivity assay

FA-J reconstituted with either vector, WT FANCJ, K141/142A FANCJ, K52R FANCJ, or S990A FANCJ were either treated with 0.5 µg/ml of melphalan (Sigma) or left untreated and incubated for 65 hr. Cells were fixed with 90% methanol in PBS and were then incubated 10 min with PBS containing 30 u/ml DNase-free RNase A and 50 µg/ml propidium iodide. 1 x 10^4 cells were analyzed using a FACs Calibur instrument (Becton-Dickinson, San Jose, CA). Aggregates were gated out and the percentage of cells with 4N DNA content was calculated using Modfit software.

The FA-J cells reconstituted with either vector, WT FANCJ, K141/142A FANCJ, K52R FANCJ, or S990A FANCJ were seeded on 24 well plates 1000 cells/well and incubated overnight. The cells were either left untreated or treated with increasing doses of MMC for 1hr and incubated for 8 days. Finally, the cells were collected by trypsinization and counted using a hemacytometer. The percentage of live cells at each concentration was calculated using the untreated controls as the baseline growth.

MCF7 cells transfected with either empty vector or the 128-158 FANCJ eGFP construct were seeded at 500 cells/well in 96-well plates and incubated overnight. The cells were either left untreated or treated with increasing concentrations of MMC for 1hr and incubated 4-5 days. The percentage of live cells was calculated by ATP content as previously described (Litman et al.2005).

Plasmid construction and in vitro translation

The WT and S990A FANCJ pLentiviral vectors were a gift of J. Chen (Yu et al., 2003). The pCDNA3-myc.his vector (Invitrogen) was digested by Not1/Apa1 and different FANCJ fragments generated by PCR and digested Not1 and Apa1 were inserted. Primers are available upon request. The WT FANCJ pOZ-FH vector was generated by PCR cloning. Specifically, 5' Xho1 and a 3' Not1 restriction sites were added by using primers: 5'-3' CGCTCGAGGCCACCATGTCTTCAATGTGGTCTGAATATACAATT and 5'-3' CAGCGGCCGCCTTAAAACCAGGAAACATGCCTTTATT. The PCR product was digested XhoI and NotI and subcloned into the pOZ-FH vector. The K52R, S990A, and K141/142A pOZ vectors were generated with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) by using the FANCJ-pOZ as a template and the following primers: (K52R) 5'-3' CCCACAGGAAGTGGAAGGAGCTTAGCCTTAGCC and 5'-3' GGCTAAGGCTAAGCTCCTTCC-ACTTCCTGTGGG; (S990A)5'-3' TCCAGATCCACAGCCCCAACTTTCAAC and 5'-3' GTTGAAAGTTGGGGCTGTGGATCTGGA. (K141/142A) 5'-3' GCAAAGTTATCTGCT GCGGCACAGGCATCCATATAC and 5'-3' GTATATGGATGCCTGTGCCGCAGCAGATAACTTTGC. The same set of primers were used to generate the K141/142 A-pCDNA3 and K141/142 A-pVL132 by using the WT FANCJ pCDNA3myc.his (Cantor et al., 2001) and

pVL132Flag tagged (Cantor et al., 2004) constructs respectively. The Q143E and S145A pCDNA3 were generated with the QuickChange Site-Directed Mutagenesis Kit(Stratagene, La Jolla, CA) by using the following primers: (Q143E)5'-3' GCTGCAAAGTTATCTGCTAAGAAAGAGGCATC CATATACAG and 5'-3' CTGTATATGGATGCCTCTTTCTTAGCAGATAACTTTGCAGC; or (S145A) 5'-3' TCTGCTAAGAAACAGGCAGCCATATACAGAGAT GAA and 5'-3' TTCATCTCTGTATATGGCTGCCTGTTTCTTAGCAGA. All DNA constructs were confirmed by DNA sequencing. MLH1 protein was synthesized in vitro by coupled transcription and translation using the T7 Quick-coupled TnT kit (Pormega) and MLH1 pCDNA3 vector as a template (Plotz et al., 2003) gift of Guido Plotz (Homburg/Saar, Germany). The WT-MLH1 pcDNA3 vector (Invitrogen) was digested Not1/Apa1 and different MLH1 fragments were generated by PCR and digested Not1 and Apa1 products were inserted. Primers are available upon request.

The FANCJ fragment (amino acids 128-158) was generated by PCR. An eGFP expression vector was created by subcloning PCR fragments of eGFP into pcDNA3.1 (Invitrogen, Carlsbad, California) (gift of Dr. Andrew Kung) to create new unique restrictions sites within the active loop of eGFP. The annealed FANCJ fragment and the empty eGFP pCDNA3 vector were digested with BamH1 and EcoR1 restriction enzymes for 1hr at 37°. The FANCJ fragment and eGFP pCDNA3 vector were ligated using Quick ligase (NEB) for 1hr at room temperature. Primers covered FANCJ sequence from 128-158Aa.

ELISA studies

Purified recombinant MLH1 protein was diluted to a concentration of 1 ng/ l in Carbonate buffer (0.016 M Na_2CO_3, 0.034 M $NaHCO_3$, pH 9.6) and added to appropriate wells of a 96-well microtiter plate (50µl/well), which was incubated at 4 ^oC. 3% Bovine serum albumin (BSA) was used in the coating step for control reactions. The samples were aspirated, and the wells were blocked for 2 hr at 30 ^oC with Blocking buffer (phosphate buffered saline, 0.5% Tween 20 and 3% BSA). The procedure was repeated. Purified recombinant FANCJ protein was diluted in Blocking buffer, and the indicated concentrations were added to the appropriate wells of the ELISA plate (50µl/well), which was incubated for 1 hr at 30 ^oC. For EtBr or DNaseI treatment, 50µg/ml EtBr or DNaseI (2µg/ml) was included in the incubation with FANCJ during the binding step in the corresponding wells. The samples were aspirated, and the wells were washed five times before addition of rabbit polyclonal anti-FANCJ antibody (Sigma, B-1310) that was diluted 1: 5,000 in Blocking buffer. Wells were then incubated at 30 ^oC for 1 hr. Following three washings, horseradish peroxidase-conjugated anti-rabbit secondary antibody (1: 5,000) was added to the wells, and the samples were incubated for 30 min at 30 ^oC. After washing five times, any FANCJ bound to the immobilized MLH1 was detected using OPD substrate (Sigma). The reaction was terminated after 3 min with 3 N H_2SO_4, and absorbance readings were taken at 490 nm.

Recombinant protein and helicase assays

Hi5 cells were infected with pVL132 K141/142A FANCJ and incubated for 72 hr. Cells were collected, lysed in Insect Lysis Buffer (Roche) containing protease inhibitors (Roche) for 30 min at 4°C, and subsequently cleared by centrifugation. The WT, K52R, and K141/142A FANCJ proteins were purified as previously described (Cantor et al., 2004). Briefly, K141/142A FANCJ-Flag was immunoprecipitated with 50 µl of FlagM2 conjugated beads for 2 hr at 4^0C. Beads were washed three times in 500 mM NETN [500mM NaCl, 0.5% NP-40, 1mM EDTA and 20 mM Tris-HCL (pH8.0)] followed by a final wash with 150 mM NETN and K141/142A FANCJ protein was eluted twice using 3x Flag peptide. Elutions were pooled and dialyzed overnight in storage buffer. Helicase assay reaction mixtures (20 µl) contained 40 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 2% glycerol, 100 ng/µl BSA, 2 mM ATP, 10 fmol of the specified duplex DNA substrate (0.5 nM DNA substrate concentration), and the indicated concentrations of FANCJ helicase. Helicase reactions were initiated by the addition of FANCJ and then incubated at 30 ^{o}C for 15 min. Reactions were quenched in the presence of a 10-fold excess of unlabeled oligonucleotide with the same sequence as the labeled strand to prevent reannealing and products resolved on nondenaturing 12% (19:1 acrylamide: bisacrylamide) polyacrylamide gels and quantitated as previously described (Gupta et al., 2005).

Acknowledgments

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CHAPTER III

An MLH1 mutation links BACH1/FANCJ to colon cancer and insight towards directed therapy

Abstract

Defects in DNA mismatch repair (MMR) proteins are the primary cause for hereditary non-polyposis colon cancer (HNPCC). Mutations in MMR genes, such as MLH1 or MSH2 often disrupt mismatch repair and/or signaling. Mammalian mismatch repair is not predicted to require a DNA helicase. Thus, we considered whether MMR-signaling required the direct interaction between MLH1 and the BRCA1-associated FANCJ helicase. We found that in response to DNA damage, FANCJ promotes MMR complex formation. As such, FANCJ-deficient cells have delayed apoptosis and greater resistance to O6-methylguanine through methylation reversal. Intriguingly, loss of the FANCJ/MLH1 interaction was identified in the HNPCC mutant, $MLH1^{L607H}$ that is proficient in mismatch repair. This link between FANCJ and colon cancer suppression provides insight towards directed therapies as cancers lacking the MLH1/FANCJ interaction are likely to be uniquely sensitive to DNA crosslinking agents.

Introduction

In the absence of DNA repair proteins, cell cycle checkpoints, and/or DNA damage, repair pathways are not properly activated. This inability to actively respond to DNA damage can lead to chromosomal instability, cell death, or cancer. The same DNA repair deficiencies that can cause cancer can also make them resistant to DNA-damaging agents used as chemotherapy agents. For example, loss of mismatch-repair (MMR) proteins is associated with hereditary nonpolyposis colon cancer (HNPCC) (O'Brien and Brown, 2006) and with resistance to the cytotoxicity of many therapeutic agents. In MMR-mutant cells DNA damage can accumulate without triggering cell death, i.e., cells become tolerant or resistant as cells “ignore” DNA damage and continue to replicate damaged DNA (Branch et al., 1993; Kat et al., 1993). These replicating cells not only resist DNA damage-induced arrest and avoid apoptosis, but also have a greatly enhanced mutation frequency. Thus, MMR-deficient cells often have a mutator phenotype, i.e., genetic information is lost (Schofield and Hsieh, 2003). This alteration of DNA sequences, which occurs in highly repetitive regions such as microsatellite DNA, is termed microsatellite instability (MSI).

MMR-mutant cells are especially resistant to alkylating agents, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU), which generate methylation at O6 in guanine of DNA to form O6-methylguanine (O6-meG) (Jiricny, 2006). In MMR-proficient cells recognition of DNA methylation, which resembles DNA mismatches, is mediated by the heterodimer

MutSα (MSH2 and MSH6) (O'Brien and Brown, 2006). The MutSα complex recruits the heterodimer MutLα (MLH1 and PMS2), which is also essential for functional MMR signaling and repair. Once mismatches or DNA damage is detected, MMR proteins signal a checkpoint response, repair, and/or apoptosis (Hawn et al., 1995). MMR-processing of DNA methylation fails to repair DNA and consequently drives apoptosis.

The MMR-induced apoptosis following DNA methylation has been proposed to evolve from one of two mechanisms. First, MMR proteins are hypothesized to facilitate misguided attempts to repair DNA methylation, ultimately leading to more severe secondary lesions, such as double strand breaks (Karran, 2001). Thus, in the absence of MMR proteins, cells escape these secondary lesions and death. Second, MMR proteins function in signaling that is hypothesized to promote checkpoint and apoptosis (Karran, 2001). Following DNA methylation damage, both the MutSa and MutLa complexes are required to recruit and activate the checkpoint kinase, ATR (Yoshioka et al., 2006). In support of the essential role of MMR signaling, separation-of-function mutants that are repair defective, but intact for signaling undergo DNA methylation-induced apoptosis. In contrast, reduced levels of expression of MMR can support repair, but not signaling. In this case, cells are microsatellite stable (MSS), but DNA methylation-induced apoptosis is reduced and cells are more tolerant to DNA damage (O'Brien and Brown, 2006).

Loss of DNA damage repair, checkpoint functions, and cancer are also

associated with defects in the BRCA1-associated helicase FANCJ (also known as BACH1/BRIP1) (Peng et al., 2006). FANCJ mutations were identified not only in patients with breast cancer (Cantor et al., 2001), but also in patients with the cancer prone disease Fanconi anemia (FA) (Litman et al., 2005). FANCJ-null FA cells are sensitive to DNA damage and undergo an abnormal checkpoint response. We previously demonstrated that FANCJ interacts directly to MLH1 and this interaction was as important as FANCJ's helicase activity for restoring a normal DNA damage response to FANCJ-null cells (Peng et al., 2007). Given that the FANCJ/MLH1 interaction is critical for a normal DNA damage response, conceivably loss of this interaction could be associated with cancer and/or the development of DNA damage resistance.

Here, we uncover that FANCJ potentiates MMR complex formation in response to DNA damage. As such, FANCJ-deficiency delays MMR-induced apoptosis. This delay facilitates O6MeG resistance through MMR-independent methylation reversal by methyl guanine methyltransferase (MGMT). Furthermore, loss of the FANCJ/MLH1 interaction is associated with hereditary colon cancer. Specifically, the leucine(L) to histidine (H) change at position 607 MLH1 ablates MLH1 binding to FANCJ. Expression of the $MLH1^{L607H}$ point mutant generates sensitivity to DNA interstrand crosslinks links (ICLs), but resistance to DNA methylation. We suggest, therefore; that a delay in checkpoint and/or apoptotic responses could explain the HNPCC familial cancers, such as MLH^{L607H}, characterized as microsatellite stable (MSS) and with intact repair.

This previously unknown link between FANCJ, MMR signaling, and colon cancer suppression also provides insight towards directed therapy as such cancers are likely to be uniquely sensitive to ICLs.

Results

FANCJ-deficiency does not resemble MMR-deficiency

Unlike prokaryotic MMR in which a DNA helicase is required, mammalian MMR has been reconstituted in vitro without a DNA helicase (Dzantiev et al., 2004). Thus, as a starting point, we tested the idea that the FANCJ helicase could participate in MMR-signaling. In particular, MNU treatment generates O6MeG lesions that when processed by MMR-signaling generates a robust G2/M checkpoint, apoptosis, and reduced cell survival. These outcomes are reduced if O6MeG lesions are instead reversed by the O6-methylguanine-DNA methyltransferase (MGMT). Thus, we inhibited MGMT by treating cells with O6-benzylguanine (O6-BZG). To examine if FANCJ functions in MMR-signaling, we sought to directly compare FANCJ-deficient cells with MMR-deficient cells. Western blot confirmed that shRNA reagents targeted FANCJ and MLH1 proteins in MCF7 breast cancer cells (Figure 3-1A). FANCJ-depletion did not alter the expression of the MutLα complex (Figure 3-1A). In contrast, shRNA-targeting MLH1 suppressed both MLH1 and PMS2 confirming reports that MLH1 stabilizes PMS2 (Figure 3-1A). Consistent with a role for MLH1 in the DNA methylation induced G2/M checkpoint, MLH1-depleted cells had a reduced

number of cells accumulating with 4N DNA content ~4-fold by 72 and 96h post-MNU as compared to non-silencing control cells (Figure 3-1B). In contrast to MLH1-depletion, FANCJ-depletion did not dramatically alter the MNU induced G2/M checkpoint (Figure 3-1B). Furthermore, unlike MLH1-depletion, which dramatically enhanced the number of surviving colonies, FANCJ-depletion had no effect on MNU-induced colony survival as compared to cells treated with non-silencing siRNAs (Figure 3-1C). Together, these findings demonstrated that MMR signaling in response to O6MeG lesions was not affected by FANCJ-depletion.

FANCJ-deficiency measurably alters the MNU response when MGMT is active

To fully characterize the consequences of FANCJ-depletion on the O6MeG-induced response, we also performed experiments without inhibiting MGMT. With MGMT active, maximal apoptosis was achieved by 72h post-MNU, whereas the apoptosis in MGMT inhibited cells continued to climb from 72 to 96h (Figure 3-1D). Consistent with reduced apoptosis, cells with active MGMT formed colonies when treated with 150 ug/ml MNU, a dose that eliminated MGMT inactivated cells (Figure 3-1E). While depletion of MLH1 reduced apoptosis irrespective of MGMT activity, depletion of FANCJ only had a measurable effect on apoptosis in MGMT-active cells ~2 fold as compared to non-silencing controls. This decreased apoptosis also correlated with enhanced

colony survival ~2 fold as compared to non-silencing controls (Figure 3-1D and E). In fact, the colony survival in MLH1- or FANCJ-depleted MCF7 cells was similar with MGMT active (Figure 3-1E).

To confirm these findings, we analyzed growth in response to MNU in non-colony forming fibroblasts either proficient- (2822 cell line) or null- (FA-J EUFA30F cell line) for FANCJ (Litman et al., 2005). Asynchronous growing cells were plated at equal numbers, left untreated, or treated with increasing doses of MNU for 0.5 h and 5-7 days later surviving cells were counted. With MGMT-active, FANCJ-null FA-J cells had a greater percent growth at all doses of MNU as compared to normal fibroblasts (Figure 3-2A) or other FA cell lines, FA-C (PD331) or FA-G (PD352) (Figure 3-2B). Reconstitution of the missing wild-type FANCJ in FA-J cells restores a normal DNA interstrand crosslink response (Litman et al., 2005) (Figure 3-2C and Supplemental Figure S3-5). If FANCJ-deficiency generated enhanced growth due to an abnormal MNU response, we reasoned that re-introduction of wild-type FANCJ should restore MNU sensitivity and reduced growth. As before, with MGMT inhibited, the presence or absence of FANCJ had no effect on MNU sensitivity (Figure 3-2C). With MGMT active, however, wild-type FANCJ, as compared to vector reconstituted FA-J cells had a reduced percent growth following MNU treatment (Figure 3-2C). In contrast, the presence or absence of wild-type FANCD2 in FA-D2 cells did not affect the percent growth following MNU treatment (Figure 3-2D). Together, these results implicate that FANCJ-null cells as compared to other FA cells have a unique

tolerance to MNU when MGMT is active that is reversed upon re-introduction of wild-type FANCJ.

FANCJ function is linked to MMR

To further understand how FANCJ could function in the response to MNU, we addressed whether the FANCJ/MLH1 interaction and/or helicase activity was required to restore MNU sensitivity to FA-J cells. FA-J cells were also reconstituted with the FANCJ mutants, FANCJ$^{K141/142A}$, which is ablated for MLH1 binding (Peng et al., 2007) or FANCJ^{K52R}, which is enzyme inactive (Cantor et al., 2004). The proteins were expressed similarly to wild-type FANCJ (Figure 3-2C). Unlike reconstitution with wild-type, however, FANCJ^{K52R}, and FANCJ$^{K141/142A}$ reconstitution did not reduce growth in response to MNU (Figure 3-2C), consistent with the idea that both MLH1 binding and helicase activities are required for FANCJ to restore a normal MNU response.

Based on our findings, we reasoned that FANCJ-deficiency could enhance MNU resistance due to reduced MMR- and/or enhanced MGMT-function. If FANCJ functions to promote MMR-function only, we reasoned that FANCJ-deficiency should not affect resistance to other DNA damaging agents that are not processed by MMR. In particular, MMR selectively processes O6-MeG lesions, generated by MNU or MNNG, but not lesions generated by methyl methanesulfonate (MMS) or N-(2-chloroethyl)-N'-cyclohexyl-N-nitrosourea (CCNU) (Kaina et al., 2007). If FANCJ normally only reduces MGMT-function,

FANCJ-deficiency should enhance resistance to CCNU, which generates DNA methylation that is reversed by MGMT. Similar to MNU-, MNNG-treatment generated greater sensitivity in FA-J cells reconstituted with wild-type FANCJ as compared to vector, whereas MMS-treatment did not generate differential sensitivity (Figure 3-3A and B). In contrast, FA-J cells were sensitive to CCNU and reconstitution of wild-type FANCJ enhanced resistance (Figure 3-3C). Together, these results implicate that FANCJ-deficiency affects MMR-signaling, not MGMT function.

Conceivably, loading or translocation of MMR proteins on damaged chromatin could be potentiated by a DNA helicase, such as FANCJ. To pursue this idea, an MNU-induced MMR chromatin complex was examined in the FA-J cells reconstituted with vector or wild-type FANCJ. Following MNU-treatment, the FA-J cells were collected and the chromatin bound MMR proteins were examined by immunoprecipiation of MSH6 as described (Hidaka et al., 2005). While the amount of MMR proteins in the cellular extracts was similar between vector and wild-type FA-J cells, a more robust MMR complex was detected in the wild-type FANCJ expressing FA-J cells (Figure 3-3D). Likewise, the MMR complex formation in response to other DNA damaging agents, such as melphalan and cisplatin was enhanced by reconstitution of wild-type FANCJ (Supplemental Figure S3-6). Together, this data suggests that DNA damage induced MMR complexes are more robust when FANCJ is present.

MLH1 clinical mutant L607H disrupts FANCJ binding and alters DNA damage responses

Could FANCJ loss of function be associated with HNPCC? To assess this idea, we sought to identify if any MLH1 missense mutations altered MLH1 binding to FANCJ. We previously demonstrated that the MLH1 amino acids 478 to 744 were required to bind FANCJ, but also PMS2 (Figure 3-4A) (Peng et al., 2007). As such, deletion mutants of MLH1 in this region disrupted both FANCJ and PMS2 binding. Thus, 13 different MLH1 clinical mutants targeting the region of 478 to 744 that were proficient for PMS2 binding were screened for ability to bind FANCJ. Wild-type or mutant MLH1 constructs were co-transfected with PMS2 into 293T or HCT116 cells that are null for the MutLα complex. MLH1 antibody precipitation revealed that most of the MLH1 mutants precipitated PMS2 and FANCJ similar to wild-type MLH1. However, mutation of MLH1 at amino acid 607, from leucine to histidine ($MLH1^{L607H}$), ablated the FANCJ interaction as compared to wild-type MLH1 that bound FANCJ when precipitated from either 293T or HCT116 cells (Figure 3-4B and C). The HNPCC associated $MLH1^{L607H}$ mutant has normal mismatch repair activity and cells from afflicted patients do not show MSI, making it unclear as to how this mutation affects MMR function (Takahashi et al., 2007).

Our previous data demonstrated that loss of the FANCJ/MLH1 interaction generates ICL sensitivity similar to FANCJ-null cells (Peng et al., 2007) consistent with this interaction being important for some aspect of the interstrand

crosslink DNA damage response. Thus, we tested if re-introduction of vector, wild-type, or MLH1^{L607H} mutant, that lacks FANCJ binding affected ICL or MNU sensitivity in HCT116 colon cancer cells. HCT116 cells were co-transfected with PMS2 and vector, wild-type, or MLH1^{L607H} and expression was determined by Western blot (Figure 3-4C). To maintain expression of transfected plasmids at a level at which wild-type MLH1 reproducibly restored DNA methylation sensitivity, cells were plated 24-30h post transfection, treated with MMC or MNU, and the percent growth was assessed five days later. HCT116 cells with vector or wild-type MLH1 had a similar sensitivity to MMC, unlike with MLH1^{L607H} that enhanced MMC-sensitivity (Figure 3-4D). Moreover, the MLH1^{L607H} failed to restore MNU sensitivity as compared to wild-type MLH1 (Figure 3-4E). Likewise, the functional affects of a defective MLH1/FANCJ-interaction was not apparent when MGMT was inhibited (Figure 3-4F). Thus, the clinical MLH1^{L607H} mutant resembles the FANCJ$^{K141/142A}$ mutant in which ICL sensitivity is enhanced and MNU sensitivity is reduced.

Discussion

The complex role of MMR proteins in the DNA damage response has complicated efforts to dissect the key function (s) required for tumor suppression. Furthermore, MMR-associated HNPCC tumors have heterogeneous defects and levels of MSI or no MSI. Here, we identify an HNPCC MSS-associated MLH1^{L607H} mutation that is intact for mismatch repair, but lacks FANCJ binding.

Our data suggest an indirect role for FANCJ in the MMR pathway based on the finding that FANCJ and MMR function in O6MeG-signaling was distinct. First, FANCJ-depletion, unlike MLH-depletion has normal G2/M accumulation. Second, the dramatic MNU-resistance generated by MLH1-depletion in MGMT-inactivated cells was not generated by FANCJ-depletion. Thus, in the absence of FANCJ cells can initiate processing, checkpoint activation, and undergo cell death, however; the time to these events appear to be delayed. With this delay, the MMR-independent methylation reversal by MGMT likely has time to enhance DNA methylation resistance. Thus, a delay in MMR-signaling could be yet another mechanism linked to cancer and/or chemoresistance.

Our findings further demonstrate a functional relationship between FANCJ and MLH1 in the DNA damage response. Similar to the DNA crosslink response, we found that the DNA methylation response required FANCJ helicase and MLH1 binding activities. Why does loss of the MLH1/FANCJ interaction enhance DNA crosslink, but reduce O6MeG sensitivity? Most likely, the normal response to these DNA damaging agents fails without FANCJ binding to MLH1. Thus, the MMR response that promotes MNU-induced apoptosis or ICL-induced checkpoint, repair, and recovery are delayed. This delay is useful when there is a competing pathway that promotes survival, such as MGMT. Consistent with this point, when MGMT is inactivated, the delay in MMR signaling is insufficient to enhance resistance because the O6MeG lesions are inevitably processed by MMR. Likewise, in response to ICLs, in which reversal by MGMT or other means

is not available, the delay in MMR signaling more likely interferes with repair or recovery.

Our data implicate that FANCJ contributes to MMR signaling by potentiating the DNA damage induced accumulation of MMR complexes on chromatin. Whether FANCJ facilitates loading, translocation of, or stabilizes MMR complexes on chromatin is not clear. Loss of FANCJ or the FANCJ/MLH1 interaction may not overtly limit mismatch repair activity given that patients with the $MLH1^{L607H}$ are characterized as microsatellite stable (MSS) (Barnetson *et al.*, 2008) and mismatch repair activity for this mutant is similar to wild-type MLH1 (Takahashi et al., 2007). It is possible, however, that the delayed MMR-signaling in L607H patient cells might provide a selective advantage in the initial stages of tumorigenesis or under high mutation stress enhance the mutation frequency. Conceivably, more DNA damage could be tolerated without activating the MMR-induced checkpoint. This lowered checkpoint barrier could enhance genomic instability and cancer.

Figure 3-1: **FANCJ deficiency enhances MNU-resistance in a MGMT dependent manner. A)** Western blot analysis of MCF7 cells stably expressing shRNA reagents targeting control, MLH1, or FANCJ is shown with the indicated Abs. **B**) DNA content was analyzed by FACs at the indicated times or **C)** colony survival was determined in the MCF7 cell lines after treatment with indicated dose of MNU in the presence of the MGMT inhibitor O6-BZG. **D)** Apoptosis was assessed by annexin V staining in the stable MCF7 cell lines treated with MNU in the presence or absence of O6-BZG. **E)** Colony survival was re-assessed in the absence of O6-BZG (MGMT-active) in the stable MCF7 cell lines and graphed as percent survival.

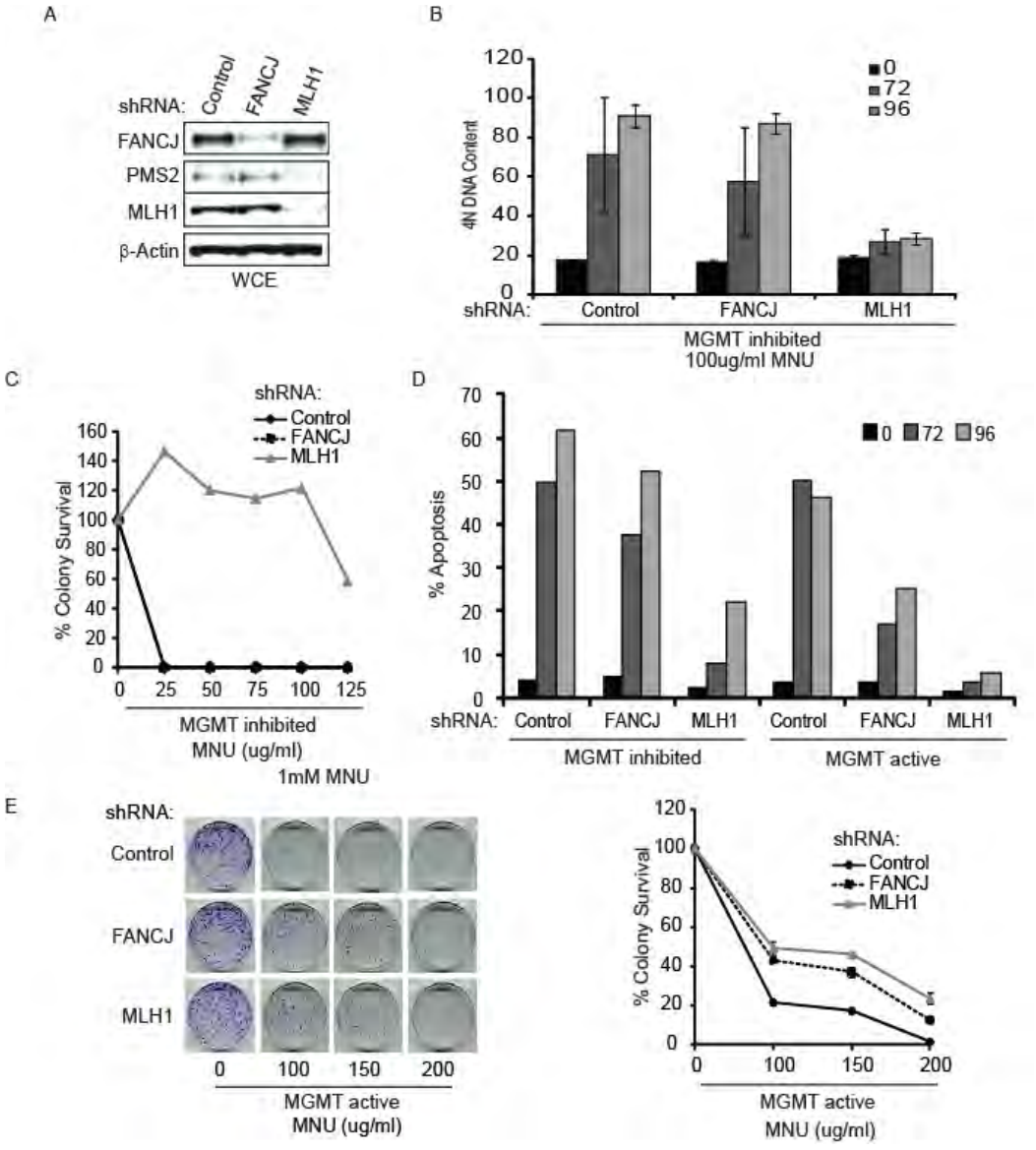


Figure 3-2. **When MGMT is active, FA-J cells are uniquely resistant to MNU and restoration of sensitivity requires FANCJ helicase and MLH1 binding activities. A)** Percent growth of fibroblast cells (2822), FANCJ-null FA-J (2833), **B)** FA-J (EUFA39F), FA-C, or FA-G was assessed following MNU treatment. **C)** Western blot analysis was performed on the FA-J (EUFA39F) cells stably reconstituted with vector, FANCJ^{WT}, FANCJ^{K52R} or FANCJ$^{K141/142A}$ with the indicated Abs. The percent growth following MNU treatment was scored in the FA-J cell lines with MGMT inhibited or active, as noted. **D)** Western blot analysis and percent growth following MNU treatment was assessed on FA-D2 (PD20) cells reconstituted with vector or FANCD2.

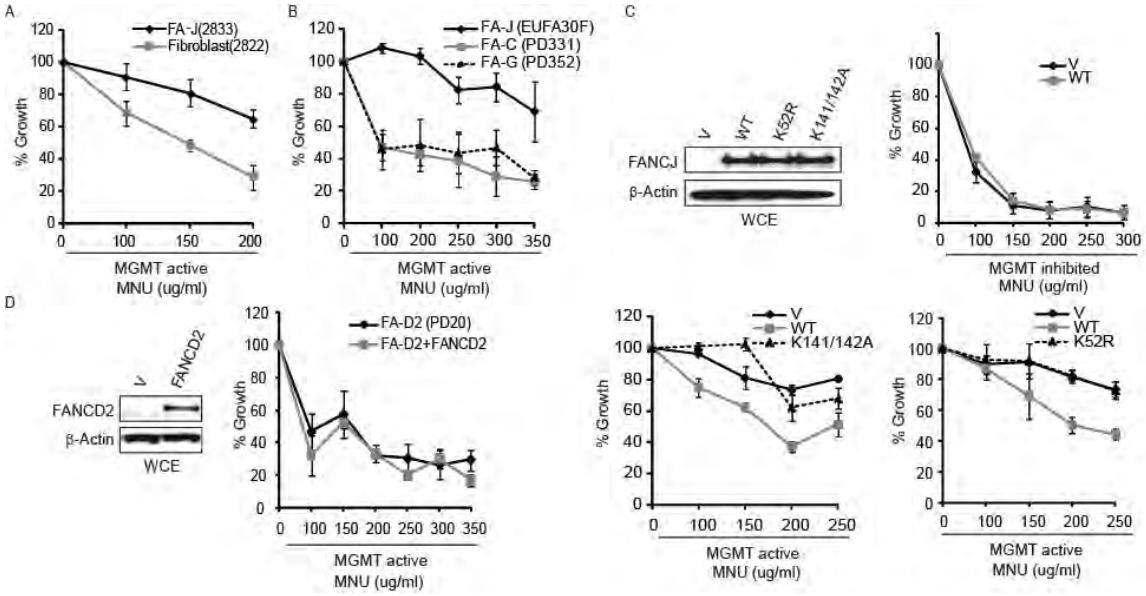


Figure 3-3: **FANCJ enhances the damage response and complex formation of MMR proteins. A)** The percent growth was assessed following MNNG, **B)** MMS or **C)** CCNU in the FA-J cells reconstituted with vector or FANCJ^{WT}. **D)** Western blot analysis was used to analyze MMR proteins in chromatin extracts or chromatin MMR protein complex formation following immunoprecipitation with Ab to MSH6 as shown with the indicated Abs.

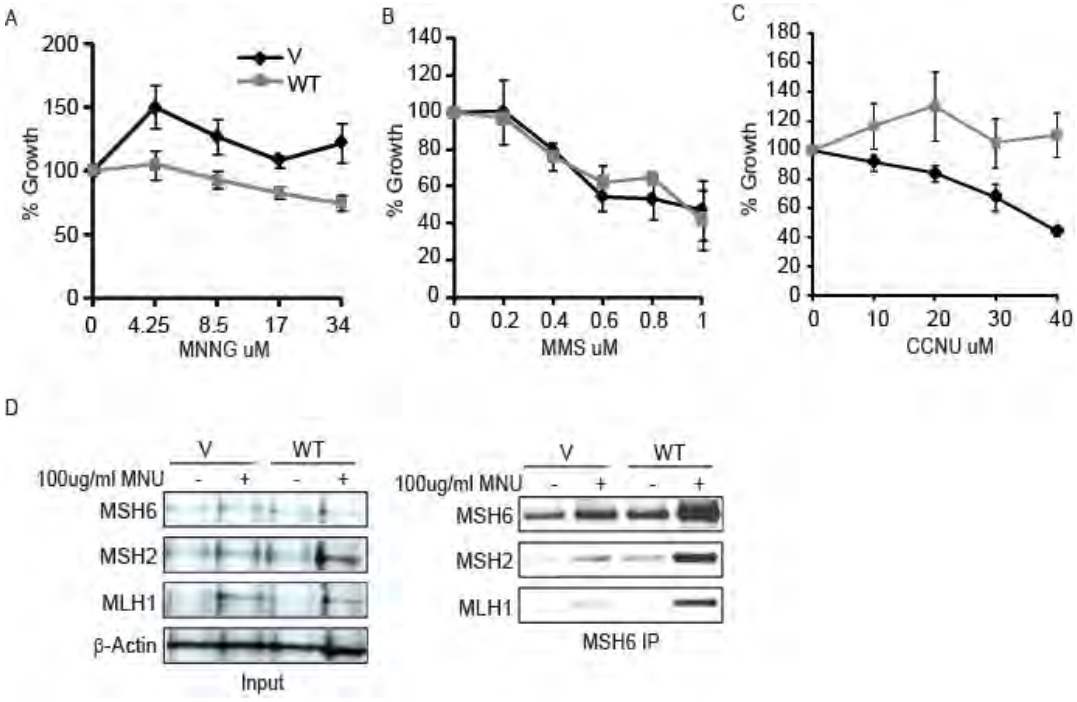
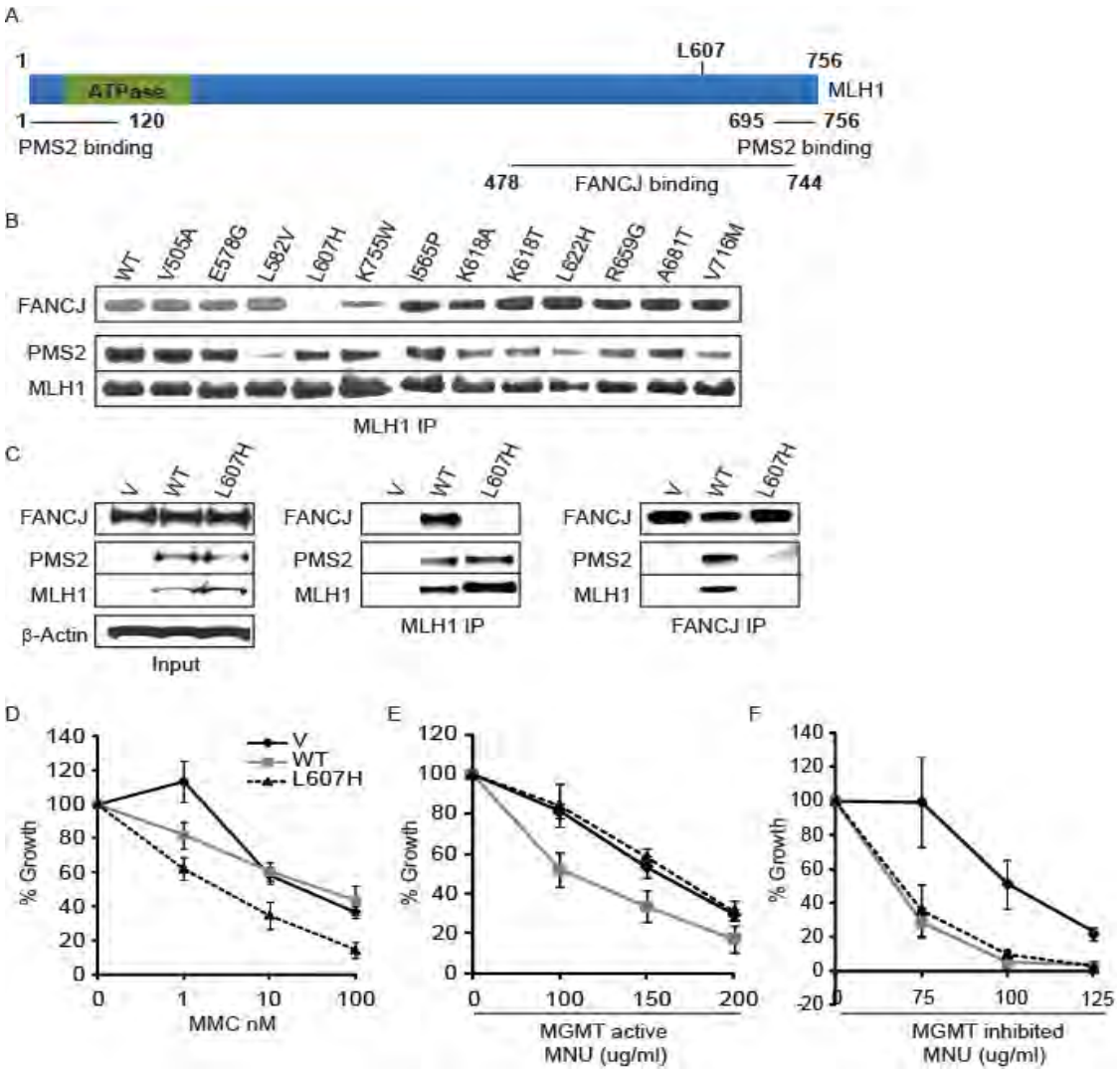
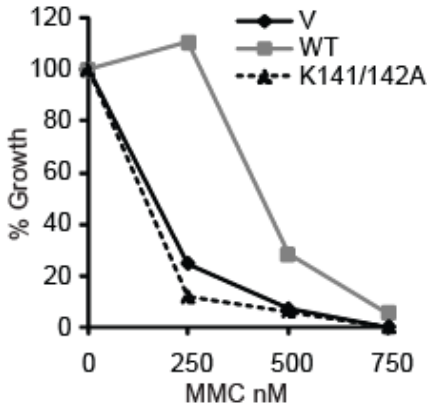


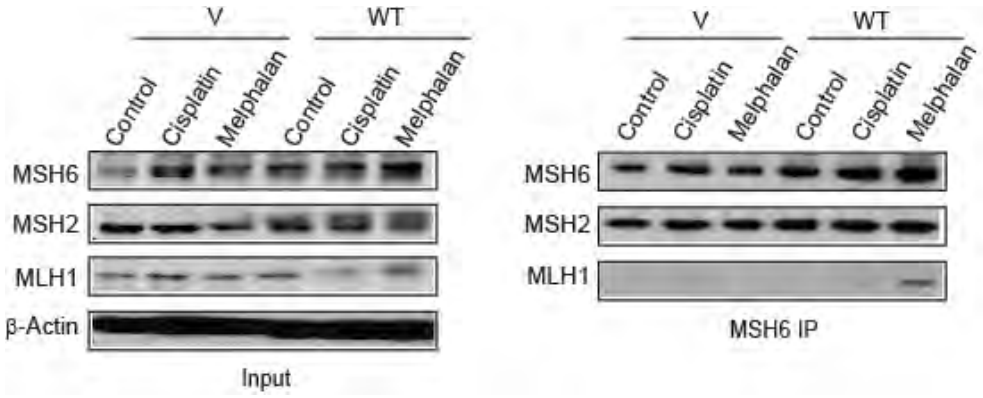
Figure 3-4. **MLH1 L607H mutant is defective for FANCJ binding. A)** Cartoon shows FANCJ and PMS2 binding region on MLH1. **B)** Western blot analysis following MLH1 immunoprecipitation from 293T (MutLα deficient) cells transfected with PMS2 and distinct MLH1 constructs. **C)** Western blot analysis of extracts or following immunoprecipitations with MLH1 or FANCJ Abs from HCT116 (MutLα deficient) cells transfected with PMS2 and vector, wild-type (WT) MLH1, or MLH1 L607H mutant. β-Actin served as a loading control. **D)** The percent growth of the transfected HCT116 cells described above was assessed following MMC, **E)** MNU with MGMT, **F)** or MNU with MGMT inhibited.



Supplemental Figue S3-5. **MLH1 binding to FANCJ is essential to correct the rescue the MMC sensivitiy of the FA-J cells.** FA-J (EUFA39F) were stably reconsituted with vector, FANCJ^{WT}, or FANCJ$^{K141/142A}$, and percent growth was assessed following MMC treatment



Supplemental Figure S3-6. **FANCJ enhances MMR complex formation in response to Melphalan.** FA-J cells stably reconstituted with vector or FANCJ^{WT} were treated with Cisplain or Melphalan. Cells were collected 24 or 48hs later and chromatin fraction was isolated (Masih et al., 2008). Western blot analysis was used to analyze MMR proteins in chromatin extracts or following immunoprecipitation with Ab to MSH6 as shown with the indicated Abs.



Material and Methods

Cell lines

MCF7 and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin (100U/mL each). HCT116 cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100U/mL each). FA and fibroblast cell lines were cultured as previously described (Litman et al., 2005). FA-J cells were infected with the POZ retroviral vector (Nakatani and Ogryzko, 2003) containing no insert, $FANCJ^{WT}$, $FANCJ^{K52R}$, and $FANCJ^{K141/142A}$ pCDNA-3myc-6xhis and POZ vectors described previously (Peng et al., 2007).

Immunoprecipitation, Western blot and antibodies

Cells were harvested, lysed, and processed for Western blot analysis as described previously (Peng et al., 2007). Abs used for immunoprecipitation and Western blot assays include FANCJ Ab, monocolonal pool (1A3, 2G7 and IG5) (Cantor et al., 2001) and polyclonal, E67 (Cantor et al., 2004), β-actin (Sigma), MLH1 (BD Bioscience), PMS2 (BD Biosciences), MSH6 (BD Biosciences), and Myc (9E10). To prepared chromatin extracts FA-J cell lines were prepared as described (Hidaka et al., 2005). Briefly cells were left untreated or treated with 1mM MNU for 1h, washed, and incubated 24h. The cells were treated with hypotonic buffer containing digitonin, collected, and sonicated.

Cell cycle progression assay

MCF7 stable cell lines were either left untreated or treated with 20mM of O6-BZG for 2hrs followed by 1mM of MNU. 1hr post MNU treatment, cells were returned to regular growth media also containing O6-BZG for 72 or 96 hr. G2/M accumulation was assayed as described (Litman et al., 2005).

Viability Assays

FA-J reconstituted cells were seeded on 6 well plates 24hr later treated with increasing dose of MNU (1hr serum free), MMS (1hr, serum free), MNNG (continuous treatment) or CCNU (1hr, serum free). After incubation for 4 to 5 days, the percent growth was measured photometrically in a model 3550 microplate reader (Perkin Elmer) as the relative growth (in luciferase units) using the Cell Titer glo-viability assay (Promega). For quantification the luciferase units of each well were normalized to those obtained from untreated cells assumed to yield 100% cell survival, and was normalized to those obtained from a well without any cells, assumed to yield 0%. HCT116 cells were transiently transfected with Fugene 6 and 24-30h after transfection, cells were seeded onto 6-well plates at 1000 cells per well, incubated overnight, and then treated with increasing dose of MNU (1hr serum free), or MMC. Ten days post-treatment, cells were counted to determine percent survival. To suppress MGMT, FA-J reconstituted or transiently transfected HCT116 cells were preincubated with

20mM of O6-BZG for 2hr followed by MNU treatment. 1hr post MNU treatment, cells were re-incubated in regular media supplemented with 20mM of O6-BZG.

Annexin V and PI Staining

MCF7 cells expressing shLuc, shMLH1, shBACH1 were plated in 60mm plates 24hr later treated with increasing dose of 1mM MNU (1hr serum free).
Cells were collected at 72 and 96hs and stained with Annexin V-FITC (5ul/10^6 cells) and propidium iodide (50ug/ml). Incubated for 15mins at room tempature in the dark and analyzed by FACs. To suppress MGMT, MCF7 cells were preincubated with 20mM of O6-BZG for 2hr followed by MNU treatment. 1hr post MNU treatment, cells were re-incubated in regular media supplemented with 20mM of O6-BZG.

CHAPTER IV

Critical role for BRCA1 binding to FANCJ to suppress lesion tolerance

Abstract

BRCA1 and the DNA helicase FANCJ (also known as BACH1 or BRIP1) have common functions in breast cancer suppression and DNA repair. However, the functional significance of the direct BRCA1/FANCJ interaction remains unclear. Here, we have discovered that BRCA1 regulates FANCJ function. When FANCJ and BRCA1 association is ablated the DNA damage repair mechanism is dramatically altered. Specifically, a FANCJ protein that cannot be phosphorylated at serine (S) 990 or bind BRCA1 inhibits DNA repair via homologous recombination and promotes polη-dependent bypass. Furthermore, the polη-dependent bypass promoted by FANCJ requires the direct binding to the mismatch repair (MMR) protein, MLH1. Together, our findings implicate that in human cells BRCA1 binding to FANCJ is critical to regulate DNA repair choice and promote genomic stability. Moreover, unregulated FANCJ function likely promotes cancer and chemoresistance.

Introduction

BRCA1 function and tumor suppression depend on the BRCA1 C-terminal (BRCT) region, which contains two discrete domains called BRCT repeats. Mutations in the BRCT domains result in defective DNA damage repair, such as failure to induce cell cycle checkpoints and sensitivity to DNA double-stranded breaks (DSBs) and interstrand cross-links (ICLs) (Kim and Chen, 2008). These BRCTs also mediate the direct binding of BRCA1 to the FANCJ DNA helicase (also known as FANCJ/BRIP1) (Cantor et al., 2001). FANCJ phosphorylation at serine 990 is required to mediate the BRCT-FANCJ interaction (Yu et al., 2003), and a serine to alanine mutation S990A ablates this interaction. Similar to BRCA1, mutations in FANCJ have been associated with hereditary breast cancer (Cantor et al., 2001; Seal et al., 2006). In addition, FANCJ is mutated in the rare childhood disease, Fanconi anemia (FA), within the FANCJ (FA-J) patient complementation group (Chandra et al., 2005; Levitus et al., 2005b; Levran et al., 2005; Litman et al., 2005).

It has been proposed that the FA pathway maintains genomic integrity by coordinating at least two DNA repair mechanisms. One of these mechanisms is homologous recombination (HR), a typically error-free DNA-repair mechanism that uses the homologous sequence in a sister chromatid for repair. HR is critical to repair DSBs, which can form at the ICL following replication fork collapse. Defects in HR characterize BRCA1-, FANCJ-, and BRCA2-deficient cells (Litman et al., 2005; Moynahan et al., 1999; Moynahan et al., 2001b). Evidence also

suggests that the FA pathway promotes ICL resolution by a mechanism engaging the error-prone translesion synthesis (TLS) polymerases. Supporting this possibility, fewer TLS-like point mutations are present in the genome of FA cells (Patel and Joenje, 2007). Moreover, the TLS polymerases REV1 and REV3 function with the FA protein FANCC to promote ICL resistance (Niedzwiedz et al., 2004). Consequently, the FA pathway has been proposed to coordinate both TLS and HR to resolve DNA ICLs as well as other DNA lesions thereby limiting the severity of mutagenesis (Hinz et al., 2006; Niedernhofer et al., 2005; Patel and Joenje, 2007).

Current models predict that TLS can function independent of HR. This pathway often does not repair lesions, but facilitates lesion tolerance or bypass (Dronkert and Kanaar, 2001). In particular, polη has been implicated in recombination-independent repair of ICLs generated by mitomycin C (MMC) (Zheng et al., 2003) as well as in the cellular tolerance to cisplatin (Albertella et al., 2005). Due to the unique structure of its active site, polη replicates through cross-linked DNA (Alt et al., 2007). Depending on the lesion bypassed, TLS can be mutagenic or error-free. For example, polη bypasses ultraviolet light (UV)-induced thymidine-thymidine dimers in an error-free manner and bypasses intra-strand crosslinks and ICLs, once unhooked, in an error-prone manner (Prakash et al., 2005; Zheng et al., 2003). Depending on the type and severity of DNA damage, TLS is activated by a RAD6-RAD18 dependent PCNA monoubiquitination (Kannouche and Lehmann, 2004) that loads different TLS

polymerases in a lesion specific manner (Barbour and Xiao, 2003; Papouli et al., 2005).

Given that both FANCJ and BRCA1 are critical for HR, genomic stability, breast cancer suppression, and cross-link resistance (Cantor and Andreassen, 2006), we hypothesized that these two proteins likely function together in DNA repair. However, recent studies support independent functions. In particular, the DNA cross-link sensitivity of FANCJ-null chicken and FANCJ-null patient (Bridge et al., 2005; Peng et al., 2007) cells was rescued with mutant versions of FANCJ that cannot interact with BRCA1. This finding leads one to wonder what is the functional role for the BRCA1-FANCJ interaction.

Here, we identify that when uncoupled from BRCA1, FANCJ functions to inhibit HR and promote polη-dependent bypass. As such, FA-J patient cells expressing the BRCA1-interaction defective mutant, FANCJ^{S990A} resist DNA damage by UV and ICLs in a polη-dependent manner. Furthermore, FANCJ^{S990A} requires the MLH1 interaction to promote TLS and retain ICL resistance. Together, our data implicate that in human cells BRCA1 binding to FANCJ is critical to regulate DNA repair and bypass mechanisms to promote genomic stability.

Results

The DNA damage response is altered in the absence of FANCJ binding to BRCA1

The mechanism for restored ICL resistance in FA-J cells expressing FANCJ^{S990A} or FANCJ^{WT} could be distinct. The restored ICL resistance in FA-J cells expressing FANCJ^{S990A} could have resulted from a reduction or disruption of the ICL-induced checkpoint response. To test these possibilities, we reconstituted FANCJ-null FA-J cells with vector, FANCJ^{WT}, or the BRCA1-interaction defective mutant FANCJ^{S990A} (Yu et al., 2003) and functional expression was confirmed by Western blot and restored ICL resistance (Figure 4-1A, C, and Supplemental Figure S4-7A). Next, the FA-J stable cell lines were treated with half the dose of melphalan (0.25 mg/ml) used previously (Peng et al., 2007) and analyzed at different times after treatment. Interestingly, the maximum G2/M accumulation in the FANCJ^{S990A} -reconstituted FA-J cells was ~25%, as compared to FANCJ^{WT} at ~40% (Figure 4-1B). Fewer FA-J cells accumulated at G2/M at all times when they were reconstituted with FANCJ^{S990A} than when reconstituted with FANCJ^{WT} or vector (Figure 4-1B and Supplemental Figure S4-7B). However, the growth of untreated cells was not measurably different (Supplemental Figure S4-7C). Thus, FANCJ^{S990A} reduced, but did not eliminate, the melphalan-induced G2/M accumulation.

To further assess whether FANCJ^{S990A}, as compared to FANCJ^{WT}, promoted a distinct DNA damage repair mechanism, the reconstituted FA-J cells lines were treated with different forms of DNA damage. FANCJ^{S990A} -reconstituted FA-J cells were slightly more resistant to cisplatin, but dramatically more resistant to ultraviolet (UV) irradiation than FANCJ^{WT}-reconstituted FA-J

cells. In contrast, FANCJ^{S990A} were more sensitive than FANCJ^{WT} or vector-reconstituted FA-J cells to zeocin, which induces double strand breaks (DSBs) (Delacote et al., 2007) (Figure 4-1C). Together, these findings suggest that the DNA damage response is distinct when FANCJ binds or does not bind BRCA1.

FANCJ^{S990A} reduces RAD51 foci and HR

The zeocin sensitivity could suggest reduced double strand break repair (DSBR) when FANCJ is uncoupled from BRCA1. Formation of DSBs corresponds with nuclear γ-H2AX foci formation. Treatment with zeocin induced the formation of γ-H2AX foci to the same extent in FANCJ^{WT} and FANCJ^{S990A} -reconstituted FA-J cells as detected by immunofluorescence (Figure 4-2A and B). In contrast, RAD51 foci were not similarly detected (Figure 4-2A and C). As compared to untreated cells, at 12 h post-zeocin treatment RAD51 foci were induced ~5 fold in FANCJ^{WT}, as compared to ~3.5 fold in vector and 2~fold in FANCJ^{S990A} reconstituted FA-J cells (Figure 4-2C). To rule out the possibility that zeocin differentially affected the FA-J cell lines and the number of cells in S-phase, in which RAD51 foci are most prominent, we measured cell-cycle distributions and found no significant differences (Supplemental Figure 4-8A).

Consistent with the possibility that HR was reduced as a result of more unbound FANCJ, we found that expression of FANCJ^{S990A} in U2OS cells also reduced HR, as found for the helicase inactive FANCJ^{K52R}, 4.5- and 3-fold, respectively, compared to that in U2OS cells with FANCJ^{WT} or vector (Figure 4-

2D). FANCJ species were expressed similarly (Figure 4-2D). FANCJ^{WT} precipitated similar ratio of BRCA1 as endogenous FANCJ and as expected the FANCJ^{S990A} did not precipitate BRCA1 (Supplemental Figure 4-9A). Despite reduced HR due to expression of FANCJ^{S990A} or FANCJ^{K52R}, only U20S cells expressing FANCJ^{K52R} were sensitive to ICLs (Supplemental Figure 4-9B). Together, these data suggest that expression of FANCJ^{S990A} in U20S or FA-J cells reduced RAD51-based HR, but not crosslink resistance.

FANCJ affects DNA damage-induced polη foci formation

We hypothesized that resistance to ICLs could be mediated by an HR-independent mechanism such as translesion synthesis (TLS), which can facilitate bypass of both unhooked ICLs and UV lesions (Kannouche and Lehmann, 2004; Nojima et al., 2005). In particular, in human cells the TLS polymerase polη can carry out bypass of cisplatin and UV lesions (Vaisman et al., 2000), (Alt et al., 2007), (Kannouche et al., 2001). Following UV irradiation polη forms nuclear foci (Kannouche et al., 2001) (Figure 4-3A). Thus, we examined whether expression of FANCJ influenced TLS by examining UV-induced polη foci formation in U2OS cells in which expression of FANCJ^{S990A} reduced HR. U2OS cells stably expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were transfected with the eGFP-polη fusion protein, UV irradiated, and analyzed for fluorescent polη foci. We were unable to examine how the helicase inactive affected polη because FANCJ^{K52R} failed to express stably and transient co-expression with polη was

toxic. Expression of FANCJ^{S990A} did not affect the number of polη foci in untreated cells, but in UV-irradiated cells expressing FANCJ^{S990A} or FANCJ^{WT}, ~30% more cells with eGFP-polη foci were found than in the vector control (Figure 4-3A).

If FANCJ normally functions in the UV-induced activation of polη, UV-induced polη foci should be less robust in cells deficient in FANCJ. To test this hypothesis, cells were co-transfected with eGFP-polη and -siRNA targeting luc, FANCJ, or Rad18. Subsequently, transfected cells were UV irradiated and assessed for polη foci (Figure 4-3B). To rule out the possibility that DNA damage differentially affects the number of cells in S-phase, in which polη foci are most prominent (Kannouche et al., 2001), we measured cell-cycle distributions before and after UV or MMC in control or FANCJ over-expressed cells and found no significant changes (Figure 4-2B). As expected, Rad18-depletion reduced the number of cells positive for polη foci (Watanabe et al., 2004) by ~4-fold. Depletion of FANCJ reduced the number of cells positive for polη foci by ~2-fold (Figure 4-3B). Together, it appears that UV induced polη foci formation is reduced with FANCJ depletion, whereas FANCJ over-expression enhanced this outcome.

FANCJ^{S990A} promotes polη dependent bypass

In contrast to UV, MMC-treated cells showed few cells with polη foci, suggesting that repair of ICLs do not readily activate polη. However, when cells

expressed FANCJ^{S990A}, cells with polη foci were readily observed (Figure 4-3A) suggesting that FANCJ^{S990A} enhances TLS in response to ICLs. Based on these findings, we hypothesized that UV and ICL resistance in FA-J cells expressing FANCJ^{S990A} was accomplished through a mechanism that involved polη. To address this possibility, the FA-J cells lines were transfected with siRNA targeting luc or polη (Figure 4-4A). Compared to FANCJ^{WT} reconstituted cells, suppression of polη reduced the viability of FANCJ^{S990A} reconstituted cells by 30%, while the viability of vector reconstituted cells was not changed (Figure 4-4B). Consistent with FANCJ^{S990A} promoting polη dependent TLS, polη-depletion uniquely reversed the MMC and UV resistance of FA-J cells expressing FANCJ^{S990A}, but not FANCJ^{WT} or vector FA-J cell lines (Figure 4-4C and D). Similar to polη-depletion, Rad18-depletion reversed the ICL resistance of FA-J cells expressing FANCJ^{S990A}, whereas vector and FANCJ^{WT} expressing FA-J cells were unaffected (Figure 4-5A and B). In contrast, Rad54- or Rev1-depletion did not affect FA-J cells expressing FANCJ^{S990A}, FANCJ^{WT}, or vector FA-J cell lines (Figure 4-5A and B). Suppression of Rad18, Rev1, and Rad54 did not cause any change in viability of the stable J cells (Supplemental Figure S4-10). Interestingly, stable over-expression of FANCJ^{S990A} in U2OS cells also promoted UV resistance in a polη dependent manner (Supplemental Figure S4-11). Together, these results implicate that in response to DNA damage, FANCJ^{S990A} promotes polη dependent bypass.

FANCJ^{S990A} requires MLH1 binding to promote polη-dependent bypass

Previously, we identified that FANCJ^{WT} binds directly to MLH1 and this interaction is required for FANCJ to promote ICL resistance (Peng et al., 2007). MLH1 binds directly to the FANCJ helicase domain through lysines 141 and 142. To address whether the MLH1 interaction with FANCJ^{S990A} was also required for ICL resistance, we replaced lysines 141 and 142 of FANCJ with alanines, which ablates MLH1 binding (Peng et al., 2007). FA-J cells were reconstituted with this triple mutant FANCJ$^{S990AK141/142A}$, FANCJ^{S990A}, FANCJ$^{K141/142A}$, vector, or FANCJ^{WT}. Expression and ablation of MLH1 and/or BRCA1 binding was confirmed by Western blot (Figure 4-6A and B). In contrast to FANCJ^{S990A}, FANCJ$^{S990AK141/142A}$ reconstituted FA-J cells were sensitive to MMC (Figure 4-6C), suggesting that FANCJ^{S990A} promotes polη dependent TLS in a MLH1 dependent manner (Figure 4-6D).

Discussion

In this study, we explore the possibility that FANCJ binding to BRCA1 is important for DNA repair in mammalian cells. This possibility was proposed based on their direct binding and common functions in breast cancer suppression, HR, and crosslink repair. We provide data that support this hypothesis by demonstrating that uncoupling FANCJ from BRCA1 alters the DNA damage response. Specifically, (1) cells expressing unbound FANCJ

(FANCJ^{S990A}), unlike FANCJ that can bind BRCA1, are sensitive to DSBs, (2) have reduced RAD51-based HR, (3) survive cross-link DNA damage with a reduced G2/M accumulation and (4) dependence on the TLS polymerase polη. These data suggest that FANCJ has anti-recombination and TLS functions that are normally regulated by BRCA1 binding. Moreover, we find that the BRCA1-bound or unbound FANCJ requires its MLH1 interaction to promote crosslink resistance.

FANCJ likely has a complex role in HR: it is required for HR when bound to BRCA1 and inhibits HR when unbound to BRCA1. If FANCJ functioned only as an anti-recombinase depletion of FANCJ would be expected to enhance HR. Instead, FANCJ-depletion reduces HR, similar to BRCA1-depletion (Litman et al., 2005). Further indicating a positive role for FANCJ in HR, reconstitution of FANCJ in FA-J cells enhances the appearance of DNA damage induced RAD51 foci (Figure 4-2A). One possibility is that the BRCA1-bound FANCJ functions to prevent other proteins from disrupting HR, such as anti-recombination helicase BLM (Bugreev et al., 2007). Instead, when unbound to BRCA1, FANCJ could also disrupt HR by unwinding recombination structures. In particular, recombinant FANCJ unwinds D-loops, but not other recombination structures such as holiday junctions (Gupta et al., 2005). BRCA1 is critical for RAD51 foci and HR (Bhattacharyya et al., 2000; Moynahan et al., 1999). Perhaps the requirement for BRCA1 is that in its absence, the anti-recombinogenic function of FANCJ is unleashed.

Conceivably, the anti-recombination activity of FANCJ^{S990A} could indirectly enhance TLS. For example, the yeast helicase Srs2 promotes TLS by binding PCNA and antagonizing HR (Papouli et al., 2005) (Barbour and Xiao, 2003). Similar to Srs2, FANCJ colocalizes at sites of replication arrest with PCNA and has been shown to translocate DNA, unwind D-loops (Dupaigne et al., 2008; Gupta et al., 2005) and displace RAD51 (Sommers et al., 2009). Moreover, the structurally and functionally related Srs2 homologue UvrD binds the MLH1 homologue, MutL (Mechanic et al., 2000). MutL helps to load and activate the UvrD helicase. Perhaps, FANCJ localization or the ability to unwind DNA also requires its MLH1 interaction. In the absence of MLH1 binding, FANCJ may not function properly, thus may fail to promote both TLS and HR. Conceivably, FANCJ could enhance TLS by not only limiting recombination, but also by potentiating TLS. For example, FANCJ could limit negative regulators of TLS, such a MMR, which detect mismatches generated by mutagenic pathways. This scenario could explain why FANCJ binding to MLH1 is required for TLS (Figure 4-5D).

Together, these findings imply that reduction or loss of BRCA1 binding to FANCJ could enable cells to survive toxic chemotherapies and provide a possible route to chemoresistance. If true, targeting FANCJ or polη bypass could reverse ICL resistance in such cancers. Likewise, a possible route to cancer in BRCA1-mutation carriers could result from excess unbound FANCJ and mutagenic bypass. Perhaps, this is why FANCJ amplification is also linked to

malignancy (Eelen et al., 2008; Sinclair et al., 2003). In fact, loss of BRCA1 binding to FANCJ could evolve from mutations in either gene, or from loss of DNA-damage signaling components that regulate the association of these two proteins. Future studies are needed to clarify the signaling pathways that participate in regulating the switch between BRCA1-bound and -unbound FANCJ.

Figure 4-1. **FANCJ^{S990A}, as compared to FANCJ^{WT}, promotes a distinct DNA damage response in FA-J cells. (A)** FANCJ-null FA-J cells were reconstituted with vector, FANCJ^{WT}, or FANCJ^{S990A}, lysates were analyzed by immunoblot with the indicated Abs. **(B)** The FA-J cell lines were treated constitutively with 0.25μg/ml melphalan, collected at the indicated times, and analyzed by FACS to determine the percentage of cells in G2/M. Data represent mean ± SD for 3 independent experiments. Asterisk indicates significant difference with melphalan treated FA-J vector reconstituted ($P < 0.05$, unpaired *t*-test) **(C)** The FA-J cells expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were plated at low density, treated with the indicated doses of cisplatin, UV, or zeocin, and allowed to grow for 5-8 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent ± SD of growth from 3 independent experiments.

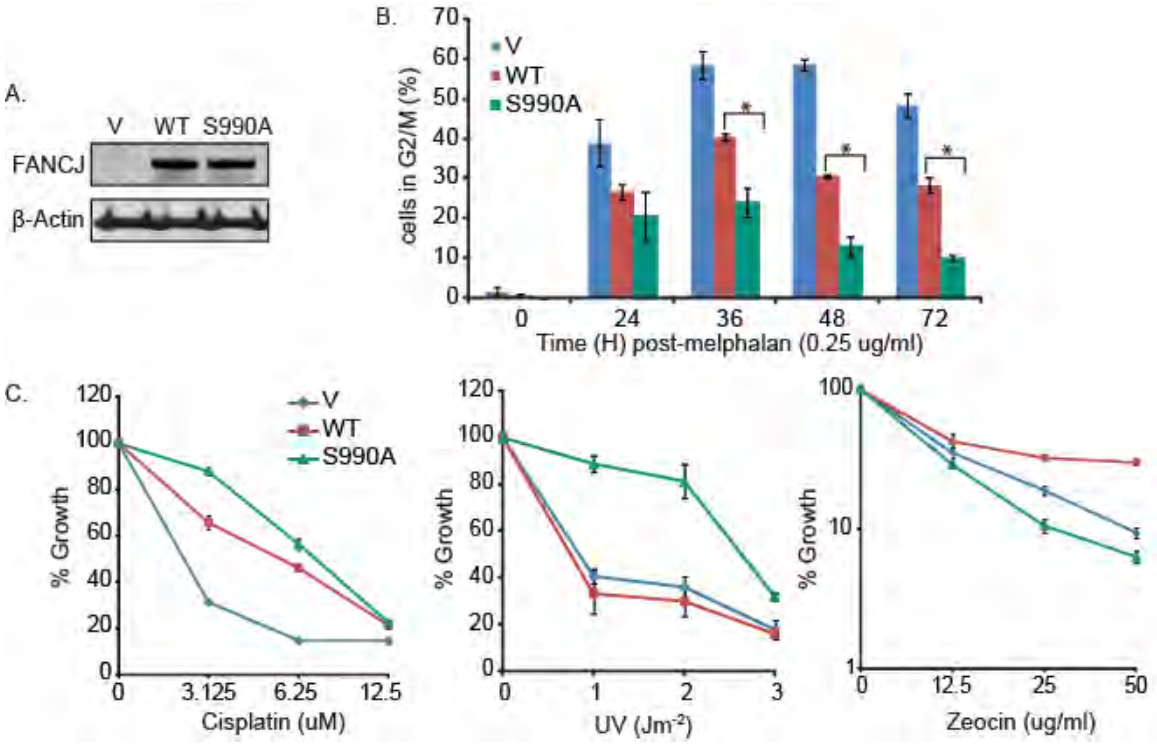


Figure 4-2. **FANCJ^{S990A} reduces homologous recombination following DSBs in FA-J cells. (A)** The FA-J cell lines were treated with 12.5µg/ml of zeocin and immunoflourescence was performed with γ-H2AX and RAD51 Abs. **(B)** The γ-H2AX and **(C)** RAD51 foci were quantitated based on a cell being positive (>10) foci per 300 DAPI positive cells from 3 independent experiments. Asterisk indicates significant difference with zeocin treated FA-J vector reconstituted ($P < 0.05$, unpaired *t*-test). **(D)** DR-U2OS cells were co-transfected with the I-Sce-1 endonuclease and vector, FANCJ^{WT}, FANCJ^{S990A}, or FANCJ^{K52R}, collected and either lysed and immunoprecipitated followed by immunoblot with the indicated Abs or analyzed by FACS. The percentage of GFP-positive cells is based on that in the vector control.

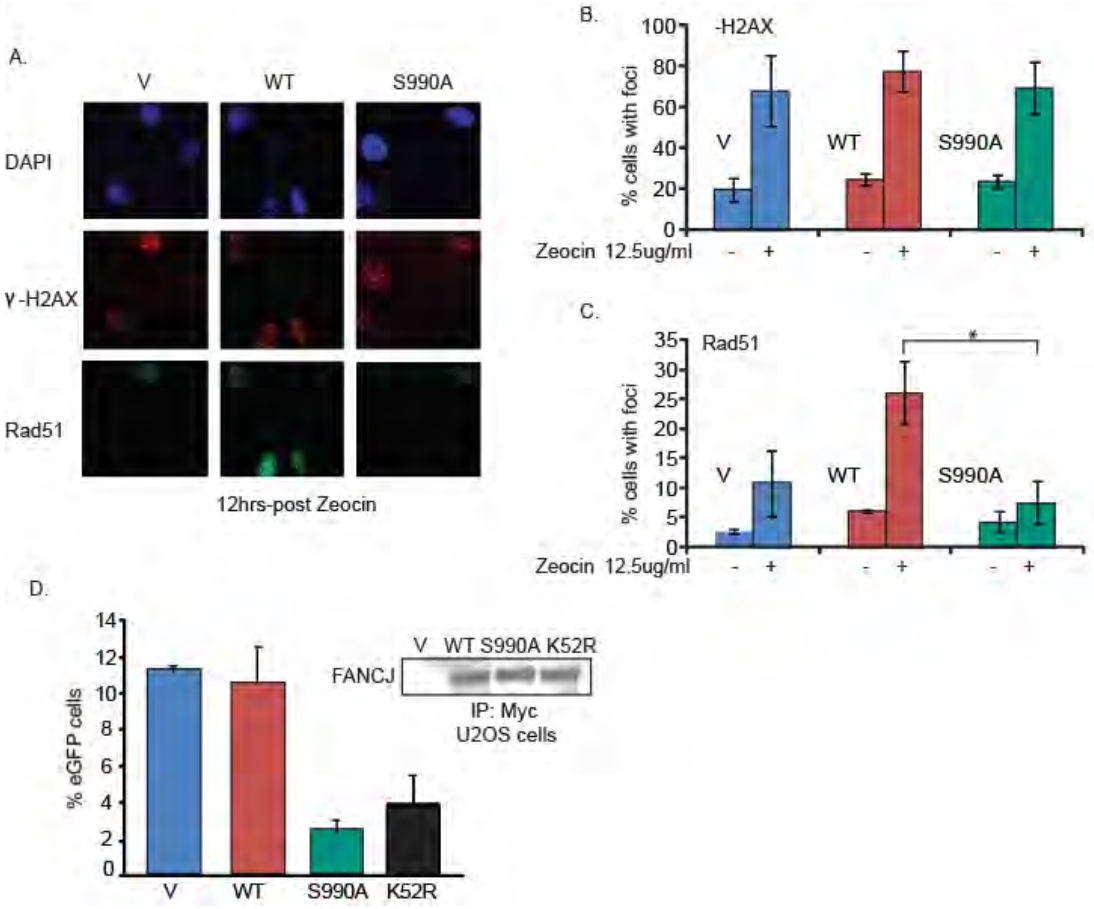


Figure 4-3. **FANCJ^{S990A} enhances polη foci, and FANCJ is required for robust UV-induced polη foci in U2OS cells**. **(A)** U2OS cells stably expressing vector, FANCJ^{S990A}, or FANCJ^{WT} were transfected with eGFP-polη and either collected for immunoblot with the indicated Abs or UV irradiated with indicated dose with 4h incubation or treated with 250nM MMC with incubation at varying times. Cells were assessed for eGFP-polη foci by autofluorescence (green staining as shown in untreated and UV-treated cells is shown as in figure). Data represent the mean percent ± SD cells positive (>10) green foci per 300 Dapi positive cells from 3 independent experiments. **(B)** U2OS cells were co-transfected with siRNA for luc, FANCJ, or Rad18 and eGFP-polη and either collected for immunoblot with the indicated Abs or UV irradiated and assessed for eGFP-polη foci as in (A). Asterisk indicates significant difference ($P < 0.05$, unpaired *t*-test).

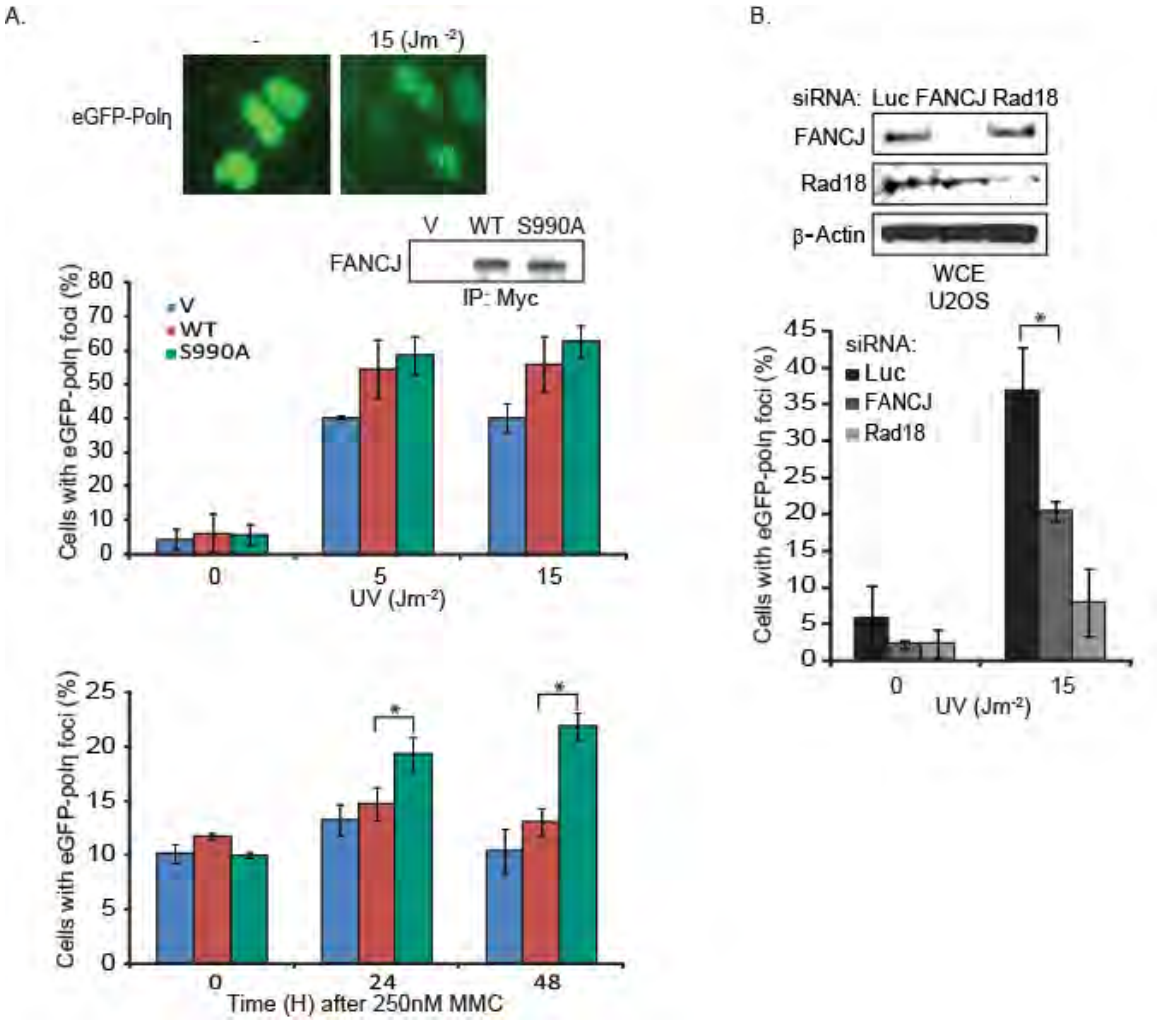


Figure 4-4. **FANCJ^{S990A} promotes ICL resistance in a polη-dependent manner.** FA-J cells stably expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were transfected with siRNA to luc or polη **(A)** Expression of polη was determined by immunoblot with the indicated Abs **(B)** Toxicity of polη knockdown was determined in vector or FANCJ^{S990A} cells normalized to FANCJ^{WT} reconstituted cells **(C)** Stable FA-J cells were treated with MMC or **(D)** UV and allowed to grow for 5-8 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent ± SD of growth from 3 independent experiments.

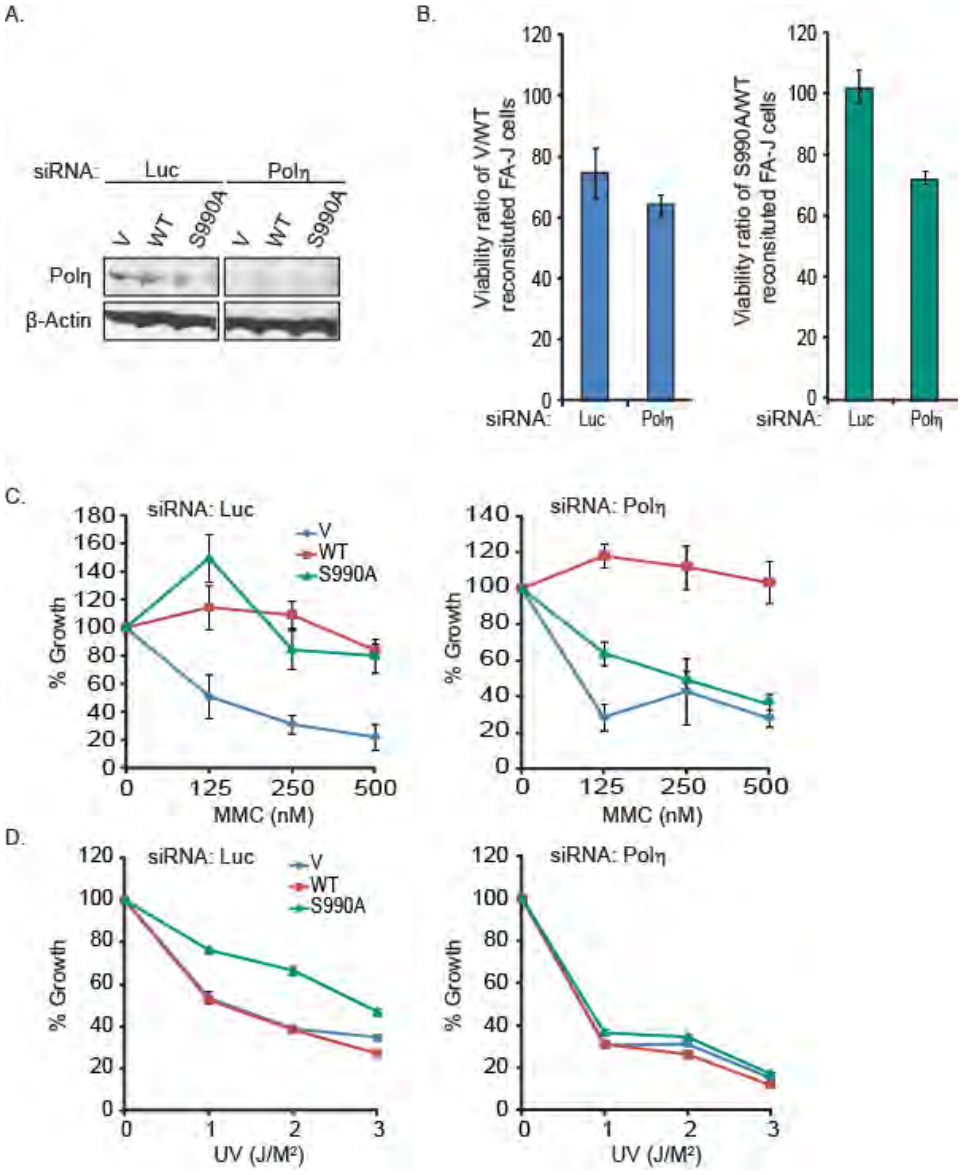


Figure 4-5. **FANCJ^{S990A} depends on Rad18, but not Rev1 or Rad54 to promotes ICL resistance.** FA-J cells stably expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were transfected with siRNA to luc, Rad18, Rev1, or Rad54 incubated for 48h. **(A)** Cells were treated with the indicated doses of MMC and allowed to grow for 5-8 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent ± SD of growth from 3 independent experiments. **(B)** Cells were collected and lysed for immunoblot with the indicated Abs.

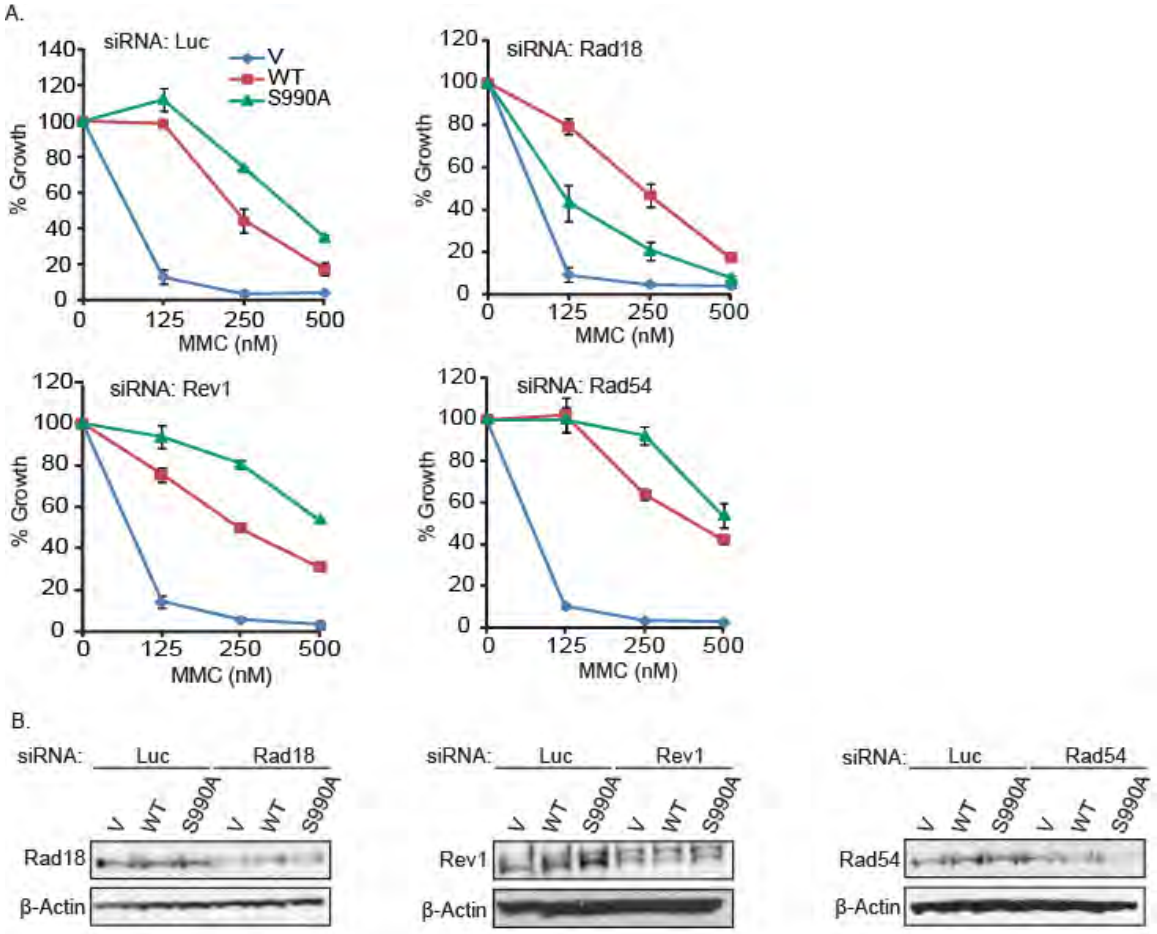
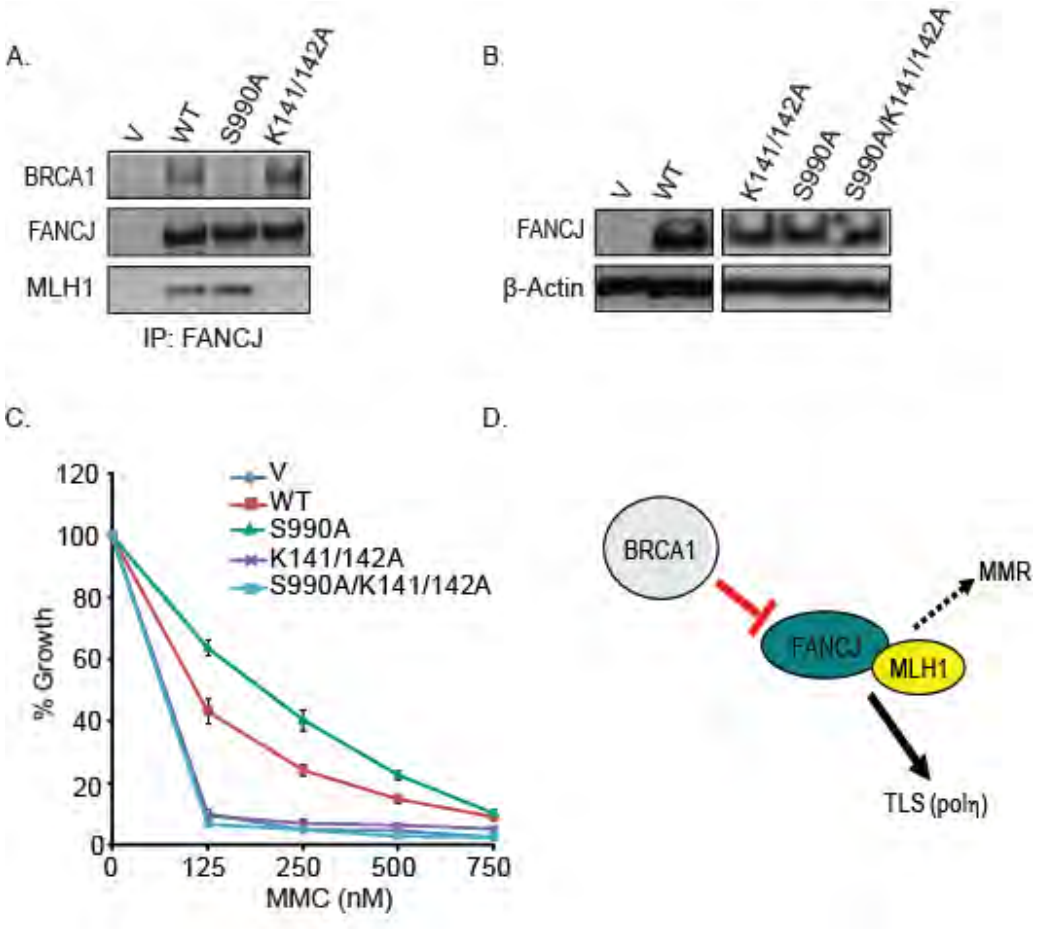
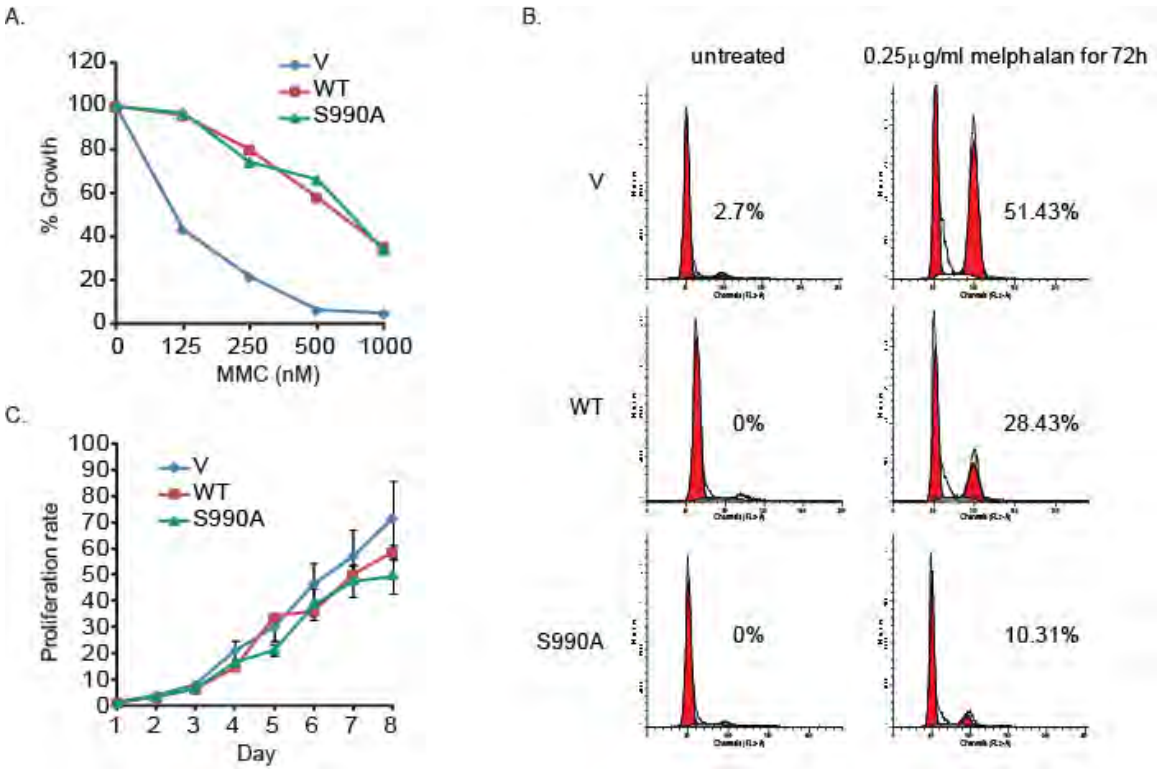


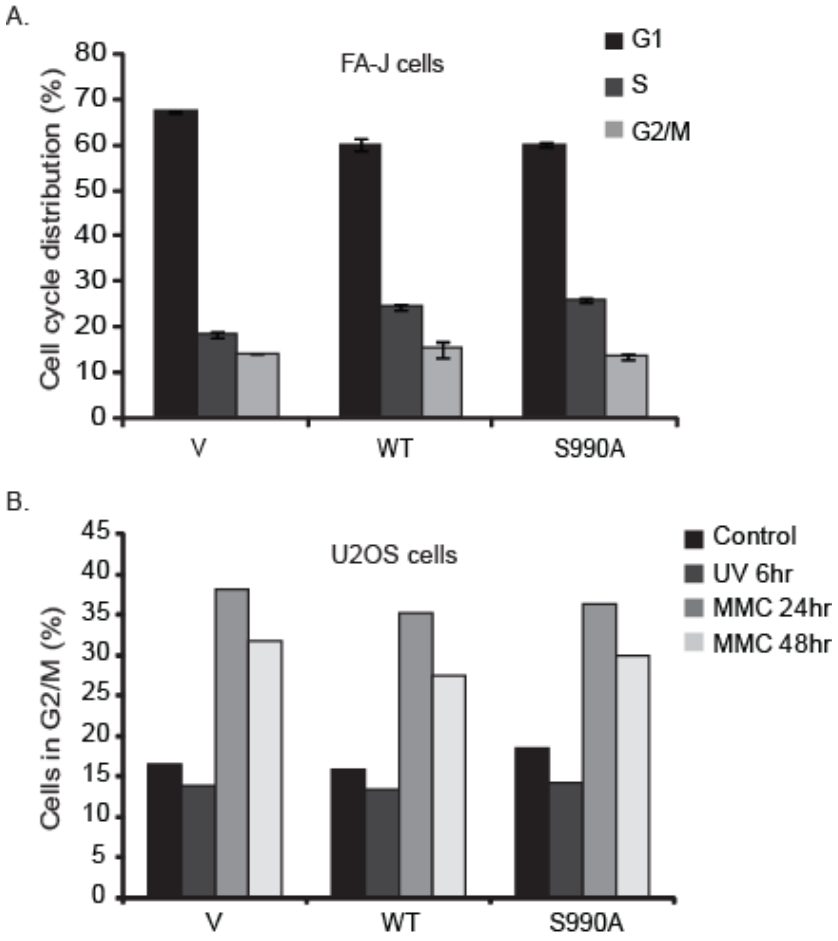
Figure 4-6. **FANCJ^{S990A} requires MLH1 binding to promote polη-dependent bypass. (A)** FA-J cells stably expressing vector, FANCJ^{WT}, FANCJ^{S990A}, or FANCJ$^{K141/142A}$ were collected and immunoprecipitated with anti-FANCJ and blotted with the indicated Abs. **(B)** FA-J cells stably expressing vector, FANCJ^{WT}, FANCJ^{S990A}, FANCJ$^{K141/142A}$, or FANCJ$^{K141/142A/S990A}$ were either collected for immunoblot with the indicated Abs or (**C**) treated with the indicated doses of MMC and allowed to grow for 5-8 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent ± SD of growth from 3 independent experiments. (**D**) Model shown summarizes observations of this study. FANCJ when uncoupled from BRCA1 promotes polη dependent TLS in a manner that requires MLH1 binding. Dotted line to MMR is added as a discussion point. To promote TLS, FANCJ could limit negative regulators of TLS, such a MMR.



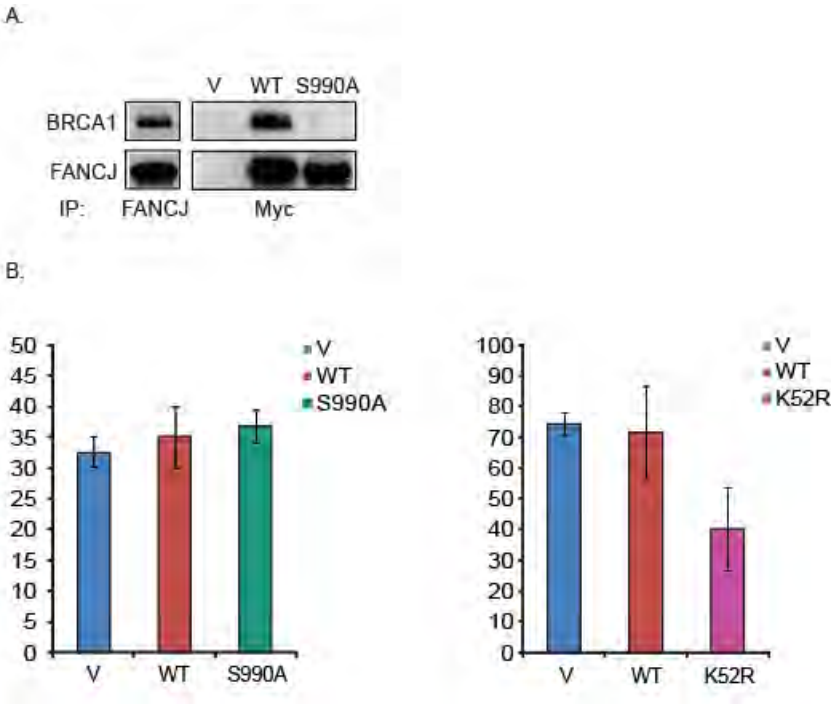
Supplemental Figure S4-7. **FANCJ^{S990A} similar to FANCJ^{WT} restores normal ICL response in FA-J cells.** (A) The FA-J cells expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were plated at low density, treated with the indicated doses of MMC and allowed to grow for 8 days. Percent growth were assessed and data represent mean percent ± SD of growth from 3 independent experiments. (B) Stable FA-J cells were treated constitutively with 0.25ug/ml melphalan, collected at the indicated times, and analyzed by FACS to determine cell cycle distribution. (C) Proliferation rate were also determine.



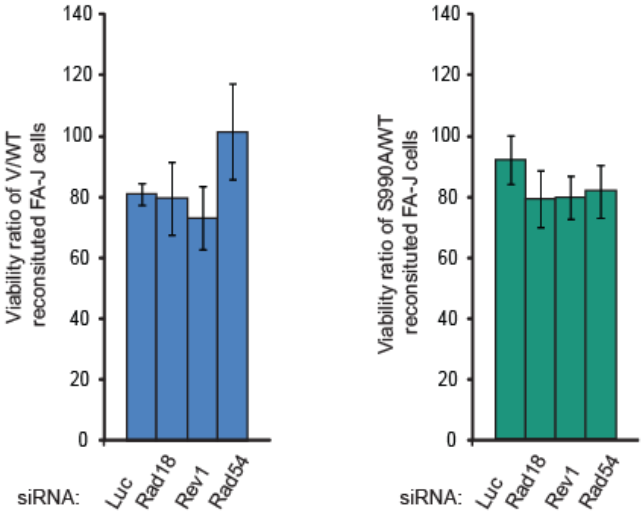
Supplemental Figure S4--8. **FANCJ^{S990A}, as compared to FANCJ^{WT} show similar cell cycle distribution in response to DNA damage.** (A) The FA-J cells expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were treated with 12.5ug/ml zeocin, cells were collected 12hs later and analyzed by FACS to determine the cell cycle distribution. Data represent mean ± SD for 3 independent experiments. (B) U2OS cells stably expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were treated with UV or MMC and collected at the indicated time points and analyzed by FACS to determine the percentage of cells in G2/M.



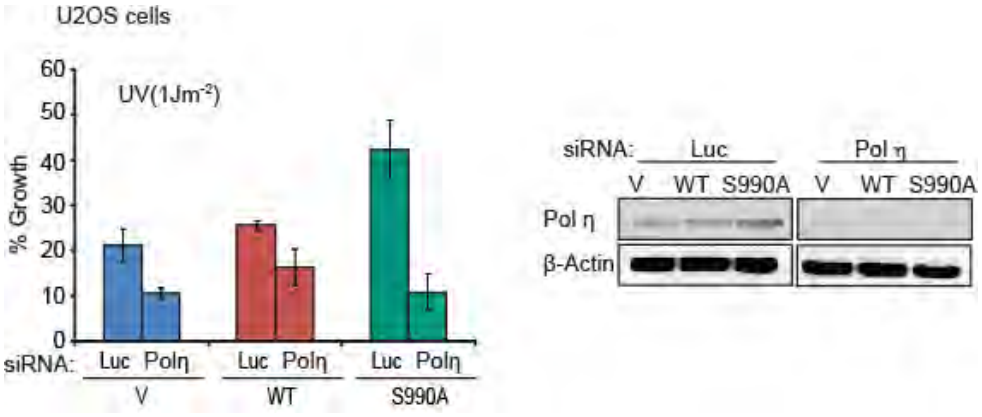
Supplemental Figure S4-9. **Exogenous FANCJ binds to similar levels of BRCA1 as endogenous FANCJ, and unlike over-expression of FANCJ^{S990A}, FANCJ^{K52R} over-expression resulted in MMC sensitivity.** (A) MCF7 cells un-transfected or transfected with vector, FANCJ^{WT}, or FANCJ^{S990A} were immunoprecipitated with anti-FANCJ or Myc respectively, and blotted with the indicated Abs. (B) U2OS or HeLa cells stably expressing vector, FANCJ^{WT}, FANCJ^{S990A} or FANCJ^{K52R} were treated with 500nM or 10nM MMC respectively. Cells were grown for 5-8 days, collected and counted to analyze percent survival. Data represent mean percent ± SD of growth from 3 independent experiments.



Supplemental Figure S4-10. **Suppression of Rad18, Rev1, and Rad54 are not toxic to vector and FANCJ^{S990A} reconstituted FA-J cells.** FA-J cells stably expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were transfected with siRNA to luc, Rad18, Rev1, or Rad54 incubated for 48h. Cells were allowed to grow for 5-8 days, then collected and counted to analyze percent growth. Toxicity was calculated by dividing vector reconstituted or FANCJ^{S990A} reconstituted cells over FANCJ^{WT} reconstituted cells.



Supplemental Figure S4-11. **FANCJS990A promotes UV resistance in polη-dependent manner.** U2OS cells stably expressing vector, FANCJ^{WT}, or FANCJ^{S990A} were transfected with siRNA to Luc or polη and incubated for 24hrs. Cells were collected and lysed for immunoblot with the indicated Abs, or plated at low density, treated with UV, and allowed to grow for 5 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent ± SD of growth from 3 independent experiments.



Materials and methods

Cell culture

U2OS cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics. Stable cells were selected with 20mg/ml G418. FA-J (EUFA30-F) fibroblasts were cultured in DMEM supplemented with 15% fetal bovine serum and antibiotics. FA-J cells were infected with pOZ vectors and selected as before (Peng et al., 2007).

Cell growth and G2/M accumulation assays

U2OS cells were transfected using Fugene or Lipofectamine for co-transfection with siRNA. Reagents for siRNA for polη, Rev1, Rad18, Rad54, and luciferase (luc) were obtained from Dharmacon. The FANCJ siRNA reagent was described previously (Litman et al., 2005). At 24 h after transfection, cells were seeded into 6-well plates at 1,500-3,000 U2OS cells/well. Stable FA-J cells infected with pOZ (Peng et al., 2007) vectors were seeded into 6-well plates at 8,000 cells /well. Seeded cells were incubated overnight and left untreated or treated with MMC (Sigma) for 1 h, UV (Stratalinker), cisplatin (Sigma) for 4h, and zeocin (Invitrogen) for 1h. Cells were counted after 5-8 days using a hemocytometer. Percent growth was calculated as (treated cells/untreated cells) X 100. G2/M accumulation was assayed as described (Litman et al., 2005), but at 0.25μg/ml melphalan.

Immunoprecipitation, Western blot, and antibodies

Cells were harvested and prepared for immunoprecipitation and Western blot as described (Litman et al., 2005). Immunoprecipitation Abs included FANCJ (E67) or Myc (9e10). Antibodies for Western blot analysis included BRCA1 (ms110) and FANCJ monoclonal (2G7 and 2C10) (Cantor et al., 2001) or polyclonal E67 (Cantor et al., 2004). Additionally, β-actin (Sigma), Rad18 **(**Santa cruz), Rev1 (H-300, Santa cruz), polη (Abcam), and Rad54 (Abcam) Abs were used.

DNA constructs

The FANCJ^{WT}, FANCJ^{K52R}, FANCJ$^{K141/142A}$ and FANCJ^{S990A} pCDNA-3myc-6xhis and pOZ vectors have been described previously (Cantor et al., 2001; Peng et al., 2007). The eGFP-polη construct was described previously (Kannouche et al., 2001). The FANCJ$^{K141/142AS990A}$ pOZ vector was generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene) using our published primers (Cantor et al., 2001; Peng et al., 2007).

Immunofluorescence

FA-J cells were either left untreated or treated with 12.5 g/ml zeocin and incubated 12h and processed for immunoflourescence as described (Cantor et al., 2001). Antibodies included RAD51 (Santa Cruz 1:200) and γ-H2AX (Upstate 1:500). Visualization of eGFP-polη foci was as described (Kannouche et al., 2001). In brief, U2OS cells were transfected with eGFP-polη, incubated for

overnight, seeded on cover slips, incubated overnight, and treated for 4 h with UV or for 24 h and 48 h with 250nM MMC. Foci counting experiments were conducted blind to the counter and in triplicate as the number of cells with 10 or more foci.

Homologous recombination

U2OS pDR-GFP cells were obtained from Maria Jasin (Pierce et al., 1999) and 1.8 X 10^5 cells were seeded per well in 6-well plates and incubated overnight. The cells were transfected with 0.5μg of pCDNA3, $FANCJ^{WT}$, $FANCJ^{S990A}$, or $FANCJ^{K52R}$ and 2.0 g of pBABE I-Sce1 using Fugene. Transfected cells were incubated for 72 h, collected by trypsinization, and analyzed by FACS. The percentage of green positive cells was calculated using Flow Jo software.

Acknowledgements

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CHAPTER V

FINAL THOUGHTS AND FUTURE DIRECTIONS

Due to the constant threat to the DNA posed by environmental carcinogens and metabolic byproducts, DDR genes are extremely important for maintaining the genomic integrity. Not surprisingly, mutations in DDR genes are associated with persistent DNA damage and cancer predisposition. For example, mutations in the DDR gene, FANCJ, are associated with various types of cancers. FANCJ directly associates with other DDR genes including BRCA1 and MLH1. Both genes pose critical functions in the DDR pathway, thus, I sought to further characterize the functional relevance of FANCJ/BRCA1 and FANCJ/MLH1 interactions in DDR.

In this thesis work, I have described several new FANCJ functions. First, I have demonstrated that FANCJ interacts with the MutLα complex heterodimer, which is composed of MLH1 and PMS2. FANCJ directly interacts with MLH1 independent of BRCA1, and PMS2 functions to stabilize this interaction (Figure 2-3 and 2-4). This interaction is facilitated by lysines 141 and 142 within the helicase domain on FANCJ, C-terminal to the nucleotide binding box 1. Importantly, the interaction between FANCJ and MLH1 is critical for ICL repair (Table 1). Second, I discovered an HNPCC associated-MLH1 mutation, $MLH1^{L607H}$ that is intact for mismatch repair, but lacks the FANCJ interaction.

Cells defective in the FANCJ/MLH1 interaction can initiate mismatch processing, checkpoint activation, and cell death; however, the progression of these events appears to be delayed. Third, I have demonstrated that FANCJ promotes error-prone bypass of ICLs when uncoupled from BRCA1. Specifically, cells expressing the BRCA1-interaction defective mutant, FANCJ^{S990A} are sensitive to DSBs and have reduced Rad51-based HR (Table 5-1). Resistance to UV and ICLs requires the post-replicative repair (PRR) pathway, including Rad18 and TLS polymerase, Polη (Table 5-1). In addition, the MLH1 and BRCA1 binding defective FANCJ is sensitivity to MMC, suggesting that the interaction of MLH1 is required for FANCJ^{S990A} to promote Polη dependent TLS (Table 5-1 and Figure 5-1). Taken together my data suggests that FANCJ functions in multiple repair pathways and its interaction with BRCA1 is critical for regulating the DDR (Figure 5-1). These observations further our understanding of the significance of FANCJ in genome maintenance and tumor suppression.

Table 5-1. **Characterization of FANCJ mutants.**

	MMC sensitivity	HR Assay	Rad51 foci	UV sensitivity	Polη foci
Vector	Hyper sensitivity	Normal	Reduced	Sensitive	Normal
WT	Normal	Normal	Normal	Sensitive	Normal
S990A	Normal	Reduced	Reduced	Resistance	Increased
K141/142A	Hyper sensitivity	NA	NA	NA	NA
S990A/K141/142A	Hyper sensitivity	NA	NA	NA	NA

The FANCJ/MLH1 complex plays a critical role in ICL response, but the specific function remains unclear. The requirement of this interaction is demonstrated by the finding that cells expressing FANCJ$^{K141/142A}$ are sensitive to ICL agents and have a prolonged G2/M accumulation. In addition, the MLH607 mutant selectively generates sensitivity to ICLs as compared to cells that lack MLH1 or express wild-type MLH1. What's more, expression of a peptide that disrupts the FANCJ/MLH1 interaction enhances the sensitivity of cells to ICLs.

Evidence is also accumulating that MMR proteins function in the ICL response. In particular, MMR proteins have been shown to act as sensors to find DNA damage, recruit ATR, and activate checkpoint responses (Yoshioka et al., 2006). This MMR function is most clear in response to methylated DNA, as opposed to ICLs. In support of the possibility that MMR proteins sense ICLs, MutSβ has been shown to bind ICLs in vitro (Duckett et al., 1996; Yamada et al., 1997; Zhang et al., 2002). Likewise, my data implicate that FANCJ facilitates MMR binding to methylated DNA, with less affect on MMR following ICLs. One possibility is that FANCJ unwinding or translocating on the DNA could serve to drive the accumulation of a robust MMR complex. Here, migration of MMR away from the O6-MeG lesion is critical so that new MMR proteins can load and repair can commence. In contrast, when a fork is blocked at an ICL, a MMR complex may not be required for repair. Instead, MMR could direct repair choice and/or regulate the fidelity of repair. Here, the FANCJ enzyme activity could be required to facilitate these processes and/or displace MMR so that replication can resume.

In future experiments, it will be important to address whether FANCJ can strip MMR proteins as it was shown to strip Rad51 from DNA (Sommers et al., 2009). It will also be critical to analyze how MMR proteins respond to ICLs in FA-J cells or in FA-J cells expressing FANCJ^{WT}, FANCJ$^{K141/142A}$, and FANCJ^{K52R} proteins.

How could cancer evolve from delayed MMR-signaling? Given that all patients with the MLH1^{L607H} are characterized as microsatellite stable (MSS) and have normal MMR function, suggest that the mechanism of tumorgenesis is unique compared to other HNPCC associated MLH1 mutations. The MLH1^{L607H} is not the only MLH1 mutant with unique mechanism of tumorgenesis, the sporadic colorectal cancer associated MLH1^{D132H} mutant have normal MMR and very low MSI (Lipkin et al., 2004). Lipikin et al. based on structural analysis, predicted that the MLH1^{D132H} mutant will attenuate ATPase activity but not eliminated thus the MMR complex is trapped in a transition state due to defect in ATP hydrolysis. The predicted defect of the MLH1^{D132H} mutant, suggests that cells expressing this mutant protein in response to DNA methylating agents may also have a defect in cell cycle signaling such as prolonged checkpoint activation. Most HNPCC patients are characterized and defined by the microsatellite instability (MSI) and MMR defective, thus patients with MLH1 mutations such as MLH1^{L607H} could be under represented due to their unique phenotypes.

Although MMR is highly conserved from *E.coli* to humans, the requirement of a helicase for mammalian MMR has not been demonstrated. Mammalian MMR proteins directly associate with multiple helicases including, WRN, BLM

and FANCJ. WRN interacts with MMR complexes including, MutSα, MutSβ, and MutLα (Saydam et al., 2007). The MutSα complex stimulates WRN helicase activity on forked structures with a 3'-singled-stranded arm (Saydam et al., 2007). In addition, the MutSα complex also enhances the unwinding activity of WRN in the presence of G:T mismatch in the DNA duplex ahead of the fork (Saydam et al., 2007). BLM like FANCJ also directly associates with MLH1 (Pedrazzi et al., 2001). The interaction of all three helicases with MMR proteins were characterized as unimportant for MMR, but since mammalian DDR is more complicated compared to *E.coli* there could be a functional redundancy. WRN, BLM and FANCJ should be suppressed simultaneously to avoid functional redundancy thus to determine if helicases are required for the mammalian MMR pathway.

FANCJ likely has a complicated role in ICL repair regulated by its binding with BRCA1, it promotes error-free repair and suppresses error-prone bypass when bound to BRCA1. As indicated by our results, when unbound to BRCA1, FANCJ promotes Pol η dependent TLS. Pol η is one of the most well characterized TLS polymerases in mammalian cells due to its association with the cancer-prone syndrome known as xeroderma pigmentosum variant (XPV). XPV patients have increased incidence of skin cancer due to increased mutation frequency caused by sunlight exposure. Sunlight is a natural source of UV damage which can be bypassed by Pol η in a relatively error-free manner, thus in the absence of polymerase η other TLS polymerases may bypass UV lesions in

an error-prone manner (Johnson et al., 1999; Waters et al., 2009). In contrast to UV lesion bypass, the bypass of ICLs by Pol η is error prone (Zheng et al., 2003). To further confirm that FANCJ^{S990A} expressing cells are promoting TLS bypass of ICLs compared to the FANCJ^{WT} expressing cells, mutation frequencies should be measured using the SupF mutagenesis assay. Compared to FANCJ^{WT} expressing cells, FANCJ^{S990A} expressing cells should have a higher ICL induced mutation frequency and lower UV induced mutation frequency.

How FANCJ promotes Pol η dependent TLS is still a question. Pol η directly interacts with PCNA through the C-terminal PCNA-binding motif (PIP box), and this interaction is enhanced by the monoubiquitination of PCNA (Kannouche et al., 2004). Although PCNA in not required for the localization of Pol η to stalled replication forks but, it is required for the accumulation of Pol η foci in response to DNA damage (Nikolaishvili-Feinberg et al., 2008). In addition to PCNA, Pol η also interacts with Rad18 which functions upstream of PCNA, thus in its absence, PCNA does not get monoubiquitinated (Watanabe et al., 2004). Our data indicate that when FANCJ is suppressed, Pol η foci accumulation in response to UV is greatly reduced similar to Rad18 suppression. This suggested that FANCJ functions upstream of Pol η similar to Rad18 and PCNA. Whether FANCJ^{S990A} affects PCNA monoubiquitination or interaction with Rad18 remains to be determined.

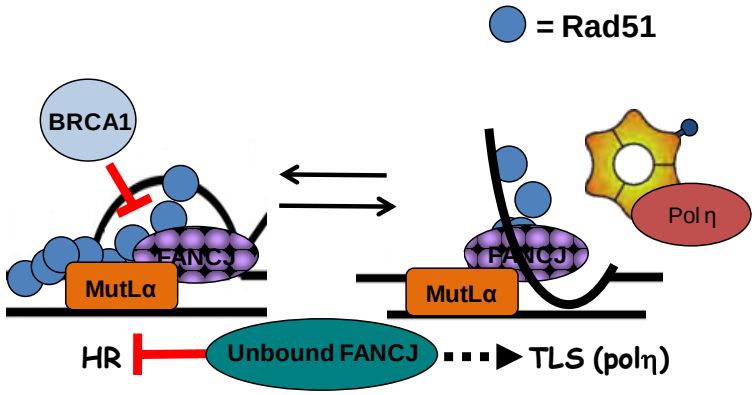
The FANCJ$^{S990AK141/142A}$ expressing cells are sensitive to ICL inducing agents compared to the FANCJ^{S990A} expressing cells (Table 5-1). How is MLH1

interaction contributing to the ability of FANCJ^{S990A} to promote TLS bypass. Pol η is among the lowest fidelity DNA polymerases on undamaged DNA and its bypass of most DNA lesions is mutagenic (Avkin and Livneh, 2002; de Padula et al., 2004; Kozmin et al., 2003; Lee and Pfeifer, 2008; Matsuda et al., 2000; Prakash et al., 2005). While MMR proteins repair nucleotide misincorporations by normal replicative polymerases, they also recognize nucleotide misincorporations induced by TLS, resulting in either removal or activation of apoptosis (Wang et al., 1999; Young et al., 2004). As such, cells deficient in a TLS polymerase, Rev3 dies of massive apoptosis (Van Sloun PP, 2002). This apoptosis is alleviated by MMR deficiency, suggesting that MMR proteins are critical for TLS associated apoptosis activation. BRCA1 mutant cells are resistant to DNA the methylating agent, 6-TG and are defective in 6-TG induced cell cycle activation (Yamane et al., 2007). All together, these data suggest that FANCJ^{S990A} may function to strip MMR proteins from TLS induced DNA mismatches thus blocking apoptosis activation. FANCJ$^{S990AK141/142A}$ expressing cells lack the interaction with MMR proteins thus FANCJ is unable to strip MMR proteins resulting in apoptosis activation. Although FANCJ$^{S990AK141/142A}$ expressing cells bypass ICLs, but eventually die from MMR dependent TLS associated apoptosis.

In conclusion, the work presented in this thesis demonstrated a new mechanism of tumorgenesis by FANCJ that is regulated by its interactions with different partners (Figure 5-1). Thus, cancers associated with mutations in

BRCA1, FANCJ or MLH1 could be due to multiple mechanisms including loss of HR, loss of MMR, loss of checkpoint control, and gain of TLS. These new findings raise the need to develop more cancer screens to determine the mechanism of tumorgenesis and more specific cancer therapies that will target the needs of each patient. It is our hope that the work presented in this thesis has promoted further understanding of tumor development and treatment.

Figure 5-1. **FANCJ functions in multiple repair pathways and its interaction with BRCA1 is critical for regulating the DDR.** FANCJ when bounded to BRCA1 promotes Rad51 dependent homologous recombination (HR) repair. However, FANCJ when uncoupled from BRCA1 promotes Polη dependent TLS which is regulated by PCNA monoubiqutination. The ability of FANCJ to promote both HR and TLS requires its interaction with the MutLα heterodimer. The choice between HR and TLS can be disrupted by the over-expression unbound FANCJ, which blocks HR and promotes TLS.



APPENDIX

In mammalian cells, 80 percent of FANCJ is unbound to BRCA1, thus raising the question of how is the “free” FANCJ regulated (Appendix Figure 1). Human TLS polymerase POLQ which contains tandem helicase and polymerase domains functions to remove the lagging strand at the replication fork providing a loading site for other TLS polymerase (Seki et al., 2003). FANCJ may also promote the loading of Pol η by removing unwanted DNA or proteins from the replication fork. The helicase activity of FANCJ could be important to remove proteins, based on the finding that FANCJ^{K52R} expressing cells are sensitive to ICL inducing agents. Regulation of FANCJ helicase activity could prevent unwanted promotion of TLS. Helicase activity of a protein can be regulated on multiple levels including protein interactions and posttranslational modifications. The contributions of BRCA1 or MLH1 interaction to FANCJ’s helicase activity need to be further investigated. There are multiple types of posttranslational modifications such as, phosphorylation, ubiquitination, sumolytion and acetylation. All of these modifications affect protein functions, interestingly; acetylation has been shown to affect the activity of many helicases including WRN and MCM. In addition, BRCA1 has been shown to associate with the acetyltransferase CBP/p300 and the deacetylase HDAC1 and HDAC2. These interactions also occur at the BRCT domain of BRCA1. All together, evidences suggested that FANCJ may be acetylated and this process could be facilitated by the BRCA1 interaction.

The ability for FANCJ to get acetylated was investigated using *in vivo* and *in vitro* assays. First, myc tagged FANCJ was co-transfected with increasing dose of CBP into 293T cell, FANCJ was immunoprecipitated with anti-myc antibody and immunoblotted with anti-AcK antibody (A). We found that FANCJ was acetylated in a CBP dose dependent manner (Appendix Figure 2A). Next, *in vitro* acetylation assays were performed by incubating the recombinant histone acetyltransferease (HAT) domains of p300 with the recombinant C-terminal domain of FANCJ in the presence of ^{3}H-acetyl-CoA. Recombinant C-terminal domain of p53 was also included as a positive control. The product of the reactions were separated by SDS-PAGE and analyzed by autoradiography. Results indicated that FANCJ is acetylated and this acetylation occurred within the C-terminal domain of FANCJ (Appendix Figure 2B). To more precisely map the domain of FANCJ acetylation, myc tagged FANCJ C-terminal truncation mutants were co-transfected with CBP into 293T cells. The nucleotides 1239 to1249 appear to be important for FANCJ acetylation (Appendix Figure 2C). Acetylations usually occur on lysine (K) residues, there are three lysines between nucleotide 1239 and 1249. All three lysine were mutated to arginine (R) individually using quick change and subsequently co-transfected with CBP into 293T cells. K1249 appears to be the dominate site for FANCJ acetylation (Appendix Figure 2C).

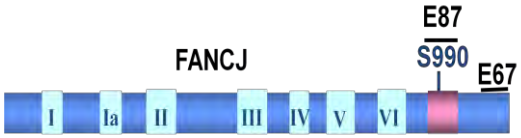
To determine the effect of acetylation status on the function of FANCJ, in addition to being mutated to R, K1249 was also mutated to glutamine (Q) which

structurally resembles the acetylated state. FANCJ null cells were reconstituted with vector, FANCJ^{WT}, FANCJ^{K1249R} or FANCJ^{K1249Q} and expression was confirmed by Western blot (Appendix Figure 3A). Acetylation modification has been shown to also affect protein localization, so reconstituted cell lines were plated, damaged with MMC and stained with FANCJ and BRCA1. Compared to FANCJ^{WT} reconstituted cells, the co-localization of FANCJ and BRCA1 in response to DNA damage in FANCJ^{K1249R} or FANCJ^{K1249Q} reconstituted cells was similar (Appendix Figure 3B). Next, reconstituted cell lines were treated with MMC or melphalan and measure for survival or G2/M acculumation. Compared to vector reconstituted cells, FANCJ^{WT}, FANCJ^{K1249R} or FANCJ^{K1249Q} reconstituted cells were all able to rescue the ICL sensitivity and the ICL induced prolonged G2/M accumulation of the FANCJ null cells (Appendix Figure 3C and D). This resulted suggested that despite the acetylation status of FANCJ, it is still able to resolve ICLs as efficiently as the FANCJ^{WT}. We know that FANCJ can process ICLs either by HR or TLS as indicated by the function of FANCJ^{S990A} thus; we treated reconstituted cells with different DNA damage agents to determine the effect of FANCJ acetylation on repair choice. Similar to FANCJ^{S990A}, FANCJ^{K1249R} also promotes Pol η dependent TLS bypass as indicated by the UV and MMC sensitivity (Appendix Figure 4A and C). In contrast, FANCJ^{K1249Q} promotes exclusively Rad54 dependent HR repair as indicated by Zeocin and MMC sensitivity (Appendix Figure 4B and C). Overall, these data indicated that the acetylation modification of FANCJ appears to be

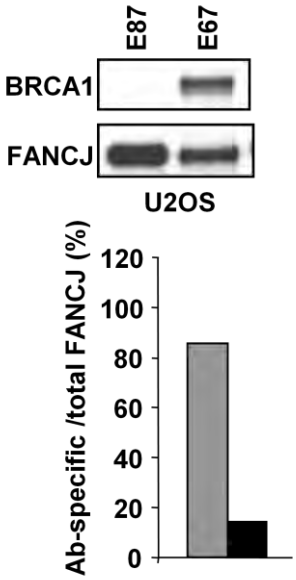
another level of regulation in addition to BRCA1 binding in the choice between HR and TLS. Endogenous acetylation of FANCJ and the contribution of BRCA1 to FANCJ acetylation still require further investigation.

Appendix Figure 1. **80% of mammalian FANCJ is unbound to BRCA1. A)** Schematic representation of the epitope of FANCJ antibodies E87 and E67. Due to the epitope specificity of E87, it only immunoprecipitates FANCJ that is unbounded to BRCA1. **B)** Lysates from U2OS cells were immunoprecipitated with E87 antibody followed by E67, and blotted with the indicated Abs. Percent of bounded and unbounded FANCJ were graphed in a bar graph.

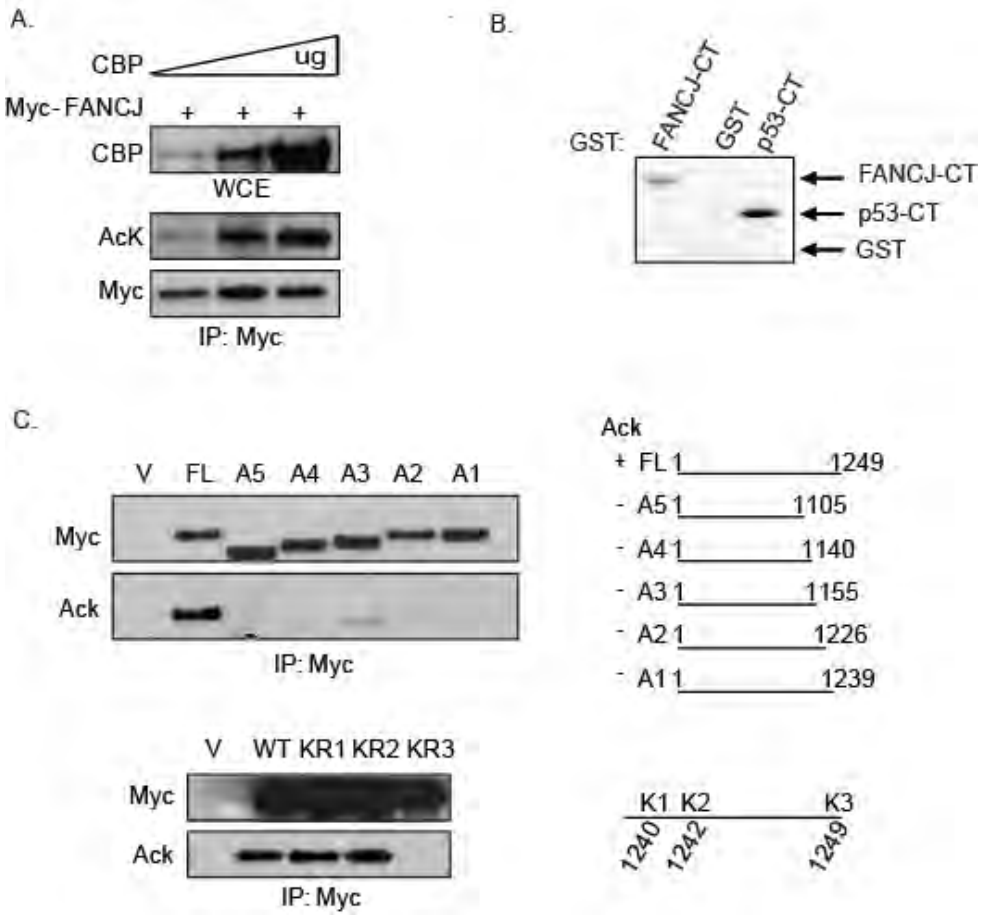
A.



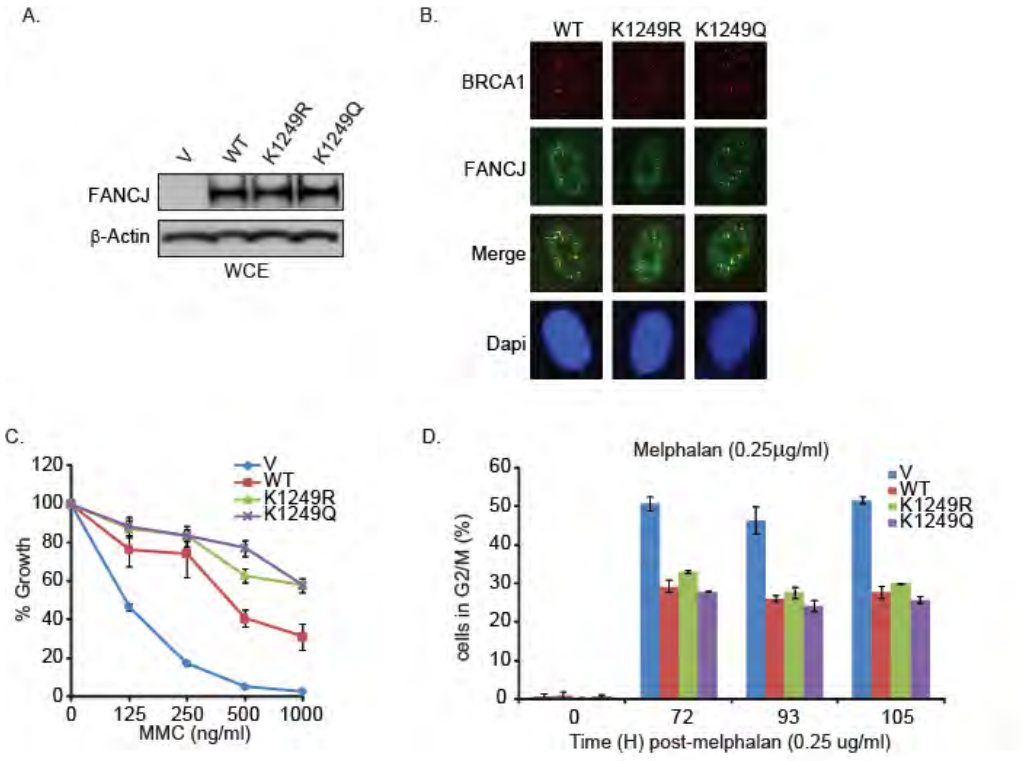
B.



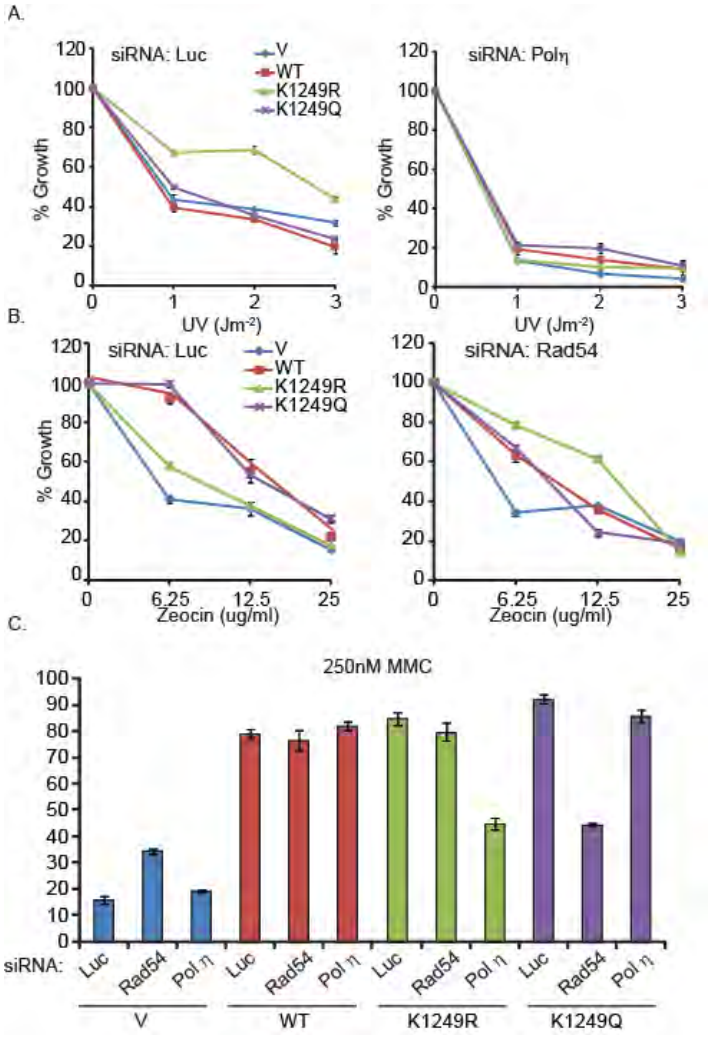
Appendix Figure 2. **FANCJ is modification by acetylation on lysine 1249. A)** FANCJ were co-transfected into 293T cells with varies concentrations of CBP. FANCJ were imminopricipitated from the lysates were with the Myc Ab and blotted with the indicated Abs. **B)** Recombinate GST tagged FANCJ-CT (888-1249) were incubated with the recombinate histone acetyltransferease (HAT) domains of p300 in the presence of ^{3}H-acetyl-CoA. Recombinant GST tagged p53-CT and empty vector was also included as a controls. The product of the reactions were separated by SDS-PAGE and analyzed by autoradiography. **C)** Varies mutant constructs of FANCJ were co-transfected with CBP into 293T cells. FANCJ were imminopricipitated from the lysates were with the Myc Ab and blotted with the indicated Abs.



Appendix Figure 3. **Hyper and Hypo acetylated FANCJ both correct the MMC sensitivity and cell cycle defect of FA-J cells. A)** FANCJ-null FA-J cells were reconsituted with vector, FANCJ^{WT}, FANCJ^{K1249R}, and FANCJ^{K1249Q}, lysates were analyzed by immunoblot with the indicated Abs. **B)** Stable FA-J cells were treated with 50nM of MMC and stained with the indicated Abs. **C)** Stable FA-J cells were treated with increasing dose of MMC, and allowed to grow for 8 days. The cells were collected and counted to analyze percent growth. Data represent mean percent ± SD of growth from 3 independent experiments. **D)** Stable FA-J cells were treated with 0.25ug/ml melphalan, collected at the indicated times, and analyzed by FACS to determine the percentage of cells in G2/M. Data represent mean percent ± SD 3 from 3 independent experiments.



Appendix Figure 4. **Hyper-acetylated FANCJ promotes Pol η dependent bypass, whereas hypo-acetylated FANCJ promotes Rad54 dependent repair. A)** FANCJ cells stably expressing vector, FANCJ^{WT}, FANCJ^{K1249R}, and FANCJ^{K1249Q} were transfected with siRNA to Luc, Pol η, and Rad54. Cells were treated with A) UV, B) Zeocin or C) MMC and allowed to grow for 5-8 days. The cells were then collected and counted to analyze percent growth. Data represent mean percent ± SD of growth from 3 independent experiments.



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