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Characterization of the BACH1 Helicase in the DNA Damage Response Pathway: a Dissertation

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**Characterization of the BACH1 helicase in the
DNA damage response pathway**

A dissertation presented

by

Rachel Litman

February 2007

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By

Rachel Litman

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.

Statement of Contribution

The works presented in Chapters 2 and 4 were collaborations with Ming Peng, a research associate in Dr. Cantor's lab. The papers that resulted from these works are Co-first author papers between Min and myself. Min also contributed immunofluorescence images and Zhe Jin conducted the FANCD2 monoubiquitination assay for Chapter 3. Jenny Xie performed the in-vitro translation assays and our collaborator Robert Brosh performed the in-vitro helicase assays and ELISA assays in Chapter 4. I am responsible for all experiments discussed in Chapter 5. We have acknowledged individuals who have made contributions to each chapter at the end of each chapter.

Characterization of the BACH1 helicase in the DNA damage response pathway

ABSTRACT

DNA damage response pathways are a complicated network of proteins that function to remove and/or reverse DNA damage. Following genetic insult, a signal cascade is generated, which alerts the cell to the presence of damaged DNA. Once recognized, the damage is either removed or the damaged region is excised, and the original genetic sequence is restored. However, when these pathways are defective the cell is unable to effectively mediate the DNA damage response and the damage persists unrepaired. Thus, the proteins that maintain the DNA damage response pathway are critical in preserving genomic stability.

One essential DNA repair protein is the Breast Cancer Associated gene, BRCA1. BRCA1 is essential for mediating the DNA damage response, facilitating DNA damage repair, and activating key cell cycle checkpoints. Moreover, mutations in BRCA1 lead to a higher incidence of breast and ovarian cancer, highlighting the importance of BRCA1 as a tumor suppressor. In an effort to better understand how BRCA1 carried out these functions, researchers sought to identify additional BRCA1 interacting proteins. This led to the identification of several proteins including the BRCA1 Associated C-terminal Helicase, BACH1. Due to the direct interaction of BACH1 with a region of BRCA1 essential for DNA repair and tumor suppression, it was speculated that BACH1 may help support these BRCA1 function(s). In fact, initial genetic screenings confirmed that

mutations in BACH1 correlated not only with hereditary breast cancer, but also with defects in DNA damage repair processes.

The initial correlation between BACH1 and cancer predisposition was further confirmed when mutations in BACH1 were identified in the cancer syndrome Fanconi anemia (FA) (complementation group FA-J), thus giving BACH1 its new name FANCI. These findings supported a previously established link between the FA and BRCA pathways and between FA and DNA repair. In particular, we demonstrated that similar to other FA/BRCA proteins, suppression of FANCI lead to a substantial decrease in homologous recombination and enhanced both the cellular sensitivity to DNA interstrand cross-linking agents and chromosomal instability. What remained unknown was specifically how FANCI functioned and whether these functions were dependent on its interaction with BRCA1 or other associated partners. In fact, we identified that FANCI interacted directly with the MMR protein MLH1. Moreover, we found that the FANCI/BRCA1 interaction was not required to correct the cellular defects in FA-J cells, but rather that the FANCI/MLH1 interaction was required. Although both the FA/BRCA and MMR pathways undoubtedly mediate the DNA damage response, there was no evidence to suggest that these pathways were linked, until recently. Our findings not only indicate a physical link between these pathways by protein-protein interaction, but also demonstrated a functional link.

CHAPTER I

INTRODUCTION

DNA DAMAGE RESPONSE

Cells are constantly under attack from both endogenous and exogenous forms of DNA damage. Endogenous DNA damage can be accrued by cellular metabolic processes including release of oxygen free radicals, base hydrolysis, and cytosine deamination. Similarly, exogenous DNA damage can be inflicted on the genome by environmental factors such as exposure to ultraviolet radiation (UV) and/or ionizing radiation (IR). Collectively, these forms of DNA damage are a threat to both cell viability and to genomic stability. Thus, cells have evolved intricate mechanisms to sense damaged DNA and signal a DNA damage response ¹.

The DNA damage response can be initiated by any of several mechanisms including changes in chromatin structure, protein modification/activation/relocalization, and/or generation of single stranded DNA (ssDNA). Once the DNA damage signal is initiated and amplified, the cell activates cell cycle checkpoints to arrest cell cycle progression, and/or repair pathways. If the damage is irreparable, the cell can activate an apoptotic response and induce cell death ². Thus, the proteins regulating cell cycle checkpoints, DNA damage repair, and apoptosis function in a coordinated manner to preserve both cell viability and genetic integrity.

The DNA damage induced checkpoints function to arrest cell cycle progression at the G1/S, intra-S, and/or G2/M checkpoints. Checkpoint activation is regulated by proteins, which can be categorized as sensors, mediators, transducers, and/or effectors. The sensory proteins are responsible for recognizing DNA damage either directly and/or indirectly. Mediators facilitate the propagation of the damage signal by bridging the activated sensor proteins to specific signal transduction proteins. These transduction proteins generally consist of protein kinases and phosphatases, which function to activate, or inactivate, the effector proteins. It is the effector proteins that function to directly regulate cell cycle progression ^{2,3}.

DNA damage-signaling and activation of cell cycle checkpoints are essential for efficient DNA damage repair. If damage is not repaired before subsequent rounds of cell division the daughter cells can acquire genetic mutations. Therefore, the cell has several distinct repair pathways to remove, or reverse, specific types of DNA modifications. Cells containing mutations in genes involved in the DNA damage response pathway become increasingly mutagenic and consequently, lose the ability to govern cell growth and/or proficient repair, enhancing the possibility of tumorigenesis ^{2,3}.

Not surprisingly, defects in DNA damage response, checkpoint, and/or repair are intimately linked to cancer susceptibility ⁴. For example, mutations in BRCA1, lead to defects in DNA damage response, checkpoint, and repair. To further understand the role of BRCA1 in the damage response and tumor suppression, research was directed at identifying BRCA1 interacting proteins (Figure 1-1). This effort led to the identification of several BRCA1 interacting proteins, including BACH1 ⁵. Further studies found

germline mutations in BACH1, in patients with early onset breast cancer^{6 7}. Moreover, BACH1 was shown to be required for efficient DNA double strand break repair (DSBR) in a BRCA1 dependent manner⁶. The aim of this thesis was to further characterize the function of BRCA1 and BACH1 in the DNA damage response and to develop our understanding of how these proteins function as tumor suppressors. To better appreciate the function of these proteins in the DNA damage response and tumor suppression, it is essential to have a basic understanding of how cells respond to different forms of DNA damage.

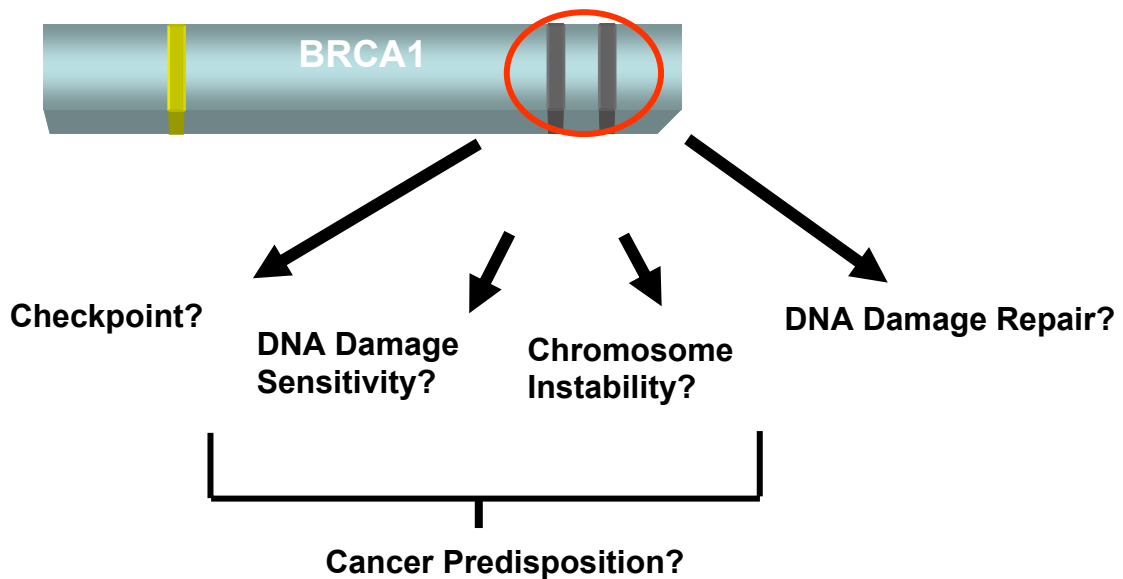
DNA DAMAGE REPAIR

DNA damaging agents are capable of inducing a range of DNA modifications. However, it is the most severe modifications, such as DSBs that threaten the overall viability of a healthy cell. Double stranded DNA breaks (DSB(s)) can be generated directly by DNA damage (i.e. IR) or by the processing of damaged genetic elements (i.e. processing of alkylating agents). If left unrepaired, these breaks threaten the overall stability of the genome and basic cell survival¹.

DNA modifications

DNA damage can be generated by several different agents. For example x-rays, alpha/beta/gamma rays, and neutrons are all forms of IR that can create a charge separation in medium. IR can damage cells both directly and indirectly depending on how the cellular components absorb the radiation particles.

Figure 1-1: **Established BRCA1 functions.** The BRCA1 protein is characterized as both a tumor suppressor and a DNA damage response protein critical for mediating cell cycle checkpoints and DNA damage repair. These functions are compromised by mutations in the C-terminal BRCT repeats of BRCA1 suggesting that this region was critical for maintaining BRCA1 function.



If the molecules within the cell absorb the radiation, an ionization reaction may result and produce electrophilic species. In a secondary or indirect reaction, these species are then capable of attacking DNA molecules and subsequently, induce a variety of genetic lesions.

Alternatively, the radiation particles can be directly absorbed by the DNA itself, resulting in the ionization of bases and/or the sugar phosphate backbone ^{1,8}. Collectively, indirect and direct ionizing radiations are capable of inducing a range of DNA modifications, however it is the formation of the DSB that is most toxic ¹.

In some cases, as with direct absorption of IR particles, the initial lesion is a DSB. Alternatively a DSB may be generated after a primary lesion is processed. For example, the processing of DNA crosslinks can lead to the formation of a DSB. DNA crosslinks can be introduced into DNA endogenously by the acidification of nitrites during cellular metabolism ^{1,9,10} or exogenously by several classes of DNA crosslinking agents including nitrogen mustards (Melphalan), platinum drugs (Cisplatin), and the natural alkylating agent Mitomycin C (MMC) ¹⁰. Complex chemical analyses have demonstrated that MMC and Melphalan are bifunctional DNA alkylating agents that predominantly result in the formation of DNA interstrand crosslinks (ICLs) ¹¹. As a consequence of DNA crosslinks, the separation of complementary DNA strands during DNA replication and transcription is inhibited. Thus, removing ICLs is critical for cell survival ^{1,2,11}. Removal of a DNA crosslink involves several repair mechanisms that are required for recognition and removal of the ICL followed by restoration of the original genetic sequence. The DNA crosslink may be initially recognized by replication

machinery actively synthesizing new DNA. When the ICL lesion is encountered, replication is arrested and the replication fork is stabilized to allow the repair machinery access to the damage site. At this time the ICL can be excised and a DSB may be formed, thus, it is the initial processing of the ICL that result in the formation of the toxic DSB¹.

Likewise, the processing of DNA methylation adducts also results in the formation of DSBs. DNA methylation is the covalent addition of a methyl group to a nucleophilic species within the DNA molecule. Although this can occur at any position within the DNA molecule, typically, the cyclic nitrogen and oxygen atoms are the most reactive. Methyl compounds are formed both endogenously as products of cellular metabolism and also introduced exogenously as chemically synthesized compounds^{1,12}. One commonly used chemical compound is N-methyl-N-nitrosurea (MNU). MNU is an SN1 type monofunctional methylating agent that is highly reactive to oxygen molecules within DNA. The major adduct formed by MNU following cellular exposure, is O⁶ methylguanine (O⁶MeG)¹². Generally, the methylguanine methyltransferase (MGMT) enzyme functions to remove the O⁶methyl adduct from the DNA prior to replication. However, in colon and other types of cancer expression of MGMT is frequently silenced by promoter hypermethylation¹³. Consequently, during DNA replication, the O⁶MeG is interpreted as an adenine and is paired with a thymine, resulting in a GT mismatch. If the mismatch is left unrepaired in the genome subsequent rounds of replication produce a GC-TA transition mutation. If the mismatch is repaired, the misincorporated base is excised and the replication machinery will inherently resynthesize DNA at the damaged

site. However, the methylated base will again be misread and the replication machinery incorporates a T across from the O⁶MeG, beginning a futile cycle of repair. If this site of misincorporation is across from another site of misincorporation, an Okazaki fragment, or the leading strand of replication a DSB can be formed¹³.

Repair of double stranded DNA breaks

One of the early events following cellular exposure to DNA damage is the phosphorylation of the histone variant γ H2AX¹⁴. γ H2AX represents the presence of DSBs and has been demonstrated to be within 1-2kb of the break site¹⁵. In eukaryotes there are two major pathways for repair of DSBs, homologous recombination (HR) and non-homologous end-joining (NHEJ). While NHEJ is an error prone pathway and results in the loss of genetic information, HR repair is error-free and generally restores the original sequence. HR requires either a homologous sister chromatid, or homologous chromosome, with identical sequence to serve as a template for accurate repair. Thus, the HR repair pathway not only repairs DNA damage, but also restores the original genetic information (Figure 1-2)¹⁶.

The initial processing of the DSB for HR requires the formation of a single stranded DNA region at the site of the break. Here, the 5' strand of the duplex is resected and leaves a 3' single-stranded DNA region. This 3' DNA overhang is initially stabilized by the single-stranded binding protein RPA, but is then replaced by the RAD51 recombination protein forming a nucleoprotein filament. The RAD51 nucleoprotein filament searches for a region of sequence homology on either a sister chromatid or a

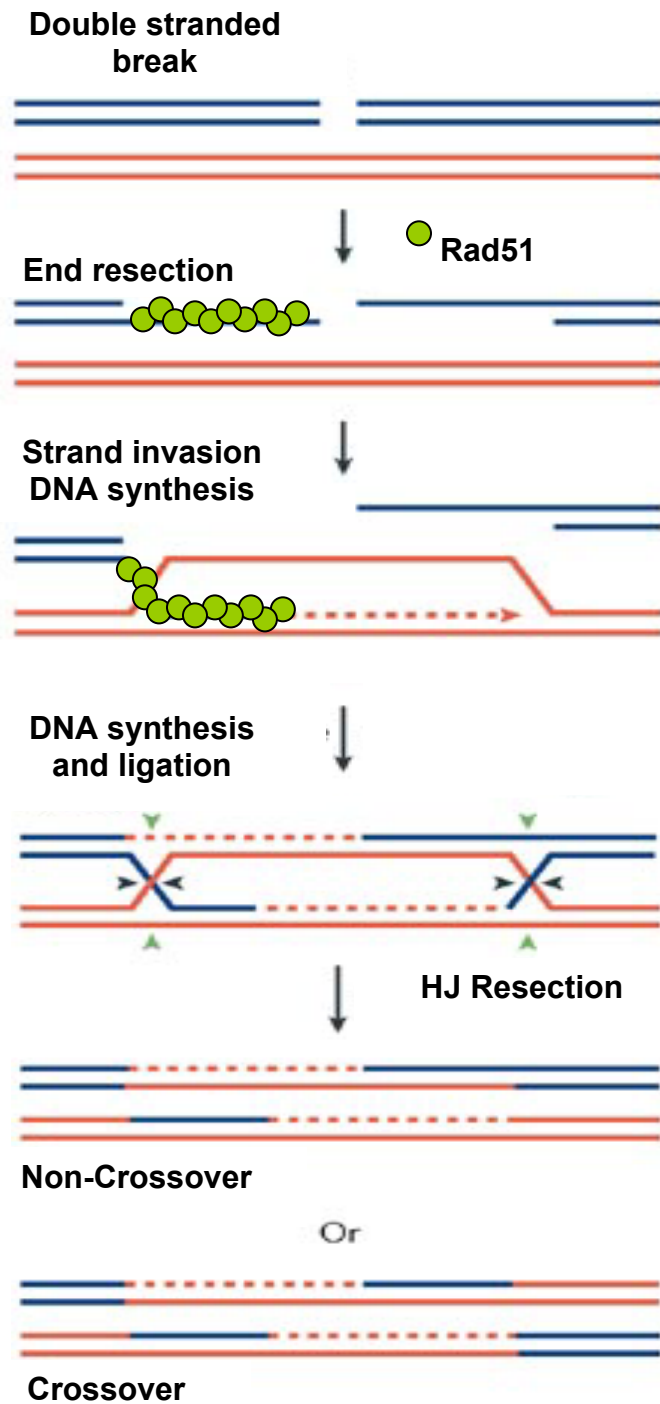
homologous chromosome and forms a paranemic joint. Once the nucleoprotein filament aligns with a homologous sequence and strand exchange occurs, new DNA is synthesized at the broken ends, and a heteroduplex DNA intermediate, or Holliday junction, is formed. Following endonucleolytic cleavage the Holliday junction is resolved and the original break has been repaired with no loss of genetic information¹⁷.

DNA DAMAGE RESPONSE PATHWAYS AND CANCER

The link between the FA/BRCA pathway and cancer

Defects in DSBR and/or pathways that help promote HR contribute to the development of cancer⁴. In particular, inherited genetic mutations in genes of the Fanconi anemia (FA)/BRCA pathway leads to reduced HR in response to DSBs and ultimately compromises cell survival. Specifically, cells from patients with FA are hypersensitive to DNA crosslinking agents such as cisplatin, MMC, melphalan, and diepoxybutane (DEB). Following exposure to any one of these clastogens, FA cells demonstrate defects in cell cycle progression and demonstrate gross chromosomal instability¹⁸. Given these defects, it is not surprising that in addition to aplastic anemia and congenital abnormalities, FA is characterized by increased cancer susceptibility.

Figure 1-2: **Schematic representation of homologous recombination.** DNA damage can result in the formation of a double-stranded break. The schematic below depicts the mechanism used to repair a DSB by HR.



Adapted from Sung and Klein *Nature Reviews Molecular Cell Biology* 7, 739–750 (October 2006).

In an attempt to understand the correlation between FA defects and cancer, researchers sought to identify the gene defects in each of the patient complementation groups. To date, 12 FA 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) have been identified and 11 of the corresponding genes have been cloned (all except FA-I). Each of the complementation groups is distinct and the genes do not share sequence homology. Since their identification, research has provided insight towards the function of several of these proteins within the FA pathway. The central feature of the FA pathway is the monoubiquitination of the FANCD2 protein by the FA core complex (FA-A, FA-B, FA-C, FA-E, FA-F, FA-G, FA-L, FA-M). This event is required for FANCD2 chromatin localization and accumulation in nuclear foci suggesting that FANCD2 aggregates at sites of DNA damage to mediate the DNA damage response and ultimately promote error-free repair (Figure 1-3) ¹⁸.

One of the first clues to the function of the FA pathway was provided by its similarity to the BRCA pathway. Specifically, the correlation between FA and BRCA cellular phenotypes suggested that these two pathways were directly connected. Loss of either FA proteins, or BRCA proteins, conferred cellular sensitivity to DNA damaging agents, defects in cell cycle checkpoints, and gross chromosomal abnormalities ¹⁹⁻²¹. Subsequent sequencing data identified that the FA-D1 gene defect was, in fact, the result of a biallelic mutation in the breast cancer susceptibility gene, BRCA2 ²². Subsequently, FA-D1 is now referred to as BRCA2/FANCD1. Further linking the two pathways, was the initial finding that BRCA1 was the E3 ubiquitin ligase responsible for

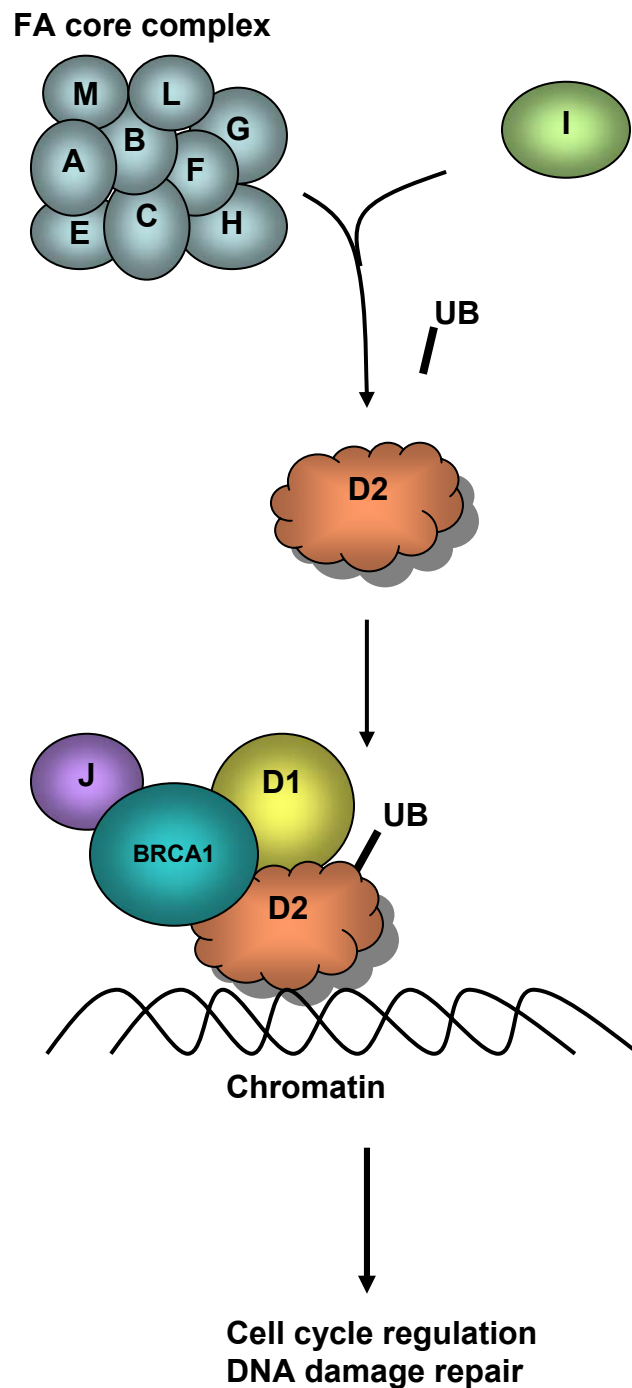
monoubiquitination of FANCD2. Initial studies conducted in vitro, suggested that the BRCA1/BARD1 E3 complex was sufficient to monoubiquitinate FANCD2. This hypothesis was further supported by studies conducted in HCC1937 cells, lacking functional BRCA1²³. However, it was later found that this result could not be reproduced in multiple cell lines, clouding the role of BRCA1 in this process. Current data supports the finding that FANCL is actually the E3 ubiquitin ligase required for FANCD2 monoubiquitination²⁴.

Although BRCA1 does not appear to be required for FANCD2 monoubiquitination, BRCA1 is required for the proper DNA damage-induced localization of FANCD2^{23,25}. Moreover, the similarity between BRCA1 and FA deficient cells further suggests that BRCA1 is linked to this pathway. Finally, research demonstrated that BRCA1 formed a direct interaction with BACH1 and that this interaction was critical for DSB repair. We later identified that BACH1 was the gene defect in the FA-J complementation group (referred to now as FANCI) and once again provided evidence of BRCA1's involvement in the FA pathway (see chapter 3).

The FA/BRCA pathway DNA damage response

The phosphatidylinositol 3-kinases Ataxia Telangiectasia mutated kinase (ATM) and the ATM Rad3 Related kinase (ATR) are initial responders and activators of the FA/BRCA pathway^{3,26,27}. Following genotoxic stress these kinases are activated and transduce the DNA damage signal downstream to a number of FA proteins, regulating their function(s)

Figure 1-3: **Cartoon of the FA/BRCA pathway.** Following DNA damage, FANCD2 is monoubiquitinated by the FA core complex and translocates into chromatin where it colocalizes in nuclear foci with FANCD1, BRCA1, and FANCD2. Monoubiquitination of FANCD2 is critical for maintaining normal cell cycle progression and effective DNA damage repair.



In particular, ATM phosphorylates FANCD2 at multiple residues *in vivo*, including S222, S1401, S1404, and S1418. However, it is the phosphorylation event at residue S222 that is critical for activation of the IR-induced intra-S-phase checkpoint^{27,29}. Like ATM, ATR has also been shown to phosphorylate FANCD2 at several residues including, T691, S717, S222, S1401, S1404, and S1418. Phosphorylation at residues T691 and S717 enhances FANCD2 monoubiquitination, increases cellular resistance to MMC, and is required for activation of the ICL-induced intra-S-phase checkpoint²⁹.

ATR is also essential for the DNA damage-induced FANCD2 monoubiquitination²⁶. Ubiquitination is a tightly regulated process and governs a multitude of pathways. In some instances, proteins are covalently modified with multiple ubiquitin moieties forming a polyubiquitin chain. This chain functions as a signal, flagging the modified protein for degradation by the proteasome. However, in other instances such as with FANCD2, a single ubiquitin moiety is added and here the modification acts as a signal activating the FA pathway³⁰. Following monoubiquitination, FANCD2 translocates into chromatin and is targeted to nuclear foci containing a number of DNA damage proteins including ATR, RPA, FANCE, FANCC, NBS1, BLM, FANCI, BRCA1, BRCA2, and RAD51¹⁸. Furthermore, in response to DNA damage many FA proteins form distinct protein-protein interactions. In particular, FANCD2 interacts with FANCE, BRCA2/FANCD1, and BRCA1 in chromatin and supports the hypothesis that FANCD2 regulates the process of recombination^{31,32}.

The DNA damage-induced monoubiquitination of FANCD2 is regulated by the FA core complex members FA-A, FA-B, FA-C, FA-E, FA-F, FA-G, FA-L, and FA-M.

This complex is required for the monoubiquitination of FANCD2 during S-phase of the cell cycle and following cellular exposure to DNA damaging agents¹⁸. Further characterization has revealed that FA-L is the catalytically active E3 ubiquitin ligase of the core subunits and is responsible for ubiquitination, and subsequent activation, of FANCD2 at lysine 561²³. FA-I is required for FANCD2 monoubiquitination, however, it is not required for stability of the core complex subunits. Thus, FA-I appears to function upstream of FANCD2 activation, but independent of the core complex³³. FANCI and BRCA2/FANCD1 are not required for FANCD2 monoubiquitination and function downstream in the FA pathway (FANCI see chapter 3)³². Monoubiquitination of FANCD2 is the central feature of the FA/BRCA pathway and loss of this biochemical transaction inactivates the pathway and abrogates an effective DNA damage response¹⁸.

FA/BRCA proteins and DNA damage repair

It is conceivable that the cellular sensitivity of FA/BRCA cells to DNA damaging agents results from defects in repair. FA/BRCA proteins have been implicated in promoting error-prone repair through non-homologous end joining and single-strand annealing, as well as error-free repair through HR^{34,35}. The evidence linking FA/BRCA proteins to HR, stems from homology directed DSB repair assays (Figure 1-4), association with other recombination proteins, and/or loss of Rad51 foci¹⁸. The exact role of BRCA1 in HR remains elusive, yet in the absence of BRCA1, RAD51 foci do not accumulate and HR is dramatically reduced^{36,37}. In contrast, the role of BRCA2/FANCD1 in HR has been well-characterized due to its direct interaction with

Rad51³⁸. The BRCA2/FANCD1 structure suggests that it loads Rad51 onto ssDNA. In particular, BRCA2/FANCD1 contains 8 BRC repeats that are each, approximately 30 amino acids in length, and span the length of the protein. These motifs directly interact with the RAD51 protein and mediate the formation of a RAD51 nucleoprotein filament, a critical step required for strand invasion during HR³⁹. At the C-terminus, BRCA2 contains a helical domain, 3 oligonucleotide/oligosaccharide binding folds, and a tower domain. The oligonucleotide/oligosaccharide binding folds (OB1, OB2, and OB3) and the tower domain bind ssDNA and dsDNA, respectively⁴⁰. Moreover, BRCA2/FANCD1 has been shown to function at stalled replication forks to provide stability and prevent fork collapse. Taken together, the structural components of BRCA2 support the notion that BRCA2/FANCD1 binds single-stranded/double-stranded DNA regions (i.e., resected end at DSB site or at stalled replication forks) and loads RAD51 protein, promoting HR and maintaining genomic integrity.

BRCA2/FANCD1 is targeted to chromatin following DNA damage where it interacts with several FA proteins including FANCD2 and FANCG¹⁸. BRCA2/FANCD1 interacts with FANCG through both its N and C-termini. This interaction mediates a ternary complex with the recombination protein XRCC3. XRCC3 is a key component in the recombinational repair pathway and facilitates the formation of the RAD51 nucleoprotein filament. FANCG can be coprecipitated with both XRCC3 and BRCA2 and it has been demonstrated that FANCG serves as a bridge stabilizing the complex⁴¹. It is the interaction between FANCG and the recombination proteins XRCC3 and BRCA2 that suggests a role for FANCG in homology directed repair.

Interestingly, ⁴² has shown that *Fancd2* ^{-/-} MEFs can be sensitized to IR by suppression of *Prkdc*, a critical component of the NHEJ pathway. *Prkdc* ^{-/-} mice are characteristically sensitive to IR, however, this sensitivity was increased in the *Fancd2* ^{-/-} *Prkdc* ^{-/-} double mutant MEFs. The double mutant MEFs also demonstrated an increase in radial chromosomes, indicative of defects in DNA damage repair. Lastly, *Fancd2* ^{-/-} *Prkdc* ^{-/-} double mutant mice had a significant decrease in survival following whole body irradiation. These findings indicate that the FA proteins function in a pathway distinct from NHEJ in repair of DNA damage and further support the role of FANCD2 in HR. Furthermore, these results suggest that the insensitivity of FA deficient cells to IR may be a result of compensation by the NHEJ pathway. In the absence of both HR and NHEJ the cell is unable to utilize two major repair pathways and accrues gross chromosomal instability, ultimately compromising cell survival.

Lastly, FANCA and FANCC have also been associated with HR ^{35,43,44}. FANCC directly interacts with FANCE ⁴⁵ and forms a ternary complex that includes FANCD2 ⁴⁶. FANCC has been speculated to function in concert with both TLS and HR to mediate DNA damage repair ⁴⁴. The role of FANCA in HR was directly assessed in mammalian cell-based homology directed repair assays. This finding was then supported indirectly by cell survival assays utilizing Poly (ADP Ribose) Polymerase (PARP) inhibitors. PARP inhibitors suppress the base excision repair pathway and thus, force the cell to repair damaged DNA by HR. Similar to BRCA1 and BRCA2 deficient cells, when FA-A cells were treated with PARP inhibitors they demonstrated an increase in cell death compared to FA-A cells reconstituted with FANCA suggesting that FANCA is in fact

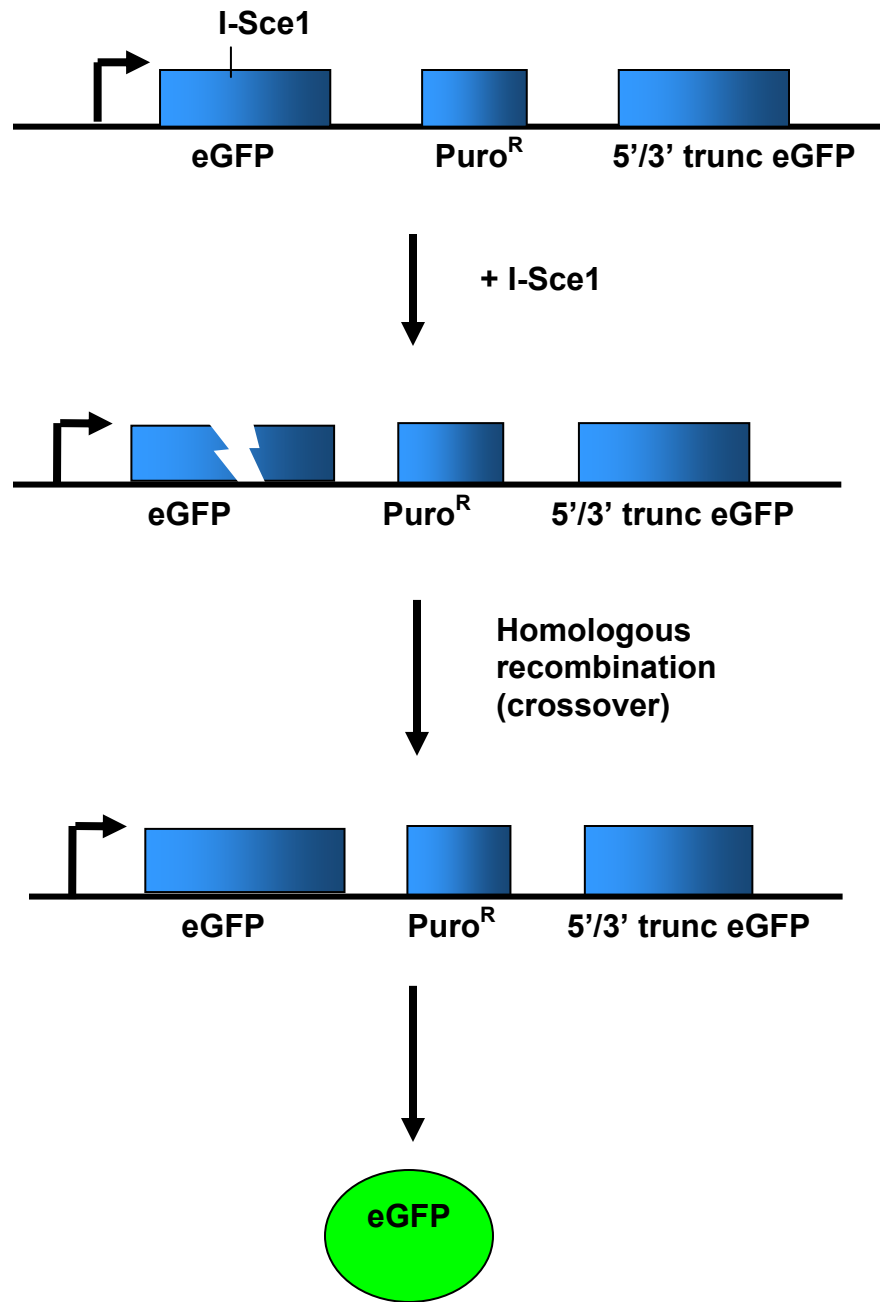
required for effective HR⁴⁷. Taken together, these findings suggest that the functions of the core complex proteins are not limited to FANCD2 monoubiquitination, but that they may extend to early stages of recombination.

To date, BRCA1, BRCA2/FANCD1, FANCD2, FANCG, FANCA, FANCC, and FANCI (see chapter 3) have all been implicated in the HR repair pathway and loss of any one protein demonstrated a decrease in the cell's ability to mediate repair by HR. In contrast, FANCC and FANCI have also been reported to have normal repair functions in DT40 chicken cells and FANCD2 cells have demonstrated both normal and aberrant repair in FA fibroblasts¹⁸. The inconsistencies in the data may be attributed to the diversity in cell type or the specific homology directed repair substrate used to assess repair functions. Although, there are discrepancies within the literature it appears that overall the FA proteins are critical for mediating some aspect of HR repair.

FA/BRCA proteins and cell cycle checkpoint

In addition to the role of the FA/BRCA proteins in DNA damage repair, they are also critical for maintaining normal cell cycle progression following DNA damage and in some cases, activating cell cycle checkpoints¹⁸. The IR inducible S-phase checkpoint is frequently characterized by observing the inhibition of replicative DNA synthesis following cellular exposure to IR. However, cells defective for this checkpoint fail to stop DNA synthesis after IR treatment and these cells are said to exhibit radio-resistant DNA synthesis, or RDS.

Figure 1-4: **Schematic representation of the DR-GFP homology directed repair substrate.** The DR-GFP substrate is one example of a reporter gene that is commonly integrated into mammalian cell lines to analyze the role of DNA damage response proteins in HR (see chapter 2 for details).



For example, cells from several FA complementation groups display a substantial RDS phenotype including FANCA, BRCA2/FANCD1, and FANCD2^{27,48}. Similarly, FANCI and BRCA1 deficient cells also display an RDS phenotype⁴⁹. The function of FANCD2 in the intra-S-phase checkpoint has been further detailed by mutational analysis. RDS assays using FA-D2 fibroblasts reconstituted with FANCD2 phosphorylation mutants, have demonstrated that serine 222, serine 717, and threonine 691 are all critical for activation of the S-phase checkpoint²⁷. Although other FA complementation groups have not been specifically tested in RDS assays, FA proteins were shown to accumulate on chromatin during replication initiation and in response to replication induced DNA lesions in *Xenopus* extracts⁵⁰ supporting a role for these proteins in regulating S-phase progression.

FA cells have also been characterized by an ICL-induced cell cycle progression defect⁵¹. In response to ICL damage, FA cells undergo growth arrest and a pronounced accumulation of cells with 4N DNA content. Reconstitution of FA cells with the appropriate FA complement restores cell growth and drastically reduces the 4N accumulation, substantiating the role of the FA proteins in maintaining normal cell cycle⁵². The true mechanism of this accumulation remains unclear; however, it may represent a failure of FA cells to cease DNA synthesis in S-phase after ICL damage.

Consequently, these cells do not have a chance to repair damaged elements until they reach G2/M and thus, show a pronounced 4N accumulation. Defective inhibition of DNA synthesis following ICL treatment has been demonstrated in FA-A, FA-B, FA-C, FA-G, FA-D2 cells⁵³⁻⁵⁵. However, it has also been suggested that the observed

accumulation at 4N may truly represent a delay in exit from S-phase and not a defect in the intra-S-phase checkpoint⁵⁶ (see chapter 4). FA cells may coordinate the completion of DNA repair and exit from S-phase, thus a delay in repair may also delay exit from S-phase^{52,57}.

Although the FA/BRCA proteins are required for normal cell cycle progression following DNA damage, it is unclear whether this progression is regulated by activation of specific cell cycle checkpoints or dictated by the repair process, or both. To further understand how FANCD1 may contribute to these functions we sought to identify FANCD1 interacting proteins. Notably, we found that FANCD1 formed a stable interaction with the mismatch repair protein (MMR), MLH1. The MMR proteins, including MLH1, have been implicated in maintenance of both cell cycle progression and the stringency of homology directed recombination. Just as in FA, mutations in MMR genes are closely associated with the development of cancer.

The link between the Mismatch repair pathway and cancer

Mutations in the MMR genes account for the majority of hereditary nonpolyposis colon cancer (HNPCC) and also contribute to the development of spontaneous colorectal cancers. HNPCC is an autosomal dominant disorder, generally resulting from the inheritance of one mutant allele and somatic mutation of the remaining allele. Similar to FA, MMR deficiency can be multigenic and mutations in 5 of the 6 genes have been identified in colon cancer patients⁵⁸⁻⁶². Interestingly, patients with mutations in one of the MMR genes, PMS2, display phenotypes similar to that of FA patients including café-

au-lait skin patches, childhood onset hematological cancers such as leukemia and solid brain tumors including, astrocytomas and glioblastomas⁶³.

Cells from patients with mutations in the MMR genes demonstrate microsatellite or genomic instability. Microsatellites are short repetitive sequences within the genome and are themselves non-threatening, however, during replication they are susceptible to slippage errors by the polymerase enzyme¹³. The MMR proteins are critical in the repair of these errors and if they are left unrepaired the microsatellite can become shorter or longer in length. Thus, MMR deficient cells are prone to generate microsatellite instability. This phenotype has been instrumental in genetic screens for patients with tumors suggestive of HNPCC, as approximately 90% of HNPCC tumors are positive for microsatellite instability⁶⁴.

In addition to surveying DNA for mismatches during replication, the MMR pathway recognizes methyl adducts incorporated into DNA by chemical agents such as MNU, MNNG, TMZ, and 6TG. Following cellular exposure to methylating agents the replication machinery creates GT mismatches (and other mismatches to a lesser extent) and triggers activation of the MMR pathway⁶⁵. Unfortunately for the cell, the O⁶ MeG is repeatedly paired with a T, resulting in a futile cycle of repair, activation of the G2 checkpoint, and induction of apoptosis. Therefore, MMR proficient cells are sensitive to methylation agents. However, MMR deficient cells have been characterized by their unique resistance to methylating agents and researchers have hypothesized that this resistance may be attributed to defects in damage recognition, G2 arrest, and/or induction of apoptosis⁶⁶.

MMR pathway activation

The MMR pathway can be divided into 4 steps, recognition, recruitment, excision, and resynthesis and is composed of the MSH2, MSH3, MSH6, MLH1, MLH3, PMS1 and PMS2 proteins. Similar to the FA/BRCA pathway, several subcomplexes within the human MMR pathway have been identified and include the MSH2/MSH6 heterodimer (MutS α), MSH2/MSH3 (MutS β), MLH1/PMS2 (MutL α), MLH1/PMS1 (MutL β), and MLH1/MLH3 (MutL γ). It's important to note that although two MutS and MutL subcomplexes exist approximately 90% of MSH2 exists as MutS α and similarly, 90% of MLH1 exists as MutL α ⁶⁵. It is believed that misincorporation or insertion loops arising during DNA replication are recognized and bound by MutS α resulting in activation of the MMR pathway. The MutS α mismatch-containing complex is then bound by the MutL α heterodimer together the complex traverses the DNA in an ATP dependent manner. This function may be critical for differentiating between the template strand and the nascent strand at the site of a mismatch. In *E. coli* the template strand is methylated and the MMR machinery exploits this characteristic to differentiate between the two strands and remove the incorrect base from the newly synthesized strand. In mammalian cells it is hypothesized that the MutS α /MutL α complex translocates along the DNA and the newly synthesized strand is discriminated from the template strand by the presence of Okazaki fragments. Additionally, MutL α has also been shown to both displace DNA polymerase and PCNA at sites of DNA synthesis and also recruit the exonuclease, EXO1, in the presence of a mismatch. Recent data supports bi-directional activity of EXO1 in either a 5'-3' or 3'-5' polarity depending on the location of the

mismatch relative to the nick, or potentially, the Okazaki fragment. EXO1 excises the nascent strand of DNA and the single stranded binding protein, RPA, stabilizes the single stranded region of the template strand for resynthesis. Finally, the gap in the nascent strand is resynthesized and the mismatch is corrected. Although, the exact polymerase responsible for this resynthesis remains unclear, Pol δ , Pol α , Pol ϵ , and Pol η have all been implicated (Figure 1-5) ⁶⁷.

In addition to misincorporation during DNA synthesis, several DNA methylators including MNU, MMNG, TMZ, and T6G activate the MMR pathway. In MMR proficient cells MNU adducts such as O⁶ MeG are removed by the MGMT enzyme. However, genetic analysis has shown that this gene is silenced by promoter hypermethylation in sporadic colon cancers resulting in the persistence of DNA methyl adducts ¹³. In vitro data has demonstrated that the MutS α complex is capable of both binding DNA methyl adducts and triggering the MMR pathway. Unfortunately, the persistence O⁶ MeG adducts causes constant misincorporation and it is this process, repeated excision and inaccurate resynthesis, that results in the futile cycle of repair ultimately leading to cell death ¹³.

MMR proteins and DNA damage repair

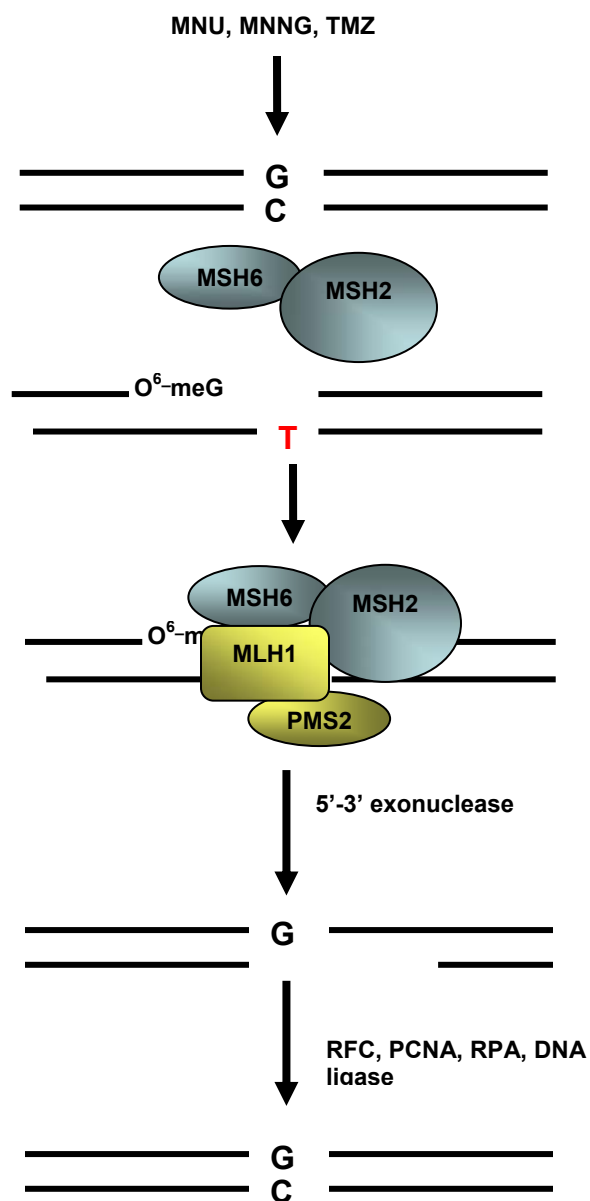
The MMR pathway is responsible for correcting DNA mismatches that arise not only during DNA synthesis, but also during genetic recombination. Specifically, the MMR proteins inhibit recombination between divergent sequences in a process called homeologous recombination. During recombination the MMR proteins are presumably

activated by the formation of heteroduplex DNA between two homologous DNA strands. When this recombination intermediate contains low sequence divergence MMR proteins process the base errors as they would if the mismatch were encountered during DNA replication. However, if the heteroduplex DNA contains highly divergent sequences the MMR proteins function to disrupt the recombination intermediate inhibiting error-prone recombination. Thus, loss of the MMR pathway causes an increase in homeologous recombination and can result in chromosomal rearrangements and/or translocations compromising genetic integrity and predisposing cells to tumor formation⁶⁶. Although the exact mechanisms for suppressing homeologous recombination are unclear several of the human MMR proteins have been implicated in this antirecombination process including MSH2, MSH3, and MSH6⁶⁸. In fact, gene targeting experiments demonstrated both MSH2^{-/-} and MSH6^{-/-} murine embryonic stem cells had a substantial increase in recombination between heterologous sequences as compared to the recombination frequencies in control cells⁶⁹⁻⁷¹.

MMR proteins and checkpoint

Similar to cells defective for FA proteins, cells deficient in the MLH1 and MSH2 MMR proteins also show a defect in the intra-S-phase checkpoint as demonstrated by RDS experiments. More specifically, loss of either protein results in both disruption of CHK2 mediated DNA damage signaling and degradation of CDC25A following treatment with IR.

Figure 1-5: **Cartoon illustration of the Mismatch Repair Pathway.** Following methylation damage and incorporation of a mismatch the MSH6/MSH2 heterodimer recognizes the mismatch and recruits the MLH1/PMS2 heterodimer. This complex then recruits the EXO1 exonuclease and the incorrect base is removed and replaced with the Watson-Crick base to restore the original sequence.



MMR deficient cells do not demonstrate decreased cell survival when exposed to IR suggesting that the cell cycle defects may be attributed to defects in signal transduction pathways ⁷².

Research has also shown that cells defective for the MMR proteins fail to activate the G2 cell cycle checkpoint following exposure to methylating agents ^{73,74}. This checkpoint is only activated following the second progression through S-phase, further supporting the hypothesis that checkpoint activation appears to require signaling and/or processing of intermediates, not simply damage recognition. Additionally, activation of the G2 checkpoint requires MLH1, ATR, and CHK1 proteins. Although controversial, it has been speculated that inactivation of the G2 checkpoint in MMR deficient cells may actually contribute to the methylation resistant phenotype these cells display ⁷⁵.

CONCLUDING REMARKS

It is clear that the DNA damage response is a complex network of pathways coordinating the reversal and/or removal of DNA damage to maintain genomic stability and ultimately, suppress cancer development. Our efforts were initially focused on deciphering the specific role of the FANCD1 helicase in the DNA damage response pathway. However, subsequent findings uncovered a novel connection between the FA/BRCA and MMR pathways. Although it was known that both FA/BRCA and MMR pathways were crucial components of the DNA damage response, the relationship between the two pathways had been previously undiscovered. Thus, it became our goal

to understand whether FA/BRCA and MMR pathways were coordinated in their DNA damage repair and genomic maintenance functions.

CHAPTER II

BACH1 is a DNA repair protein supporting BRCA1 damage response

Abstract

The link between defects in BRCA1 and breast cancer development may be best understood by deciphering the role of associated proteins. BACH1 interacts directly with the BRCA1 C-terminal BRCT repeats, which are important for BRCA1 DNA repair and are mutated in the majority of BRCA1 familial cancers. Thus, BACH1 is a likely candidate for mediating BRCA1 DNA repair and tumor suppression functions. Although previous evidence using overexpression of a dominant negative BACH1 has suggested that BACH1 is involved in BRCA1-DNA repair function, our results using BACH1 deficient cells provide direct evidence for involvement of BACH1 in DNA repair as well as for localizing BRCA1. Following DNA damage BACH1 is modified by phosphorylation, displays a BRCA1-like nuclear foci pattern and co-localizes with γ -H2AX. Given that the BACH1/BRCA1 complex is unaltered by DNA damage and the intensity of BRCA1 foci is diminished in BACH1 deficient cells, BACH1 may serve to not only facilitate DNA repair, but also maintain BRCA1 in DNA damage foci.

Peng*, M., R., Litman*, Z. Jin, G. Fong and S.B. Cantor. BACH1 is a DNA repair protein supporting BRCA1 damage response. *Oncogene* (2006) **25**, 2245–2253.

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Introduction

The breast cancer susceptibility gene, BRCA1, is a nuclear phosphoprotein with an N-terminus that contains a zinc finger RING domain, and a C-terminus that contains two BRCT (BRCA1 C-Terminal) repeats. BRCA1 has many protein partners and numerous ascribed functions including ubiquitin ligase activity,^{76,77} transcriptional activity,^{20,78-80} DNA repair, cell cycle control, and chromatin remodeling functions⁸¹. However, it is not certain which protein partners contribute to BRCA1 function(s) and it is not clear which BRCA1 function(s) contribute to BRCA1 tumor suppression.

BRCA1 is phosphorylated after genotoxic stress in a manner dependent upon the DNA damage signaling proteins ATM, ATR, Chk1, and Chk2⁸²⁻⁸⁵. During S-phase or after treatment with DNA damaging agents, BRCA1 is in nuclear foci with several other DNA repair proteins including BRCA2, Rad51, and NBS1/Mre11/Rad50 complex^{36,86-88}. Recruitment and localization of BRCA1 in these foci is dependent on phosphorylation of the histone variant, H2AX-phosphate (γ -H2AX)⁸⁷ and the MDC1 protein⁸⁹. In the absence of functional BRCA1 cells exhibit spontaneous and induced chromosomal aberrations, are sensitive to DNA damaging agents, and demonstrate loss of cell cycle checkpoints²⁰. Interestingly, BRCA1 mutations that affect the ability of BRCA1 to localize in nuclear foci also affect the ability of BRCA1 to mediate an effective DNA damage response⁹⁰ suggesting that BRCA1 foci formation is critical for BRCA1 function.

Evidence suggests that the C-terminus of BRCA1, including the BRCT domains, is important for BRCA1 focus formation^{90,91}. The integrity of the BRCA1 BRCT

domains has also been shown to be critical for both BRCA1 DSB repair function and HR^{21,37,92,93}. Moreover, nearly all BRCA1 germline mutations involve either truncation or loss of the C-terminal BRCT domains⁹⁴. These data suggest that BRCA1 DNA repair and tumor suppression functions are linked and highlight the importance of the BRCT domain.

Proteins that bind the BRCT domains may participate in some or all of these BRCA1 BRCT domain specific activities including BRCA1 focus formation, DSB repair, HR, and tumor suppression. In particular, a candidate mediator of BRCA1 function is BACH1, which was identified by its direct binding to the BRCT domains⁵. This binding was shown to be dependent on BACH1 phosphorylation at serine 990 and critical for BRCA1 to mediate the G2/M checkpoint^{95,96}. BACH1 makes physical contacts with residues located at the interface of the both BRCTs^{90,97,98}, substantiating the finding that multiple clinical BRCT mutations disrupt BRCA1 binding to BACH1.

We hypothesized that BACH1 is critical to mediate BRCA1 DNA damage response given its direct binding to BRCA1 and its helicase function. In fact, overexpression of a BACH1 helicase mutant disrupted the kinetics of DSB repair in a BRCA1 binding dependent manner⁵. In addition, BACH1 helicase activity may also contribute to BRCA1 tumor suppression function given the identification of hereditary breast cancer patients with helicase disrupting mutations in BACH1⁶. In this report, we provide evidence that BACH1 not only participates in the DNA damage response, but also supports BRCA1 localization in DNA damage foci.

Results

Resolution of DSB is delayed in BACH1 deficient cells

To directly test whether BACH1 function is required for efficient repair, we evaluated the ability of BACH1 deficient cells to repair IR induced DNA breaks. We suppressed BACH1 protein expression with an shRNA vector targeting BACH1⁹⁹. Infection of MCF7 or SKOV3 cells with either vector led to substantial suppression of BACH1 expression (Fig. 2-1a and b). However, shRNA directed against enhanced green fluorescent protein (eGFP) target sequence had no effect on the level of BACH1 protein expression (Fig. 2-1a and b). Moreover, the suppression of BACH1 expression with the shRNA vectors is stable for as many as four months in culture with no detectable alterations in BRCA1 levels or in levels of β -actin (Fig. 2-1a). IR induced DSBs can be visualized by scoring phosphorylation with an antibody (Ab) specific to the variant histone H2AX at serine 139 (γ -H2AX)¹⁴. Phosphorylation of γ -H2AX occurs rapidly after DSBs occur¹⁰⁰ and resolution of DSBs results in loss of γ -H2AX phosphorylation similar to the timing of resolution of DSBs imaged by pulse field gel electrophoresis¹⁰¹. Here, we use γ -H2AX foci to detect the presence of IR-induced DSBs. MCF7 cells were plated, either left untreated or treated with IR, collected at different time points post-IR, and prepared for immunofluorescence (IF) using a γ -H2AX Ab (Upstate). We quantified the kinetics of repair by scoring the percent of cells containing damage induced γ -H2AX foci over total DAPI positive cells at several time points after a sublethal dose of IR, .5 Gray (Gy). γ -H2AX irradiation-induced foci (IRIF) were detected initially 30 minutes (min) and 1 hour (hr) post IR at similar levels in both cells

stably expressing shRNA for BACH1 or eGFP control, suggesting that the accumulation of γ -H2AX at the sites of DNA breaks was not affected by BACH1 deficiency (Fig. 2-2a). However, BACH1 deficient cells maintained enhanced IR-induced foci at 4hrs, 6hrs and 8hrs (Fig. 2-2b) compared to control cells. In the BACH1 deficient cells, γ -H2AX foci were not only found in more cells but these foci were brighter and larger (Fig 2-2a). This enhanced γ -H2AX staining was reverted back to untreated cells 10-24 hrs post IR. These results suggest that there is a delay in the resolution of DSBs generated by IR in cells deficient in BACH1 protein expression, but that breaks are eventually repaired. Consistent with a delay in DSBR, the BACH1 deficient cells demonstrated mild radiation sensitivity upon treatment with IR in a four-day survival assay (Fig. 2-2c). Suppression of BACH1 with RNAi reagents in SKOV3 and 293T led to similar IR sensitivity compared to their respective control cells containing luciferase RNAi (data not shown).

BACH1 is phosphorylated after DNA damage and remains bound to BRCA1

The delay in DSBR detected in the BACH1 deficient cells is consistent with BACH1 functioning in a DNA damage repair. In order to further evaluate BACH1's role in DNA repair, we began by addressing whether BACH1 protein responded to DNA damage. First, we examined whether BACH1 demonstrated a post-DNA damage response similar to BRCA1^{82,83,102}. We hypothesized that if BACH1 was active in DNA repair similar to BRCA1, it would respond to DNA damage. We found that multiple forms of genotoxic stress previously shown to lead to BRCA1 induced phosphorylation⁸¹ resulted in the slower migration of BACH1 (Fig. 2-3a). The change in BACH1 migration

returned to its normal pre-damage migration as detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after treatment with serine/threonine phosphatases (λ Ppase) suggesting that the observed modification was a result of phosphorylation. The reversal in gel migration as a result of λ Ppase treatment occurred irrespective of the type of DNA damage (Fig. 2-3b). Moreover, inclusion of phosphatase inhibitors (λ Ppase inhib.) with the serine/threonine phosphatases prevented the return of BACH1 to its normal pre-damage migration further supporting the finding that DNA damage induced change in BACH1 migration was due to phosphorylation (Fig. 2-3b). The DNA damage induced phosphorylation appears to be both dose and time dependent. Maximum gel retardation is obtained by treating cells with 50 J/m² of ultraviolet light (UV), 1mM hydroxyurea (HU), or 20 Gy of IR, whereas 12 Gy of IR generates intermediate BACH1 migration that reaches maximal at 8 hrs post-IR (Fig. 2-3c). The post-DNA damage BACH1 gel retardation was present in multiple cell lines including HeLa, SKOV3, and 293T as well as in cells that lack functional BRCA1 (HCC1937 cells) (data not shown) indicating that this is not a cell specific event and that BRCA1 is not required for BACH1 post-damage processing. We next addressed whether DNA damage changed the equilibrium of the BRCA1-BACH1 complex. Immunoprecipitation (IP) experiments followed by Western blot analysis demonstrated that co-precipitating BACH1/BRCA1 proteins were not noticeably changed by DNA damage (Fig. 2-3c). Quantification of the percent BRCA1 in a BACH1 IP showed that the percentage of BRCA1-bound BACH1 was unchanged at 15 min to 8 hrs post-IR (Fig. 2-3c) suggesting that neither DNA damage nor the DNA damage-induced phosphorylation of BRCA1 or

BACH1 affected the complex. Consistent with this finding, phosphorylation of BACH1 at S990, the site critical for BRCA1 binding, is not changed after DNA damage (data not shown). Moreover, co-localizing BRCA1 and BACH1 nuclear foci are easily detected before and after IR (Fig. 2-3d).

BACH1 and γ -H2AX foci co-localize after DNA damage

To better assess the role of BACH1 in the DNA repair response, we addressed if and when BACH1 localized to sites of DNA damage by testing whether BACH1 IRIF co-localize with sites of DSBs as has been shown for BRCA1⁸⁷. MCF7 cells irradiated with 12Gy IR demonstrated BACH1 IRIF pattern that overlapped with the γ -H2AX IRIF pattern. Irradiation-induced co-localization of BACH1 and γ -H2AX was detectable from 5min to 24hrs post-IR (Fig. 2-4). These results suggest that BACH1 response to IR is similar to the published BRCA1 response⁸⁷ in that BACH1 and BRCA1 IRIF diffuse and reform with similar kinetics and both proteins form early and late IRIF co-staining with γ -H2AX. In addition, the pattern of BACH1 and BRCA1 foci was similar after DNA damage (Table 2-1). As reported previously, we found that the BRCA1 IRIF were disrupted within 1 hr and then gradually reassembled into bright foci¹⁰³. By 6 to 8 hrs after IR 70% to 90% of nuclei were positive for BRCA1 IRIF and remained until 12 hrs⁸⁸. BACH1 foci pattern resembles the BRCA1 foci pattern overall. In untreated cells BACH1 and BRCA1 foci are 35.9% and 36.5% respectively and BACH1 and BRCA1 IRIF reach maximum 6 hrs post-IR at 78.9% and 75.3% respectively (Table 2-1).

BACH1 deficient cells demonstrate reduced BRCA1 foci

Since loss of BACH1, leads to enhanced cellular sensitivity to DNA damage and defects in DSBR kinetics, two consequences of BRCA1 deficiency, we reasoned that this result could be due to loss of BRCA1 at sites of DNA damage. In fact, BACH1 has been proposed to localize BRCA1 into nuclear foci given the finding that BRCT mutations in BRCA1 disrupt both BACH1 binding and BRCA1 nuclear foci formation^{90,98}. These results are complicated by the fact that these BRCT mutations may also disrupt the binding of other BRCT interacting proteins like CTIP or RNA helicase A¹⁰⁴, making it unclear whether BACH1 is truly directing BRCA1 to foci or whether other BRCT interacting proteins are directing BRCA1 to foci.

To directly examine the contribution of BACH1 to BRCA1 localization, we analyzed BRCA1 foci formation in BACH1 deficient cells. BRCA1 foci were present, but appeared less intense overall in BACH1 deficient cells compared to control cells while γ -H2AX signal was unaltered (Fig. 2-5a). Nevertheless, these BRCA1 foci present in the BACH1 deficient cells colocalized with γ -H2AX IRIF (Fig.2-5a). The reduced BRCA1 foci present in BACH1 shRNA containing cells was not due to a decrease in BRCA1 protein levels (Fig. 2-1a) and BRCA1 post-damage phosphorylation was unchanged (Fig. 2-5b). Thus, we reasoned that the reduced BRCA1 staining was not due to changes in BRCA1 expression, we hypothesized that BACH1 deficiency may effect the kinetics of BRCA1 localization in IRIF. Cells expressing shRNA for BACH1 or eGFP control (Fig. 2-5c) were not treated or treated with low-dose IR of 2.5Gy, a sub-lethal dose that generates DNA damage induced BRCA1 and γ -H2AX IRIF immediately

post-IR. Cells were collected at the indicated time-points post-IR and stained with BRCA1 and γ -H2AX Abs (Fig. 2-5c). As before, a visible reduction in BRCA1 staining was detectable in all BACH1 deficient cells (Fig. 2-5d). In addition, a measurable reduction in cells positive for BRCA1 foci (> 10 foci/ cell) was clearly detected from 8-30 mins post-IR (Fig 2-5c and d). Although complete recovery in BRCA1 foci intensity was not achieved in BACH1 deficient cells, the number of cells positive for greater than 10 BRCA1 foci per cell was similar to control cells by 1hr post-IR (Fig. 2-5d). Together these results suggest that BRCA1 foci are dependent on BACH1 at early times post-IR for nucleation at sites of DNA damage and for maintenance of BRCA1 foci.

To confirm our finding that BACH1 deficiency disrupted normal BRCA1 foci formation, BRCA1 foci were characterized in the BACH1 deficient cell line, EUFA30-F. This cell was derived from an FA patient previously assigned to the FA-J complementation group³³. Recently, several groups have identified the gene defect in the FA-J complementation group as BACH1^{99,105-107}. The FA-J cells, EUFA30-F, have a homozygous point mutation in the BACH1 gene encoding a premature stop codon resulting in undetectable BACH1 protein expression. Re-introduction of WT BACH1 corrects the post-DNA damage G2/M accumulation in these cells⁹⁹. Here, we have also re-introduced FA-J cells with either WT BACH1 or vector alone using lentiviral vectors⁹⁹. Cells stably expressing the vectors were drug selected and analyzed by Western blot to confirm BACH1 expression (Fig. 2-6a). To investigate whether BACH1 is important for BRCA1 foci formation in these cells, we analyzed FA-J cells reconstituted with WT BACH1 or vector alone for the ability of these cells to form BRCA1 foci. FA-J cells with

BACH1 or empty vector were seeded on coverslips, either left untreated or treated with 2.5Gy IR, and cells positive (> 10 foci/cell) for BRCA1 foci were counted. The cells reconstituted with WT BACH1 had a greater percentage of cells positive for BRCA1 foci than the cells reconstituted with empty vector alone, both before and after DNA damage. Approximately, 50% of FA-J cells reconstituted with WT BACH1 were positive for BRCA1 foci, however, only 25% of the FA-J cells reconstituted with the empty vector scored positive for BRCA1 foci (Fig. 2-6b untreated). Following exposure to IR, both WT BACH1 and empty vector FA-J cells contained BRCA1 foci that colocalized with γ -H2AX suggesting that BACH1 is not required for BRCA1 colocalization with γ -H2AX (Fig. 2-6c). However, WT BACH1 reconstituted cells maintained more detectable BRCA1 foci at all time points tested (Fig. 2-6b and c) up to 24hrs following IR treatment (data not shown). Perhaps the FA-J cells, unlike the shRNA BACH1 containing cells, have a greater disruption in BRCA1 foci formation due to the total lack of BACH1 protein in the FA-J cells. Thus, similar to BACH1 deficient cells established by RNAi reagents, BACH1 mutant cells also demonstrate defects in the number and intensity of BRCA1 foci.

Discussion

In this report we have shown that BACH1 deficient cells are sensitive to DNA damage and are delayed in the repair of DSBs. BRCA1 foci formation is not only diminished in BACH1 deficient cells, but also BRCA1 IRIF formation is delayed.

BRCA1 eventually localizes with γ -H2AX at sites of breaks suggesting that BACH1 only contributes to recruitment of BRCA1 in IRIF or serves to maintain BRCA1 in IRIF.

Our findings demonstrate that BACH1 is a DNA repair protein and suggest that BRCA1 and BACH1 functions are linked. The same DNA damage-signaling pathways that modify BRCA1 also modify BACH1. Similar to BRCA1, BACH1 post-DNA damage modification is most likely phosphorylation since λ Ppase treatment of post-DNA damage BACH1 IPs led to a reversion of BACH1 post-DNA damage slower gel migration to a pre-DNA damage gel migration. Second, similar to BRCA1, BACH1 IRIF form immediately after DNA damage and maximum IRIF is obtained at 6hrs post-IR. Third, BACH1 mimics BRCA1 post-damage colocalization with γ -H2AX. Given that BACH1 DNA damage response mimics BRCA1 DNA damage response, it is not surprising that BACH1 binding to BRCA1 is unaltered by DNA damage.

While BRCA1 binding to BACH1 may be important for localization of BRCA1 in foci,^{90,98} BACH1 may only contribute to BRCA1 localization since both N-terminal and C-terminal portions of BRCA1 have been shown to be required for BRCA1 localization⁹¹. Our results in BACH1 deficient cells suggest that BACH1 contributes to BRCA1 localization, but is not ultimately required. We predict that BACH1 serves to maintain BRCA1 in IRIF and not directly recruit BRCA1 to IRIF. Unlike MDC1 deficient cells in which BRCA1 foci do not form in the absence or presence of DNA damage⁸⁹, in BACH1 deficient cells BRCA1 foci have reduced intensity, but are present. Moreover, BRCA1 IRIF reform in BACH1 deficient cells by 1hr post-IR. In contrast to BACH1 deficient cells in which BRCA1 foci returned by 1hr post-IR, in FA-J cells BRCA1 foci

did not fully return even at later times post-IR. This difference may result from residual BACH1 expressed in the BACH1 RNAi treated cells compared to the FA-J cells used in this study, which have no detectable BACH1 protein⁹⁹. Alternatively, differences in the cell type or transformation state of the transformed MCF7 cells compared to immortalized FA-J fibroblasts may influence the BRCA1 foci formation. Finally, BRCA1 appears to be appropriately modified in BACH1 deficient cells following IR unlike in MDC1 deficient cells in which BRCA1 post-IR modification is disrupted⁸⁹. Perhaps the mild radiosensitivity found in BACH1 deficient cells reflects the finding that BACH1 deficient cells are capable of eventually localizing BRCA1 and repairing breaks. BACH1 may unwind the DNA in the vicinity of the DNA damage and facilitate access of BRCA1 to these sites.

Previous results demonstrated that BACH1 is absent from nuclear foci in cells devoid of intact BRCA1 suggesting BRCA1 is upstream of BACH1 in the DNA damage response pathway. In these same cells, BACH1 foci will form when WT full-length BRCA1 is reconstituted⁵. One explanation for these findings may be that BRCA1 serves as a scaffold for which multiple repair proteins are localized and stabilized at the sites of breaks. Thus, in the absence of BRCA1, BACH1 and other repair proteins fail to form distinct repair foci. BACH1 may in turn stabilize BRCA1 at these sites. BRCA1 is a dynamic protein that relocates after DNA damage and associates with multiple repair proteins in multiple complexes. In doing so, BRCA1 may cycle between bound and unbound sites of DNA damage, replication, or transcription to mediate multiple functions. If BACH1 participates in maintaining BRCA1 at any of these sites, BRCA1

focus formation would be reduced overall as we detected. In addition, after low dose IR, BRCA1 IRIF would be reduced, but not impaired if BACH1 participates with other repair proteins to maintain BRCA1 binding to sites of DNA damage in BACH1 deficient cells.

The direct and highly specific interaction between BACH1 and BRCA1 suggests that they are functionally linked. Consistent with this idea, our findings show that BACH1 resembles BRCA1 post-DNA damage response in its localization pattern, immediate presence at sites of DNA damage, and in the DNA-damage induced phosphorylation pattern. Moreover, our findings suggest that BACH1 function is required for the timely arrival of BRCA1 into DNA damage foci, radioresistance, and for normal kinetics of DSBR. Recently, we demonstrated that BACH1, similar to BRCA1, is required for repair of DSBs by HR, MMC resistance, and for maintenance of chromosomal integrity⁹⁹. In addition, BACH1 is the gene defect in the FA-J complementation group of the FA disease in which patients are plagued by leukemia and sarcomas¹⁰⁸. This finding clearly demonstrates that BACH1 is a tumor suppressor gene. The next critical question is whether the BACH1-BRCA1 interaction is required for these mutual repair functions, whether BACH1 connects BRCA1 to the FA pathway, and whether ultimately BRCA1 and BACH1 tumor suppression functions are linked. Future experiments in which the BACH1-BRCA1 interaction is disrupted directly will clarify the importance of this complex formation for DNA repair and tumor suppression.

Figure 2-1: **BACH1 protein is suppressed.** A) Effects of siRNA on protein expression in MCF7 and SKOV3 cells are shown by Western blot, whole cell lysates (wce) (top) and BACH1 IPs (bottom). B) MCF7 cells containing shRNA for eGFP or BACH1 were stained with BACH1 Abs.

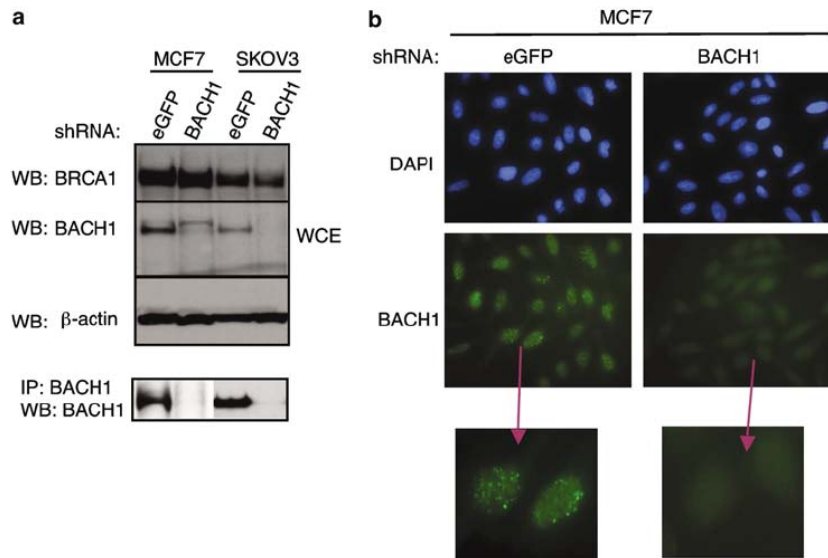


Figure 2-2: BACH1 deficient cells are delayed in DSBR and have sensitivity to IR.

A) MCF7 cells containing shRNA for eGFP or BACH1 were either left untreated or treated with IR (0.5 Gy) and were fixed at the indicated time points. Cells were then stained with γ -H2AX Ab. The arrow denotes a single cell positive for γ -H2AX (>10 foci per cell), which displays the variation in intensity of the γ -H2AX signal. B) The percent of MCF7 cells positive for γ -H2AX foci are indicated for each time point with standard deviation based on three independent experiments. Over 500 cells were analyzed from each time point. C) MCF7 cells containing shRNA for either eGFP or BACH1 were treated with increasing dose of IR. Cell growth was assessed 4 days later using the CellTiter glo viability assay (Promega). Each point represents an average of three data points from three independent experiments and a composite graph is shown.

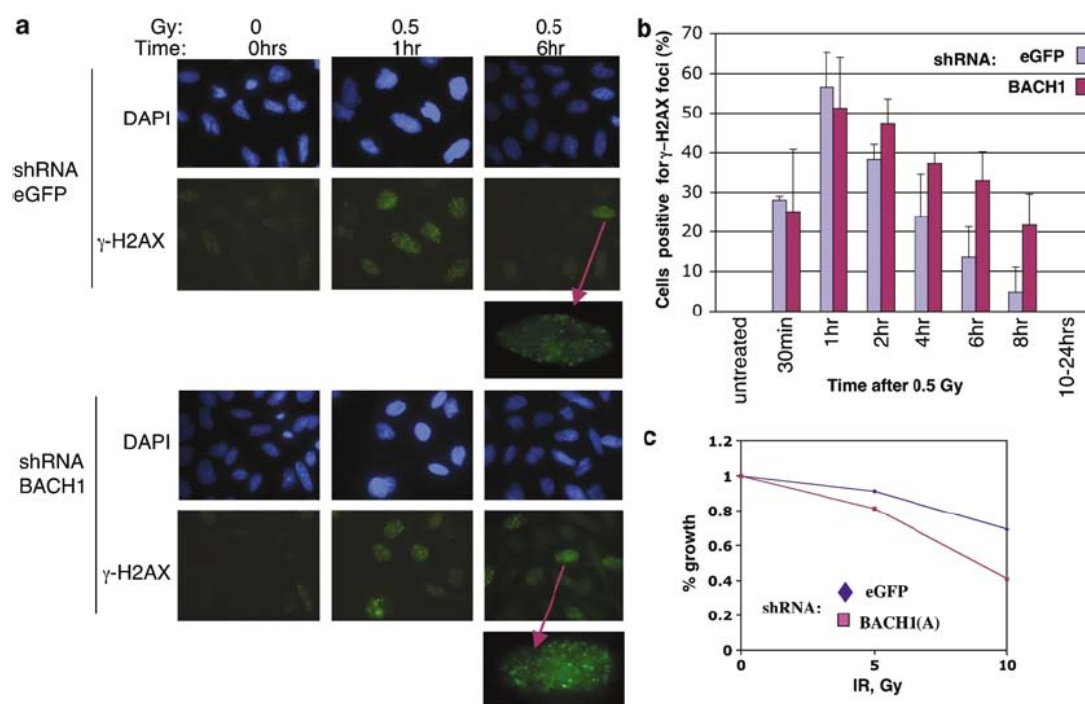


Figure 2-3: **BACH1 is modified after DNA damage.** A) MCF7 cells were either left untreated or treated with the indicated type of DNA damage. Protein mobility was analyzed by Western blot with BRCA1 or BACH1 specific Abs. B) MCF7 cells were either left untreated or treated with 50 J/m² UV and subsequently BACH1 IPs were treated with λ phosphatase and/or λ phosphatase inhibitors as indicated. (C) For quantitative analysis of the BRCA1/BACH1 complex, MCF7 cells were either left untreated or treated with 12Gy IR and collected 15min, 4hr, and 8hr post-IR. The percentage of BRCA1 bound to BACH1 was quantified using a LAS-3000 image reader and Image Gauge software. D) SKOV3 cells were either left untreated or treated with IR (12Gy), fixed 4 hrs later, and stained with anti-BACH1 and anti-BRCA1 Abs as indicated.

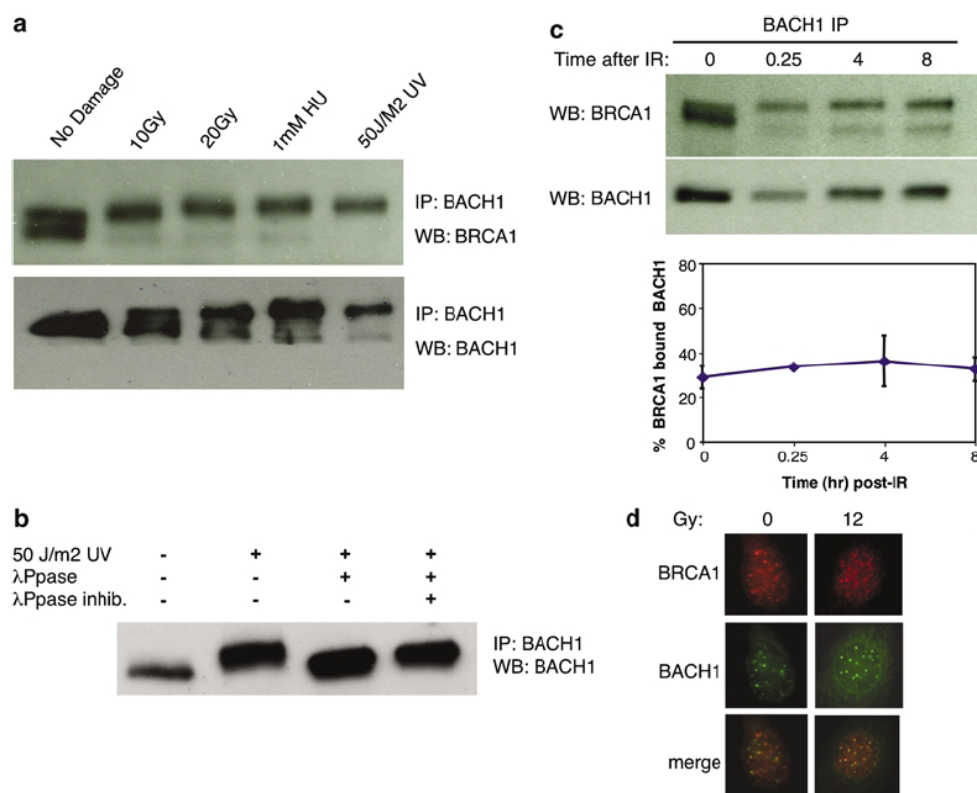


Figure 2-4: **BACH1 colocalizes with γ -H2AX after IR.** MCF7 cells were either left untreated or treated with IR (12Gy). At the indicated time points, cells were stained with anti-BACH1 and γ -H2AX Abs.

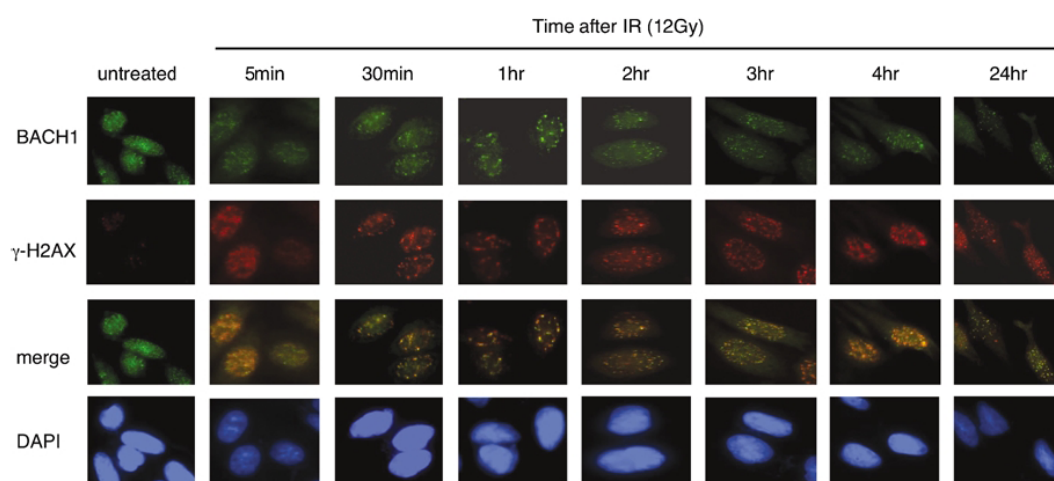


Table 2-1: Focus formation of BRCA1 and BACH1 after IR. MCF7 cells were left untreated or treated with IR (12Gy), allowed to recover for the indicated time, and stained with anti-BRCA1 or anti-BACH1 Abs. A cell nucleus displaying >10 BRCA1 or BACH1 foci was counted as a foci positive cell. At least 500 cells were analyzed for each time point, +/- represents the standard deviation from the mean.

Time after IR (hours)	Foci containing cells(%)	
	BRCA1	BACH1
0	36.5 +/- 5.6	35.9 +/- 4.9
1.0	17.7 +/- 3.7	24.8 +/- 2.9
2	44.7 +/- 5.1	43.2 +/- 6.5
4	60.2 +/- 5.4	60.6 +/- 8.6
6	75.3 +/- 7.0	78.9 +/- 6.1
12	69.0 +/- 6.6	52.0 +/- 6.8

Figure 2-5: BRCA1 foci are diminished in BACH1 deficient cells. A) SKOV3 cells containing shRNA for either eGFP or BACH1 were either left untreated or treated with IR (2.5Gy), were fixed 4hrs later, and stained with Abs for γ -H2AX and BRCA1. BRCA1 foci colocalize with γ -H2AX foci after DNA damage (merge). B) MCF7 cells containing shRNA for either eGFP or BACH1 were either left untreated or treated with IR (10Gy or 20Gy) and collected 1 or 3 hrs later, respectively. C) The percent of SKOV3 cells positive for BRCA1 foci are indicated for each time point with standard deviation based on three independent experiments. A cell nucleus displaying >10 BRCA1 foci was counted as a foci-positive cell. Over 500 cells were analyzed from each time point. D) Representative cell images from each time point are shown.

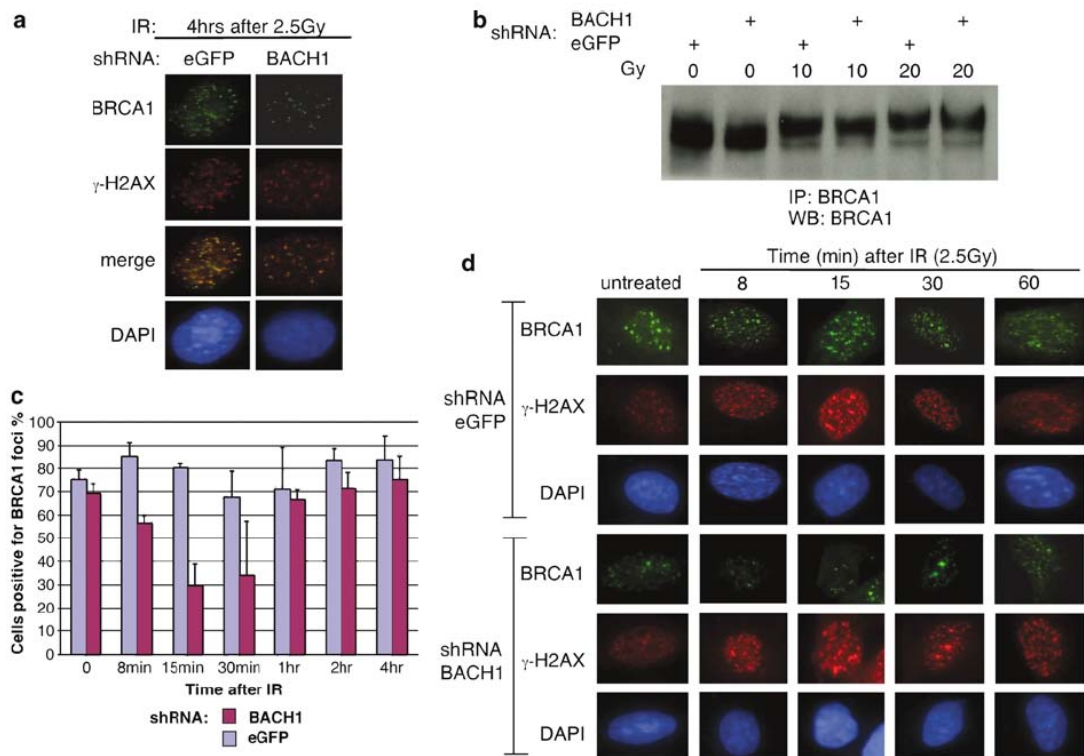
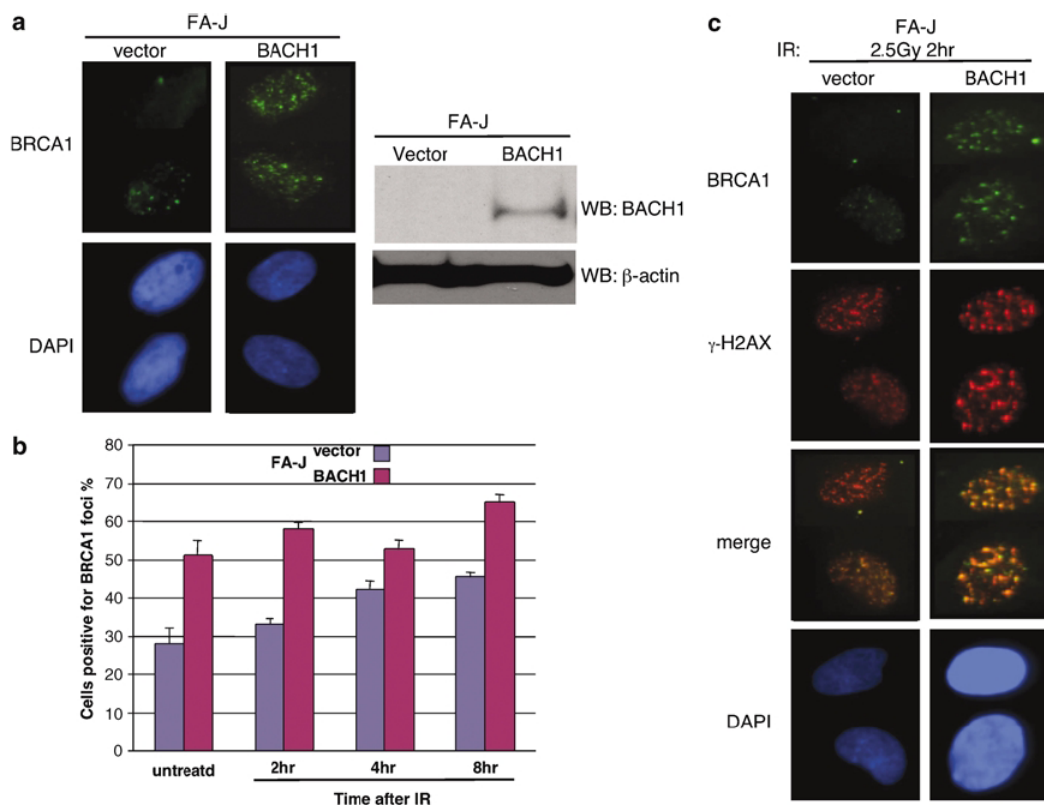


Figure 2-6: **BRCA1 foci are diminished in BACH1 null cells.** A) FA-J cells containing either empty vector or WT BACH1 were stained with BRCA1 Abs for IF or analyzed by Western blot using BACH1 and β -actin Abs. B) The percent of FA-J cells positive for BRCA1 foci are indicated for each time point with standard deviation based on three independent experiments. A cell nucleus displaying >10 BRCA1 foci was counted as a foci-positive cell. Over 600 cells were analyzed from each time point. B) Representative cell images from the 2hrs post IR time point are shown.



Materials and methods

Cell cultures

MCF7, SKOV3, and 293TD cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and antibiotics. FA-J fibroblasts (EUFA30-F)¹⁰⁶ containing either empty pLenti vector or WT BACH1 pLenti vector⁹⁵ were grown in DMEM (Gibco) supplemented with 15% FBS, 7 μ g /mL Blasticidin (Sigma) and antibiotics.

Antibodies

The monoclonal BACH1 Abs 2C10 and IG5 used for IF or as a monoclonal pool for Western blot⁵ and polyclonal Ab E67 was used as previously described⁶. The phosphospecific BACH1 serine 990 Ab (pser990) was generously provided by the Chen laboratory and was used as previously described⁹⁵. The monoclonal BRCA1 Abs SD118 and MS110 were used as previously described¹⁰³ and the polyclonal BRCA1 Ab was purchased from (Stressgen Bioreagents). The mouse monoclonal and polyclonal Anti-phospho-Histone H2AX (Ser139) Abs were purchased from Upstate. Rhodamine Red-X conjugated AffiniPure Goat anti-rabbit IgG and Fluorescein (FITC)-conjugated AffiniPure Goat Anti-mouse IgG were purchased from Code Jackson ImmunoResearch Laboratories Inc..

Immunoprecipitations and Western blot assays

Cells were harvested and lysed in lysis buffer (20mMTris[pH8.0],150mM NaCl, 1mMEDTA, 0.5%NP-40,1mM phenylmethylsulfonyl fluoride,10 μ g of leupeptin per ml, 10 μ g of aprotinin per ml) for 30 min on ice. The extracts were clarified by centrifugation for 10min at 13,200rpm. The cell lysates were incubated with 3 μ l E67, 30 μ l protein A beads (Amersham) for 2hr at 4°C and washed three times in IP washing buffer (20mMTris[pH8.0], 150mMNaCl, 1mMEDTA, 0.5%NP-40). IPs were boiled in SDS loading buffer and proteins were separated using SDS-PAGE. Proteins were electrotransferred to nitrocellulose membranes and Western blot assays were carried out with the indicated primary Abs over night at 4°C followed by horseradish peroxidase-conjugated secondary Abs for 1hr at room temperature. All blots were detected with ECL-plus Western Blotting Detection System (Amersham Biosciences) using Biomax MR or XAR film (Kodak). Bradford assays were used to determine protein content (Bio-Rad). For the DNA damage treatment experiments cells were treated with IR of 10 Gy, 20 Gy, 1mM HU, and 50J/M² UV and then collected 1, 3, 24, and 3 hrs later, respectively. Proteins were separated on 4% tris-glycine gels. λ -phosphatase inhibitors included sodium orthovanadate and sodium fluoride (NEB),

Cell survival assays

MCF7 cells were either untreated or damaged with 5 Gy or 10 Gy IR and plated at low density. A baseline reading was taken at the initial cell plating and total ATP was quantified as absorbance units after addition of Cell titer Glo reagent (Promega). Cells

were allowed to grow for 4 days and an endline reading was taken to quantify total cellular ATP, which was detected as absorbance units. Total absorbance units were used to assess relative viability.

Immunofluorescence

Cells were grown on coverslips in a 6 well plates. Before immunostaining, cells were irradiated or left untreated. At different time points, cells were fixed using 3%paraformaldehyde/2% sucrose in PBS for 10 min, and permeabilized with 0.5% Triton X-100. Cells were then incubated with both primary and secondary Abs in 6 well plates for 1hr at RT. Cells were washed five times for 5min each in PBS after incubation with both primary and secondary Abs. Coverslips were mounted using Vectashield with DAPI and sealed with nail polish, then stored in the dark at 4°C. IF was performed by an inverted Olympus 1X71 fluorescence microscope.

Retroviral or Lentiviral vectors and infection

The lentiviral shRNA expression vector FSIPPW, (eGFP) targets the sequence 5'-AAGAACGGCATCAAGGTGAACTT-3' and was described previously¹⁰⁹. The FSIPPW targets BACH1 sequence 5'-AAAGCUUACCCGUCACAGCUU-3'. The FSIPPW vectors were a generous gift of Dr. Andrew Kung. The WT BACH1 pLenti vector was a generous gift of Dr. Junjie Chen and Xiaochun Yu. 293TD retroviral packaging lines were used to produce all shRNA encoding lentivirus. 293TD cells were transfected with 2-4µg of plasmid DNA using FugeneTM (Roche) transfection reagent 48

hrs prior to harvesting retroviral or lentiviral supernatants. Supernatants containing 8 μ g/mL polybrene were added to recipient cell lines. Cells were transduced with lentivirus and were selected with either 2 μ g /mL of puromycin (Sigma) for cells containing FSIPPW shRNA vectors or 7 μ g /mL Blasticidin for cells containing pLenti vectors (Sigma).

Acknowledgements

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CHAPTER III

BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCF

Abstract

We show in this study that cells deficient of the BRCA1-associated BACH1 helicase fail to elicit HR after DNA DSBs. BACH1 deficient cells were also sensitive to MMC and underwent MMC-induced chromosome instability. Moreover, we identified a homozygous nonsense mutation in BACH1 in a FA-J patient-derived cell line and could not detect BACH1 protein in this cell line. Expression of wild type BACH1 in this cell line reduced the accumulation of cells at G2/M phases following exposure to DNA crosslinkers, a characteristic of FA cells. These results support that BACH1 is FANCF.

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Significance

A functional link between BRCA1 and FA proteins has been controversial. The putative identification of the BRCA1-associated helicase, BACH1, as FANCD1 suggests that BACH1 could link BRCA1 to the FA pathway. In addition to a role for BACH1 in breast cancer, we predict a wider role for BACH1 in disease mechanisms, including the bone marrow failure and predisposition to leukemia associated with FA. These results suggest that BACH1 may function as a FA gene product by a direct role in HR.

Determination of the mechanistic relationship of BACH1 to other FA gene products now becomes an important topic for future investigation.

Introduction

DNA repair pathways are critical for the maintenance of genomic stability and defects in these processes are frequently associated with cancer. For example, inheritance of germline mutations that affect one allele of either of the breast cancer susceptibility genes, BRCA1 or BRCA2, confers susceptibility to breast and ovarian cancer. In the absence of functional BRCA proteins, cells are sensitive to DNA damaging agents, and exhibit spontaneous and DNA damage-induced chromosomal aberrations²⁰. Additionally, numerous chromosomal fragility disorders are associated with cancer susceptibility¹⁰⁴. Among these is FA. FA is associated with chromosome instability, both spontaneously and in response to DNA crosslinkers such as MMC, and also with an increased risk of leukemia and other cancers¹¹⁰.

Unlike BRCA1 or BRCA2 associated breast cancer, FA is a multigenic disorder, with at least eleven distinct complementation groups ¹¹¹. Nine FA genes have been cloned, but FANCI and FANCIJ, which are associated with the FA-I and FA-J complementation groups, respectively, have remained unidentified ³³. The FA proteins are organized into a pathway in which a complex that includes seven of the identified FA gene products, FANCA, B, C, E, F, G, and L, are required for the monoubiquitination of the FA protein, FANCD2 ²³. Monoubiquitination of FANCD2 is required for resistance to MMC, maintenance of chromosome stability, and for the assembly of FANCD2 foci, which colocalize with proteins such as BRCA1 and Rad51 following exposure to DNA damage ^{23,27,112}. Neither, FANCD1, which is BRCA2 ²², nor FANCIJ are required for FANCD2 monoubiquitination, either with or without exposure to DNA damage ^{22,32,33}. Consequently, it has been suggested that BRCA2/FANCD1 and FANCIJ function downstream in the FA pathway, following activation of FANCD2 by monoubiquitination.

In addition to the identification of BRCA2 as the FA gene, FANCD1 ²², other observations suggest a potential relationship between BRCA and FA proteins. Among these, deficiency in either BRCA1 or BRCA2 results in hypersensitivity to MMC, similar to that observed in FA cells ^{22,113-115}. Also, BRCA1 is required for FANCD2 foci formation ²⁵, and BRCA1 was shown to immunoprecipitate (IP) with FANCD2 in DNA damaged cells ²³. Moreover, BRCA1 and BRCA2 colocalize with FANCD2 in DNA damage induced foci ^{23,116}. But, whether BRCA1 is required for DNA damage-induced FANCD2 monoubiquitination has been controversial ^{23,25}.

Several lines of evidence indicate that the BRCA and FA pathways influence HR-mediated DNA repair. BRCA1 and BRCA2 deficient cells are defective for DSB induced HR (reviewed in ^{20,81}). Likewise, in chicken DT40 cells, *fancec*, *fancg*, and *fancd2* mutants show reduced HR ^{44,117,118}. DNA damage that activates HR can be measured by the induction of Rad51 foci ¹¹⁹. Deficiency of BRCA1 or BRCA2 attenuates the DNA damage-inducible assembly of Rad51 foci ^{93,120}. It has been proposed that through monoubiquitination of FANCD2, FA proteins serve to stabilize broken replication forks making Rad51-mediated recombination more efficient ¹²¹. While the specific contribution of FA proteins to HR is not clear, it appears that FANCD2 monoubiquitination is required for efficient HR ^{35,43,111}.

BACH1, which was identified by its direct binding to the BRCA1 BRCT domains, was shown to participate in DNA DSB repair with BRCA1 ⁵. In this report, we have generated mammalian cells in which expression of BACH1 has been suppressed to better characterize the role of BACH1 in the DNA damage response. We provide direct evidence that BACH1 is critical for the repair of DNA DSBs by HR. BACH1 deficient cells are defective for HR, hypersensitive to MMC, and display increased levels of MMC-induced chromosomal instability, suggesting a potential relationship to the FA pathway. Indeed, we find that a FA-J cell line lacks detectable BACH1 protein and maintains a biallelic truncating mutation in BACH1. Further, we find that FA-J cells, like other FA cells display a pronounced G2/M accumulation following exposure to DNA interstrand crosslinkers ¹²²⁻¹²⁷, and this phenotype is corrected by expression of BACH1.

Together these results suggest that BACH1 is the FANCD2 gene functioning in HR downstream of FANCD2.

Results

HR is disrupted in BACH1 deficient cells

BACH1 function in DSBR was identified by the finding that overexpression of a helicase dead version of BACH1 delayed DSBR kinetics⁵. To more directly address the role of BACH1 in DSBR, we examined the biological consequence of BACH1 deficiency on the repair of DSBs by HR. Using a similar assay system in which BRCA1 has been shown to support HR³⁷, we examined the ability of BACH1 deficient cells to support HR in response to DSBs. We established an MCF7 cell line with an integrated copy of the pDR-GFP reporter as described previously¹²⁸. Puromycin-resistant cell lines were isolated and screened for the ability of an I-Sce1 induced DSB to generate HR. If HR occurs, GFP is expressed and GFP positive cells can be quantified by flow cytometry¹²⁸. Twenty-four hours after transfection with short interfering RNAs (siRNA) targeting BACH1 or BRCA1, the MCF7 DR-GFP cells were subsequently transfected with pCMV-I-Sce1. MCF7 DR-GFP cells transfected with luciferase (luc) siRNA then with pCMV-I-Sce1 were used as a control. Three days after I-Sce1 transfection, the cells were analyzed by flow cytometry to determine the percentage of green fluorescent cells relative to the total cell number. Transient transfection of the I-Sce1 endonuclease demonstrated that MCF7 DR-GFP cells exhibit I-Sce1 inducible HR, producing GFP positive cells quantified by flow cytometry (Figure 3-1A). The percentage of I-Sce1 induced GFP

positive cells was greatly reduced in cells previously transfected with siRNA to BRCA1 or with either of two different siRNA to BACH1 (A or B) than cells transfected with siRNA against luc. The I-Sce1 induction of GFP positive cells (~ 0.5%) was similar to what has been observed previously¹¹³. In contrast, the I-Sce1 induction in cells with BACH1 siRNA led to an average of ~0.048% cells that were positive for GFP, which is, ~10 fold lower than the GFP positive cells containing the luc control siRNA (average p-value .00051) (Figure 3-1B). The number of GFP positive cells were reduced to 0.07%, that is ~7-fold lower in cells with siRNA to BRCA1, as compared to luc controls (p-value .00029) (Figure 3-1B). The siRNA-mediated suppression of BACH1 and BRCA1 was specific to their respective target proteins whereas siRNA directed against luc had no effect on the levels of BACH1 or BRCA1 protein (Figure 3-1C). The overall transfection efficiency with pCMV-I-Sce1 was not affected by the siRNA transfection (data not shown). These results demonstrate that BRCA1 and BACH1 deficiency lead to a defect in DSB repair by HR.

BACH1 deficient cells are sensitive to MMC

The reduction in HR frequency in BACH1 deficient cells prompted us to test whether BACH1 deficient cells were also sensitive to MMC. MMC induces DNA interstrand crosslinks, which are repaired by a mechanism relying in part on HR¹²¹. To examine the effects of MMC treatment, we suppressed BACH1 protein expression with BACH1 siRNA. Previous reports have demonstrated that BRCA1 deficient cells are sensitive to MMC^{113,114}. Thus, we also suppressed BRCA1 protein expression with

siRNA to compare the effects of MMC on survival in BACH1 or BRCA1 deficient cells. Transfection of MCF7 cells with the BACH1 or BRCA1 siRNA led to substantial reduction of BACH1 or BRCA1 protein levels, respectively, as compared to control cells transfected with luc siRNA. Transfections with these siRNA did not induce detectable alterations of β -actin protein levels (Figure 3-2B). Photometric assays showed that BACH1 and BRCA1 deficient cells were extremely sensitive to MMC as compared to control cells (Figure 3-2A). Representative results from one of three independent experiments are shown in which the SF_{50} (dosage at which 50% of cells survived) for MMC was ~10-100 nM for BACH1 and BRCA1 -deficient MCF7 cells, as compared to 1000 nM for MCF7 cells transfected with luc siRNA. Similar MMC sensitivity results were obtained with suppression of BACH1 or BRCA1 in HEK cells (data not shown).

In order to determine whether MMC led to chromosomal abnormalities in BACH1 deficient cells, we compared BACH1 deficient and control cells treated with 100 nM MMC for four days. For these experiments, shRNA lentiviral vectors targeting BACH1 (A) or enhanced green fluorescent protein (eGFP), used as a control, were designed and used to infect HMEC cells. BACH1 protein level was efficiently reduced in cells expressing BACH1 shRNA (Figure 3-2D). BACH1 deficiency led to chromatid breaks and more complex chromatid aberrations such as triradial and quadriradial chromosomes. At the same dose, such aberrations were rarely seen in control eGFP shRNA HMEC cells (Figure 3-2C). These findings suggest that BACH1 deficiency leads to a failure to repair DSBs by HR. As suggested previously, other mechanisms to repair

DSBs such as single-strand annealing and/or non-homologous end joining most likely fail to maintain chromosome integrity^{129,130}.

DNA damage-induced FANCD2 monoubiquitination and Rad51 foci formation are intact in BACH1-deficient cells

Inactivation of BACH1 yields defects in HR, hypersensitivity to MMC, and increased MMC-induced chromosome instability similar to inactivation of FA genes^{35,110,117}. This suggested a possible relationship of BACH1 to the FA pathway, although inactivation of genes that have not been identified as FA genes, such as BRCA1 and Rad51, also result in similar defects^{113,114,131}. To pursue the relationship of BACH1 to the FA pathway, we investigated whether BACH1 function was required for FANCD2 monoubiquitination. FANCD2 monoubiquitination, as identified by a HU-induced increase in a form of FANCD2 which migrates slower by SDS-PAGE (FANCD2-L), was the same in BRCA1 and BACH1 deficient cells as in control cells infected with shRNA to eGFP (Figure 3-3A). Suppression of protein expression by shRNA is shown (Figure 3-3C). In contrast, FA-A (PD6914) cells do not support FANCD2 monoubiquitination unless reconstituted with the FANCA gene, as previously reported^{23,43}. FA-D2 cells served as a control for lacking FANCD2 protein²³ (Figure 3A). FANCD2 monoubiquitination was also intact in HeLa cells in which BACH1 was suppressed with siRNA (data not shown).

The formation of Rad51 foci is critical for efficient HR¹¹¹. DNA damage-induced assembly of Rad51 foci is defective in FANCD1 cells expressing C-terminally

truncated BRCA2^{32,132} and in BRCA1 deficient cells⁹³. Based on the finding that HR was defective in BACH1 deficient cells, we hypothesized that similar to BRCA1 deficient cells, DNA damage induced Rad51 foci would be diminished in BACH1 deficient cells. However, in response to HU we found that RAD51 foci were present in both BACH1 deficient cells and in control cells (eGFP). In contrast, Rad51 foci were not formed in cells deficient for BRCA1 treated with HU (Figure 3-3B). Similar results were obtained in MCF7 BACH1 siRNA cells treated with MMC, as well as in BACH1 deficient SKOV3 cells treated with HU (Supplemental Figure 3-6). The fact that Rad51 foci form in BACH1 deficient cells suggests that BACH1 functions independently of BRCA1 and potentially downstream of Rad51 (Figure 3-3B). In contrast, γ -H2AX foci form normally in both BRCA1 and BACH1-deficient cell lines following treatment with HU (Figure 3-3B). This suggests that the DNA damage response is initiated normally in the absence of BACH1.

FANCD2 cells lack detectable BACH1 protein expression and contain a biallelic BACH1 truncating mutation.

There are two FA complementation groups in which the gene defect is not currently known. Given our findings that BACH1 functions in the HR pathway and is likely to be downstream of FANCD2, we considered whether BACH1 is the gene defective in the FA-J complementation group. To test this possibility, we analyzed cell extracts from MCF7, hTERT immortalized normal (PD846F), or FA-J (AG656) fibroblasts for BACH1 protein expression. Unlike the MCF7 and normal cells, full-length

BACH1 was not detected in FA-J cells (Figure 3-4A). Moreover, IP (Immunoprecipitation) with two different BACH1 antibodies, or pull-down with the GST-BRCT fusion protein that binds directly to the serine 990 region of BACH1⁹⁵, failed to precipitate full-length BACH1 in the FA-J cells (Figure 3-4B). Western blot analysis using the I82 Ab, which was a polyclonal antibody generated using the full-length BACH1 protein, did not reveal any detectable BACH1 species in cell extracts or in IP experiments (data not shown). Consistent with the possibility that BACH1 is the gene defective in FA-J cells, FA-J cells maintained Rad51 and γ -H2AX foci after HU treatment (Supplemental Figure 3-6) similar to the MCF7 cells in which BACH1 was suppressed with shRNA (Figure 3-3B).

We then investigated whether BACH1 was mutated in the FA-J cells. RNA from the FA-J cells was purified and RT-PCR was performed using a series of BACH1 sequence primer sets (Experimental procedures). The RT-PCR generated the expected size products as determined using agarose gel electrophoresis (Figure 3-4C). These products were then purified from the agarose gel and subjected to DNA sequencing analysis. A C to T alteration was identified at nucleotide 2392 in exon 17 (Figure 3-4D). This alteration changes the codon 798 from CGA encoding for arginine to a stop codon, TGA. This BACH1 mutation has not previously been reported. The remainder of the sequence was intact and read as a homozygous sequence except at nucleotide 2755, in which both a T and a C were detected (Figure 3-4D). This polymorphism at nucleotide 2755 has previously been reported⁵. The point mutation at nucleotide 2392 was confirmed by sequencing genomic DNA (Supplemental Figure 3-8). The identification of

both a C and a T at nucleotide 2755 indicates that the FA-J cells used in this study contain two alleles of BACH1 gene and that both alleles contain a C to T mutation at nucleotide 2392.

BACH1 cDNA corrects interstrand crosslink-induced G2/M accumulation in FA-J cells.

Characteristically FA cells show an increased accumulation in G2/M following exposure to DNA interstrand crosslinkers¹²²⁻¹²⁷. Consistent with this finding, we found that FA-J cells accumulated in G2/M after treatment with the interstrand crosslinker, melphalan. In contrast, this accumulation in G2/M was not detected in the normal cells (PD846) (Figure 3-5A). Next, we stably infected FA-J cells with cDNA encoding the full-length wild-type BACH1 protein⁹⁵ using lentiviral infection (Figure 3-5B). To test the ability of wild-type BACH1 cDNA to correct FA-J G2/M accumulation, we treated FA-J + vector or FA-J +BACH1 with melphalan. The FA-J + vector cells had an increase in G2/M content similar to the parental FA-J cells, 46.3% and 45.4 % respectively. Whereas, in FA-J + BACH1 cells G2/M accumulation was decreased to 28.2% (Figure 3-5A). The G2/M accumulation generated in FA-J cells with MMC was also corrected with BACH1 cDNA (data not shown). A comparable correction of the accumulation of FA-C cells in G2/M was obtained by reintroduction of FANCC cDNA¹²⁴ and recently this correction of melphalan induced G/M accumulation was demonstrated by reconstitution of FA cells with their respective FA cDNAs¹²⁷. Taken together, these results indicated that BACH1 is the FANCI gene.

Discussion

In this study, we have characterized the role of BACH1 in HR mediated DSBR using mammalian cells deficient for BACH1. BACH1 was first identified by its interaction with the BRCT domain of BRCA1⁵. Consistent with the possibility that the interaction of BACH1 and BRCA1 is of functional significance, we find that HR repair stimulated by DSBs is compromised in both BACH1 deficient cells and BRCA1 deficient cells. Further supporting a role for BACH1 in HR-dependent repair, BACH1 deficient cells, like BRCA1 deficient and FA cells are extremely sensitive to MMC^{22,113-115}. BACH1 deficiency also results in increased MMC induced chromosome damage, suggesting a possible relationship to the FA pathway. Analysis of human FA patient derived cells and deletion of various FA genes in chicken DT40 cells has led to the conclusion that FA genes also function in HR^{35,44,117,118,133}.

FANCD2 monoubiquitination, the central feature of the FA pathway^{23,28}, is unaffected in BACH1 deficient cells. This is of interest since there are two FA complementation groups in which FANCD2 monoubiquitination, either spontaneously or in response to DNA damage, is not compromised. One of these groups is BRCA2/FANCD1^{22,24}. BRCA2 has an important function in HR, and appears to regulate the assembly of the Rad51 filament involved in strand invasion^{134,135}. The other FA complementation group in which FANCD2 monoubiquitination is normal is FA-J³³, for which the defective gene, FANCI, had not been previously identified. Our results suggest that BACH1 is FANCI, since BACH1 protein is not detectable in FA-J cell lysates, a homozygous BACH1 truncating mutation was identified in FA-J cells, and

expression of wild-type BACH1 corrects the drug induced G2/M accumulation found in FA-J cells. These results suggest that BACH1/FANCD1 and BRCA2/FANCD1 have critical functions in HR downstream of FANCD2 monoubiquitination.

Since FANCD2 monoubiquitination is normal in FA-J cells³³, it would appear that BACH1/FANCD1 functions independently of the seven identified FA proteins that are present in the FA nuclear complex required for FANCD2 monoubiquitination^{23,24,136} and downstream of monoubiquitinated FANCD2, like BRCA2/FANCD1. Potentially consistent with this possibility, we find that BACH1 and FANCD2 show partial colocalization after treatment with MMC (data not shown). However, we also find that BACH1 and FANCD2 do not co-immunoprecipitate before or after DNA damage (data not shown). Understanding the functional relationship of BACH1/FANCD1 to other FA proteins will be an important focus of future work.

Our inability to detect a truncated BACH1 protein by Western blot analysis, suggests this truncated BACH1 species is not stably expressed in the FA-J cells. Mutations of FANCA predicted to yield premature truncation and an absence of FANCA protein are frequently found in human FA-A patients^{137,138}. Alternatively, the Ab reagent used to detect BACH1 may lack the appropriate epitopes to efficiently bind the truncated BACH1 species.

Interestingly, BACH1 and BRCA1 function are distinct with respect to their relationship to Rad51 foci. In BRCA1 deficient cells, as previously reported⁹³, we found that Rad51 foci are greatly diminished, placing BRCA1 upstream of Rad51 in the HR pathway. In contrast, we found that DNA damage induced Rad51 foci formation was

maintained in BACH1 deficient cells. Similarly, *fancd2* mutant DT40 cells are deficient for DSB-initiated HR but Rad51 foci formation were shown to be intact after MMC treatment¹¹⁸. Still, it is possible that the assembly of RAD51 foci may merely be delayed in BACH1 deficient cells, so examination of the kinetics of the assembly of RAD51 foci in these cells will be necessary to completely exclude the possibility that BACH1 may regulate RAD51 assembly.

Our results suggest that there is no correlation between the capacity for I-SceI induced HR and Rad51 foci formation. This is also supported by results obtained by deletion of *fancd2* in DT40 cells¹³³. We conclude that unlike BRCA1, BACH1 is dispensable for the assembly of Rad51 foci in response to DNA damage. Importantly, the DNA damage-induced assembly of Rad51 foci is greatly diminished in FA-D1 cells expressing C-terminally truncated BRCA2/FANCD1^{24,132}. Because Rad51 foci formation is normal in an FA-J cell line (Supplemental Figure 3-6), and in cells in which BACH1 has been suppressed (Figure 3-3), BRCA2/FANCD1 and BACH1/FANCD1 likely have distinct functions in the FA pathway.

During genetic recombination and the recombinational repair of chromosome breaks, DNA molecules become linked at points of strand exchange. Branch migration and resolution of these crossovers, or Holliday junctions (HJs), completes the recombination process. While BACH1 demonstrates no ability to unwind HJ substrates *in vitro*^{6,139} like BLM or WRN helicases¹⁴⁰, BACH1 binds HJ substrates (Cantor unpublished). Given that BLM is a suppressor of HR^{141,142}, one possibility is that BACH1 could promote HR by blocking the access of BLM to the HJ and prevent BLM

from unwinding the HJ substrate. Alternatively, BACH1 could promote branch migration similar the E.coli RuvA protein. After strand invasion and the formation of the HJ, RuvA (a HJ specific DNA binding protein) recruits RuvB, which promotes branch migration. Similar to RuvA, BACH1 could serve as a scaffold for assembly of other proteins and/or promote branch migration of the HJ.

Alternatively, BACH1 could participate in processes that affect the dynamics of Rad51 filament extension and disassembly like the Srs2 helicase in yeast. Mutants of the Srs2 helicase display enhanced Rad51 foci, as the Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments on single stranded DNA¹⁴³. We have shown that BACH1 deficiency disrupts HR without disrupting the assembly of DNA damage-induced Rad51 foci. Thus, BACH1 could function in HR by disrupting Rad51 nucleoprotein filaments and promote HR by enabling recycling of Rad51 for subsequent rounds of HR. If so, in BACH1 deficient cells, Rad51 could be locked on DNA. Given that BACH1 unwinds in a 5'-3' direction, relative to the DNA to which it is bound^{6,139}, and Rad51 filaments extend in a 5'-3' direction relative to the single stranded gap, BACH1 unwinding could directly serve to strip Rad51 protein filaments upon completion of HR.

The development of gross chromosomal abnormalities in MMC treated BACH1 deficient cells supports the role of BACH1 in maintaining chromosome stability. The sensitivity of BACH1 deficient cells to MMC is most likely due to an overload of DNA damage that forces cells to rely on the error-free HR pathway¹⁴⁴. In the absence of functional BACH1, HR is defective, thus these crosslinked replication forks may persist

and be lethal. Deficiencies for other proteins involved in HR, including BRCA1, BRCA2, and Rad51, all result in hypersensitivity to MMC^{87,113,114}. The repair of DNA ICLs, which are induced by MMC, is a multi-step process⁴⁴. Translesion synthesis (TLS) and HR appear to cooperate in repair of crosslinks¹⁴⁵, and one way in which HR may be involved is in restarting the replication fork following resolution of the damage⁴⁴. It has been reported that FANCC is epistatic with TLS in chicken DT40 cells⁴⁴. This would suggest that the upstream FA pathway, culminating in FANCD2 monoubiquitination, might have only a modest role in HR³⁵ and with a greater role in TLS. In contrast, the downstream FA pathway functioning after FANCD2 monoubiquitination may be directly involved in HR. This is best supported by the direct role of BRCA2/FANCD1 in HR. Our paper's findings now place BACH1, which appears to be FANCD1 directly in the HR pathway as well. At present, it is unknown whether the upstream pathway leading to FANCD2 monoubiquitination simply regulates the involvement of the downstream FA proteins in HR, or whether it has a distinct function that precedes HR.

Transient or prolonged suppression of BACH1 in multiple tissue culture cells including human mammary epithelial cells, in most cases, led to increased or unchanged cell numbers compared to BRCA1 siRNA or siRNA controls (Cantor unpublished). The ability of cells to tolerate prolonged BACH1 deficiency in tissue culture cells suggests that BACH1 function is not required for proliferation or viability. Since the extreme HR defect in BACH1 deficient cells does not affect cell viability, the cells must be able to process DSBs by alternative pathways.

The identification of BACH1 mutations in breast cancer patients⁵ highlighted the possibility that BACH1 is a tumor suppressor. The finding that BACH1 likely is the gene defect in FA-J patients not only links BACH1 to the FA pathway, but also indicates that BACH1 function is critical for normal human development as well as for disease prevention. Patients in the FA-J complementation group have several symptoms including cafe-au-lait spots, thumb abnormalities, abnormal kidney, microphthalmia, microcephaly, growth delay and aplastic anemia, which are typical of other FA complementation groups³³. Since such patients typically die at an early age, it is unknown whether breast cancer would have developed.

Our findings suggest that BACH1 function is intimately linked to maintenance of chromosome stability and HR. The HR DNA repair defect reported here and previously reported checkpoint defects⁹⁵ found in BACH1 deficient cells could set the stage for genomic instability and eventual transformation. Therefore, we predict that BACH1 will be targeted in multiple cancer syndromes in addition to breast cancer. This prediction was borne out by the connection of BACH1 to FA, an autosomal recessive disorder that encompasses a range of cancer syndromes including leukemia, sarcoma and medulloblastoma¹⁴⁶. Interestingly, while kindred heterozygous for BRCA2 mutations are predisposed to breast cancer, FA-D1 patients with biallelic mutations of BRCA2 develop early onset leukemia¹¹⁶. Given the prior association of BACH1 mutations with breast cancer⁵ this suggests the possibility that FA-J patients with biallelic mutations of BACH1 will similarly have a different tumor spectrum than the heterozygous carriers.

Future experiments using mouse models or human cancer molecular genetics will ideally reveal the true role of BACH1 in tumor suppression.

Figure 3-1: **BACH1 deficient cells have a defect in DSB induced HR.** Gene conversion of the pDR-GFP construct by HR is observed as GFP positive cells. The percentage of cells with I-SceI induced HR events was quantitated by flow cytometry in MCF7 DR-GFP cells, also transiently transfected with either BACH1 (reagent A or reagent B), BRCA1, or luciferase siRNA. A) Representative flow cytometric profiles of viable MCF7 DR-GFP cells transfected with the indicated siRNA reagents. B) Percent GFP positive cells are indicated for each siRNA reagent, with standard deviation based on three independent experiments. P-values calculated using the Student's t-test ($P = .00051$ and $.00029$ for BACH1 and BRCA1, respectively). A total of 250,000 events were analyzed for each experiment, and experiments were performed in triplicate. C) Western blot of whole cell extracts were prepared from MCF7 DR-GFP treated with indicated siRNA using indicated antibodies.

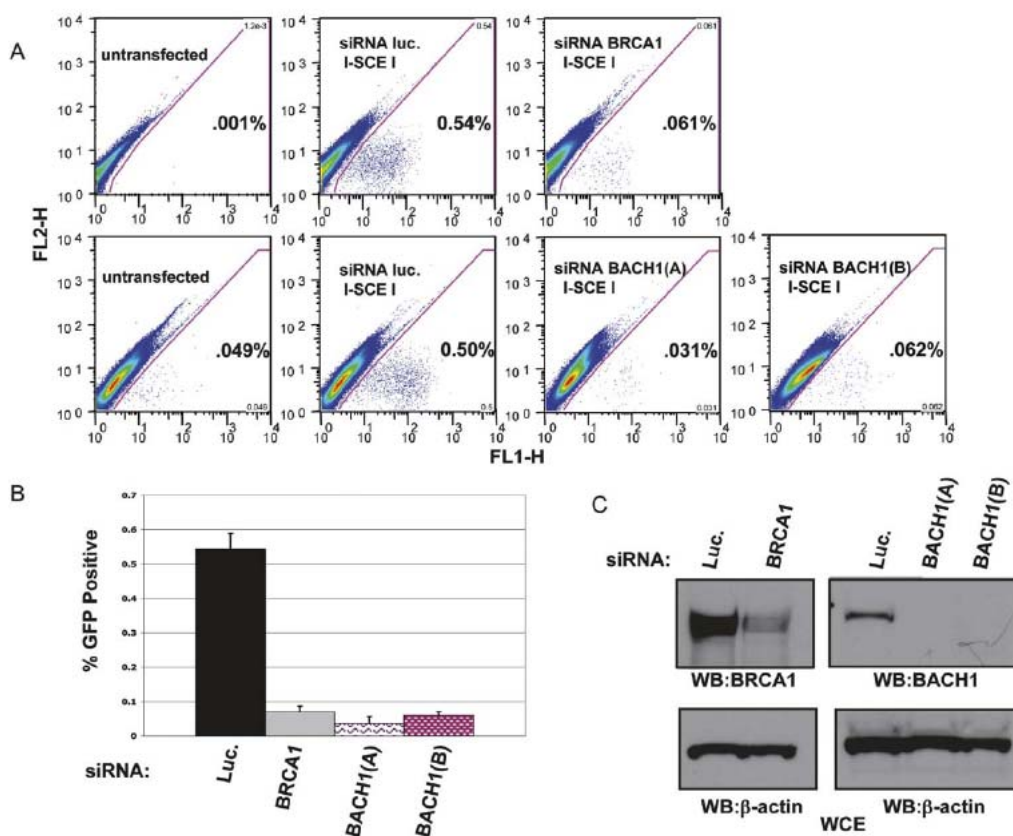


Figure 3-2: **BACH1 deficient cells are sensitive to MMC.** A) MCF7 cells transfected with luciferase siRNA (diamonds), BRCA1 siRNA (squares), or BACH1 siRNA (triangles) were treated with increasing concentrations of MMC. Cell growth was measured by ATP content after 4-5 days of MMC exposure. Experiments were performed in triplicate and a representative experiment is shown. B) Effects of siRNA on protein expression in MCF7 cells are shown by Western blot. C) HMEC cells containing shRNA to either eGFP or BACH1 were untreated (left) or treated (right) with 100 nM of MMC and genetic instability was analyzed by chromosome spreads. Representative defects from various BACH1 deficient cells treated with MMC are shown on far right, a, chromosome break, b, quadriradial, and c, triradial. Arrow shows a radial chromosome. D) Effects of shRNA on BACH1 protein expression in HMEC cells are shown by Western blot

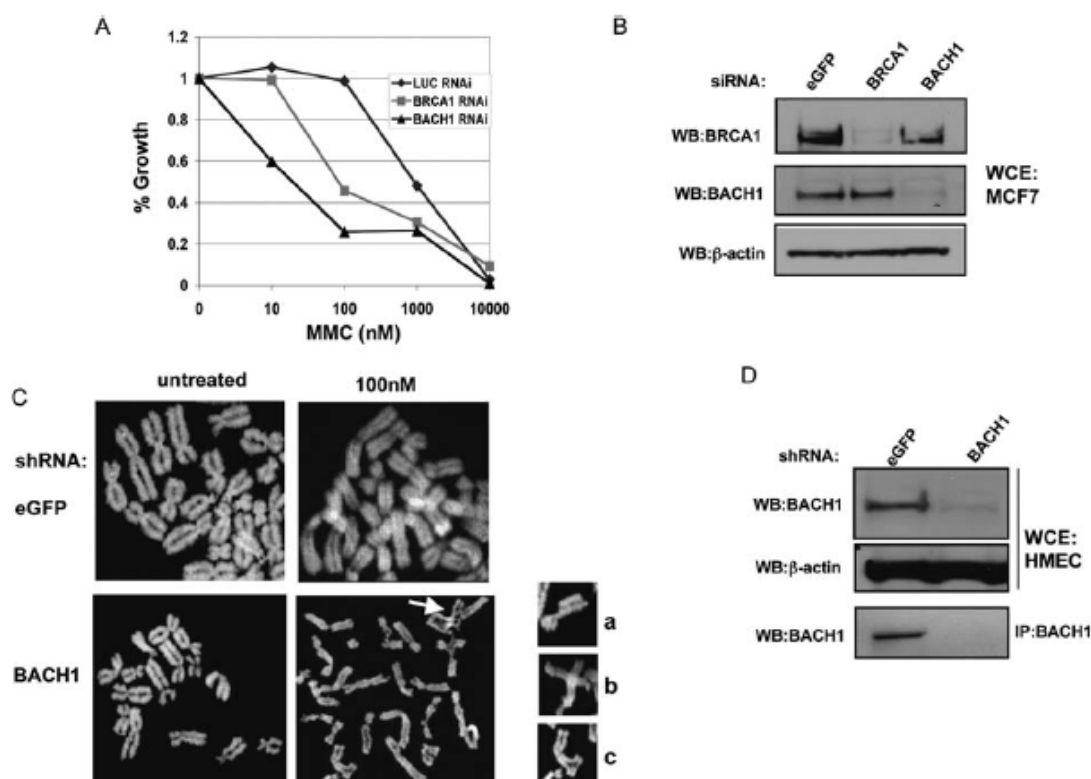


Figure 3-3: BACH1 is dispensable for FANCD2 monoubiquitination and Rad51 foci formation after DNA damage. A) FANCD2 protein in whole cell lysates from untreated or HU-treated (24 hours) MCF7 cells transduced with lentivirus expressing indicated shRNA was detected by Western blot. FA-A (a FANCA mutant cell line PD6914) and FA-A+FANCA cells (PD6914 expressing wild type FANCA) were used as negative and positive controls for monoubiquitination of FANCD2. FA-D2 (a FANCD2 mutant cell line PD20) served as a negative control for the FANCD2 Western blot. FANCD2-L and FANCD2-S represent monoubiquitinated and non-ubiquitinated FANCD2, respectively. Asterisk (*) denotes a non-specific band present in all cell lines analyzed, including PD20 cells lacking FANCD2. C) MCF7 cells containing shRNA for eGFP, BRCA1, or BACH1 were treated with 1 mM HU for 18 hours and stained with either γ -H2AX or Rad51 antibody as indicated, or with 4',6-diamidino-2-phenylindole (DAPI). C) Western blot analysis of BACH1, BRCA1, and β -actin in whole cell lysates from MCF7 cells expressing indicated shRNA

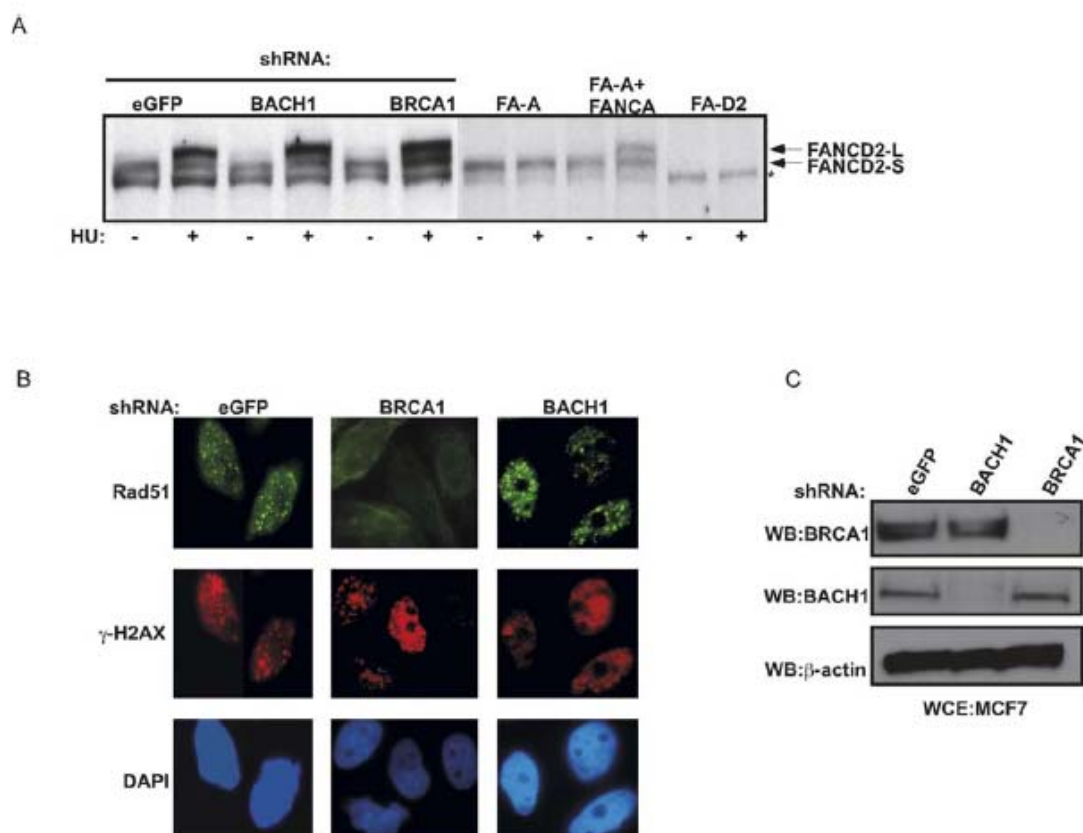


Figure 3-4: **BACH1 protein expression is absent from a patient-derived FA-J cell line.** A) Whole cell lysates from MCF7, PD846F (normal), and AG656 (FA-J) cells were probed with BACH1 and β -actin Abs. B) BACH1 in cell lysates prepared from PD846F and AG656 cells was immunoprecipitated using I82 (lanes 1 and 3) or E67 antibody (lanes 2 and 4), or was precipitated using GST-BRCT (lanes 5 and 6) and was then detected by Western blot using the BACH1 antibody E87. C) A schematic representation of the BACH1 coding region shows the PCR products created by the specified primer sets (top). Roman numerals show the relative location of the conserved helicase motifs in the helicase domain (dark blue). RT-PCR was performed on FA-J cells using overlapping primers sets and cDNA products were analyzed by gel electrophoresis (bottom). D) Mutations in the BACH1 gene in the FA-J cell line AG656 were confirmed by direct DNA sequencing. The chromatograms shown indicate a C-T point mutation at nt2392 (above), and a polymorphism at nt2757 (below). Together, this indicates a biallelic mutation of BACH1 at nt2392 in this human FA-J cell line.

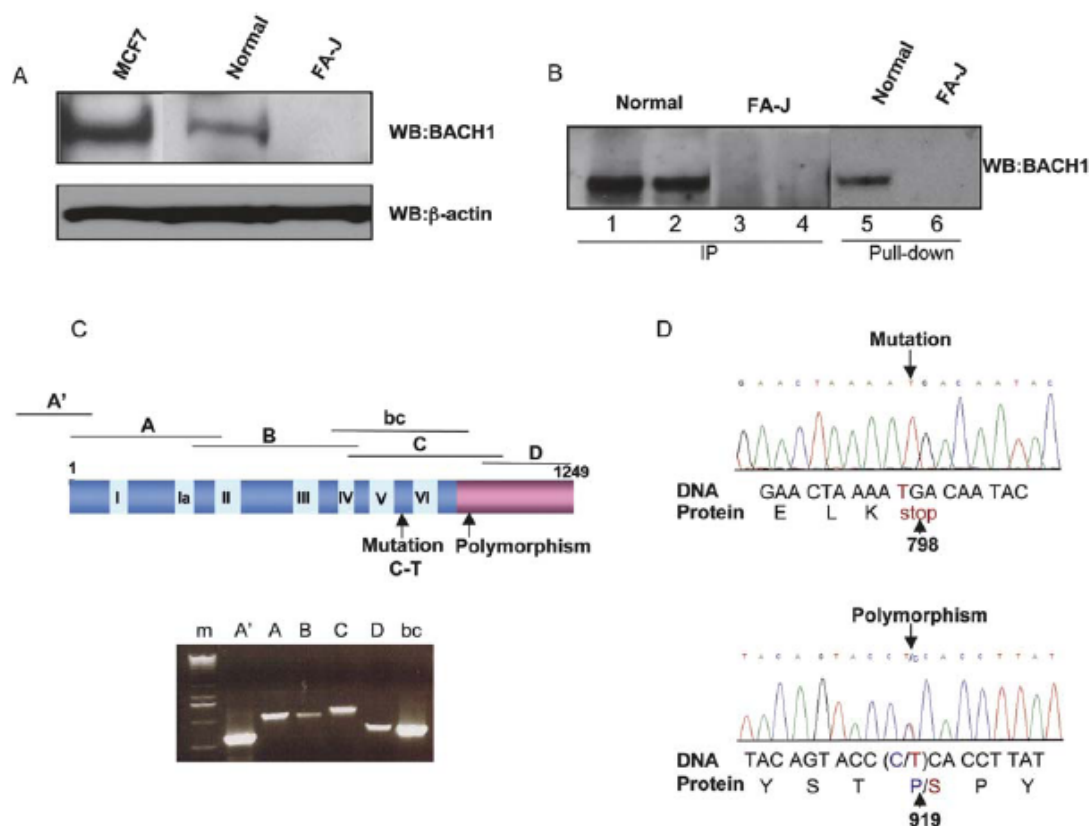
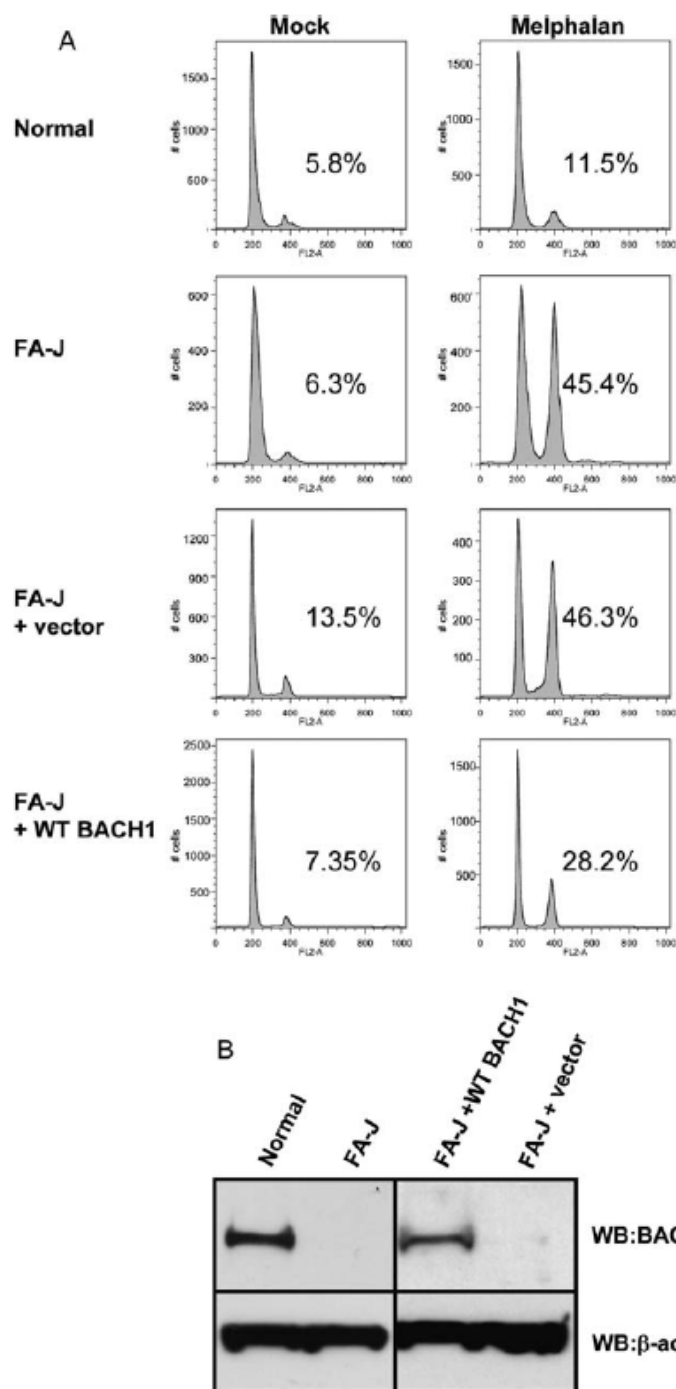
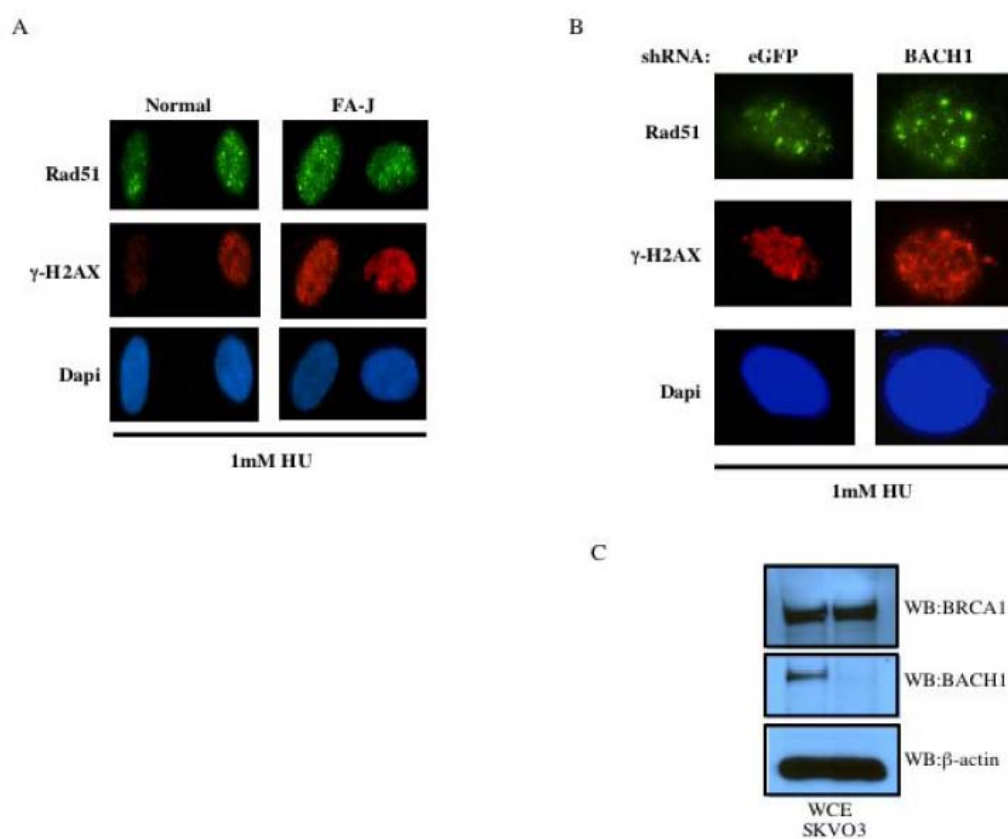


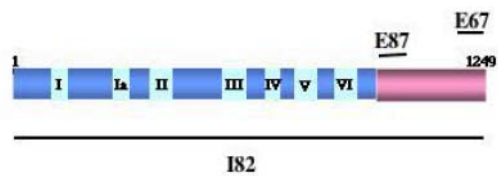
Figure 3-5: **Restoration of BACH1 cDNA corrects the melphalan induced G2/M accumulation of FA-J cells.** A) PD846F (normal), FA-J, FA-J + vector, and FA-J + WT BACH1 cells were either mock treated or treated with 0.5 μ g/mL melphalan for 65 hr. Cells were then collected, stained with propidium iodide, and analyzed by flow cytometry to assess DNA content. Three independent experiments were performed and a representative experiment is shown. B) Western blots confirm BACH1 expression in FAJ cells infected with lentivirus expressing WT BACH1.



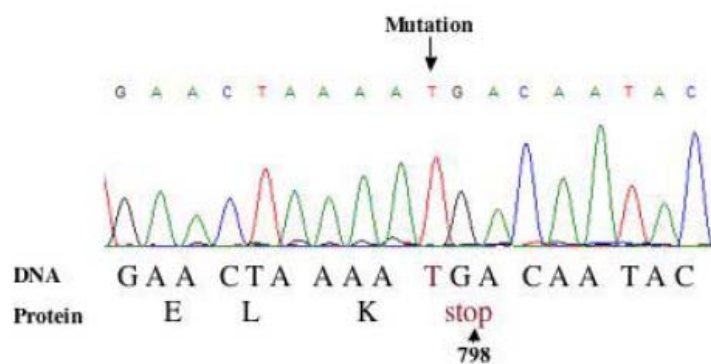
Supplemental Figure 3-6: **RAD51 foci formation is intact in FA-J cells.** A) Normal and FA-J cells were treated with 1mM HU for 18 hours and stained using polyclonal Rad51 and monoclonal γ -H2AX Abs. B) SKOV3 cells containing shRNA to eGFP or BACH1 were treated with HU for 18 hours and stained as in A. C) Effects of shRNA on BACH1 expression in SKOV3 cells are shown by Western blot.



Supplemental Figure 3-7: **Relative location of BACH1 protein, or fragments, used to make BACH1 antibodies.**



Supplemental Figure 3-8: **Sequencing of FA-J genomic DNA.** The mutation in the BACH1 gene in the FA-J cell line AG656 was verified by sequencing a PCR product generated from genomic DNA from these cells. The chromatogram shows a C-T point mutation at nt 2392 and the respective encoded protein sequence with stop codon.



Materials and Methods:

Homologous Recombination Assay and DNA constructs

The plasmid, pDR-GFP, utilizes two modified GFP genes to create a recombination reporter. The I-Sce1-GFP gene is a GFP gene that is mutated by an 11 bp substitution to contain the 18 bp recognition sequence for the I-Sce1 endonuclease. Downstream of I-Sce1-GFP is the 0.8-kb GFP fragment iGFP, which is a wild type GFP gene truncated at both its 5' and 3' ends. Expression of I-Sce1 in cells that contain the pDR-GFP substrate integrated into their genome results in a DSB in the chromosome at the position of the I-Sce1 site. Repair of the induced DSB in I-Sce1-GFP by a non-crossover gene conversion with iGFP reconstitutes a functional GFP gene, expression of which can be scored by cellular fluorescence. Although other DSB repair events at the I-Sce1 site are possible, they are not detected, because the 11bp substitutions in the I-Sce1-GFP gene cannot be restored to the wild-type GFP sequence except through a templated gene conversion event. Molecular analysis of the pDR-GFP substrate in sorted GFP-positive cells after I-Sce1 expression has verified that cellular green fluorescence, as measured by flow cytometry, results from repair by gene conversion¹²⁸. MCF7 DR-GFP cells were generated by transfecting $\sim 1 \times 10^6$ MCF7 cells in p100mm dish with 4 μ g DNA plasmid (pDR-GFP) with lipofectamine and Plus reagents (Invitrogen) following the manufacturers guidelines. After 24-hours cells were split into fresh dishes at different cell densities. After 48-hour fresh media containing 1.0 μ g/ml puromycin was added to select for puromycin resistant colonies.

MCF7 DR-GFP stable cells were seeded at low density and transfected with either siRNA directed against BACH1 (Reagent A, 5' AGCUUACCCGUCACA 3' or Reagent B, 5' GUACAGUACCCCACCUUAU 3'), BRCA1 (siRNA BRCA1 pool, Dharmacon), or a luciferase control (Dharmacon). Twenty-four hours after siRNA transfection, cells were transfected (Fugene) with 10 µg of the pCMV-I-Sce1 vector. Cells were collected 3-4 days following the final transfection and analyzed by flow cytometry. In each case, the percentage of GFP positive cells was scored out of a total of 250,000 total viable events. The shRNA lentiviral vectors were made by placing the BACH1 (A) sequence and the BRCA1 sequence 5' AGTACGAGATTTAGTCAAC) into the FSIPPW lentiviral vector as described previously¹⁰⁹.

MMC Sensitivity Assay and Chromosome Spreads

MCF7 cells were transiently transfected as described above. Twenty-four hours after transfection, cells were seeded onto 96-well plates at 1×10^3 cells per well, incubated overnight, and then treated with a concentration range of MMC (Sigma). 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 quantitation, the luciferase units of each well were normalized to those obtained from untreated cells assumed to yield 100% cell survival, and were normalized to those obtained from a well without any cells, assumed to yield 0%.

Human mammary epithelial cells (HMEC) were infected with either eGFP or BACH1 (A) shRNA (see description above), treated with 0.05 $\mu\text{g}/\text{mL}$ colcemid (Sigma) and incubated at 37°C for 1 hr. Cells were collected, resuspended in 75 mM KCl, and incubated in a 37°C water bath for 18 minutes. Swollen cells were then fixed in 3:1 acetic acid and methanol for 45 minutes on ice. The final fixation was repeated and fixed cells were dropped on coverslips, exposed to steam from an 80°C water bath, and dried cell-side-up on a 70°C heat block. Coverslips were stained with 0.3 $\mu\text{g}/\text{mL}$ propidium iodide for 30 minutes, washed for 30 minutes, and mounted on glass slides. Metaphase spreads were captured using an Olympus IX 70 Inverted Light microscope. Data were collected as a Z series for deconvolution with 0.2 μm between planes. Images were deconvolved using MetaMorph software, no neighbors algorithm.

Cell Culture

MCF7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and antibiotics. MCF7 DR-GFP, FA-A fibroblasts (PD6914 + pMMP empty vector), FA-A reconstituted fibroblasts (PD6914 + FANCA), and FA-D2 fibroblasts (PD20) were grown as above with the addition of 1 $\mu\text{g}/\text{mL}$ puromycin. MCF7-DR-GFP cells, resistant to puromycin, were screened for the ability to show I-SceI induction of HR, since pooled populations would contain a number of cells that would not contribute to the HR assay. Normal human skin fibroblasts (PD846F)²⁶ and FA-J fibroblasts (AG656)³³ immortalized with hTERT were grown in DMEM supplemented with 15% FBS and antibiotics.

Immunoprecipitation and Western blot assays

Cells were harvested and lysed in lysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin) for 30 min on ice. For FANCD2 monoubiquitination assays cells were treated with 1 mM HU (Sigma) for 24 hours and lysed as above, but with 600 mM NaCl. Cell extracts were clarified by centrifugation for 10 min at 13,200 rpm. The cell lysates were boiled in SDS loading buffer and DTT. For precipitation assays, cells lysates were incubated with either protein A beads for I82 and E67 Abs, or with glutathione conjugated beads for BRCT-GST fusion protein, and incubated at 4°C for 2 hrs. Beads were subsequently washed and boiled in SDS loading buffer. Proteins were separated using SDS-PAGE and electrotransferred to nitrocellulose membranes.

Western blot assays were carried out with a BRCA1 monoclonal Ab (MS110), BACH1 monoclonal Ab pool (1A3, 2G7 and IG5)⁵ or BACH1 polyclonal Abs I82 made vs. full-length BACH1-Flag protein⁶, (and generated by R. Drapkin), E67⁶, and E87 generated against epitopes 983-997 of BACH1, β-actin (Sigma) monoclonal Ab, and E35 rabbit anti-human FANCD2 antibody²³ for 2 hrs at 4°C. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 1 hr at RT. All blots were detected with ECL-plus Western Blotting Detection System (Amersham Biosciences) using Biomax MR or XAR film (Kodak). Bradford assays were used to determine protein content (Bio-Rad).

Immunofluorescence Microscopy

Cells were fixed with 2% paraformaldehyde in PBS for 20 min at RT, and were subsequently permeabilized with 0.2% Triton X-100 in PBS for 3 min at RT, and incubations with antibodies and washes were as previously described⁵. For immunostaining of γ -H2AX or Rad51, MCF7 or FA-J cells were treated with 1 mM HU for 18 hrs and stained as above. Mouse monoclonal anti-phospho-Histone γ -H2AX (Ser139) antibodies were purchased from Upstate. Polyclonal Rad51 Abs were purchased from Santa Cruz. Secondary Abs were either Rhodamine Red-conjugated AffiniPure Goat anti-mouse IgG or FITC-conjugated AffiniPure Goat Anti-rabbit IgG purchased from Code Jackson ImmunoResearch Laboratories INC.

Sequencing

Total RNA was purified from FA-J cells (Qiagen RNeasy) and RT-PCR was performed as outlined by the manufacturer (Qiagen one step RT-PCR) using overlapping primers. The thermocycling conditions for amplification of cDNA were as follows: 50°, 30 min, 95°, 15 min, 40 cycles each with a denaturing step at 94°C for 1 min, an annealing step at 50°C for 1 min, and extension step at 72°C for 1 min. The reaction was completed with a final extension at 72°C for 10 minutes and a 4°C soak. Genomic DNA was purified from FA-J cells (Qiagen DNeasy) and PCR was performed using intronic primers that flank the mutated region observed in exon 17. The thermocycling conditions for amplification of genomic DNA were as follows: 95°C, 2 min, followed by 30 cycles each with a denaturing step at 95°C for 30 seconds, an annealing step at 50°C for 1 min,

and extension step at 72°C for 4 min. The reaction was completed with a final extension at 72°C for 10 minutes and a 4°C soak. Both RT-PCR and PCR products were separated on 1% agarose gels containing ethidium bromide and visualized with UV light. PCR primer combinations were as follows (primers written in 5'-3' orientation):

A') CCGCTTTATTTGCTCTCAGAAG and

CTGTTCCAAAGCAATGACGTTTTCTAATCTGCTGT;

A) ATGTCTTCAATGTGGTCTGAATATAACAATT and

ACACAGCCCGAGAACTAATACAA

B) TTGCTAGATGGGAAAAACGG and AGAGATTCCGACCCTTGGGGCC

C) GTTGGTACCATTGGGTCAGGC and TGATCTCCGCCCGTTTCAAAA

D) ACAAAGAGAGTTAGCTGGTCAAGCTTTA and

CAGCGGCCGCCTTAAAACCAGGAAACATGCCTTTATT

bc) ATGGCAAAGTTCAGACCATTG and

AAGTCCAGATATATAGCGACTTGGGTTATTCCT

Forward Intron: ATCTCTACCCTAAAAATATGTATATTC

Reverse Intron: CCAGTTCCTATGGTTCCAGTTAAATA

PCR products were sequenced (Nucleic Acid Facility, UMMS) on both strands with the primers used to create the amplicon. Additional primers were used for sequencing the region containing the noted mutations listed below:

Forward 1: AGGAATAACCCAAGTCGCTATATATCTGGACTT

Reverse 2: AAGTCCAGATATATAGCGACTTGGGTTATTCCT

Forward 2: CTTAATGTATCCATAAAGGAC

Reverse 2: AAGGGCCCACTTGGTAGAGGTGAATTTTTGGT

Lentiviral Infection and G2/M accumulation assay

FA-J cells were infected with either pLENTI6/v5-DEST (Invitrogen) empty vector or pLENTI6/v5-DEST + WT BACH1 (gift of J. Chen) and stable cells were selected with 7 $\mu\text{g}/\text{mL}$ Blasticidin (Invitrogen). PD846F (Normal), FA-J, FA-J + vector, and FA-J + WT BACH1 cells immortalized with hTERT were either mock treated or treated with 0.5 $\mu\text{g}/\text{mL}$ of melphalan (Sigma) and incubated for 65 hrs. 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 $\mu\text{g}/\text{ml}$ propidium iodide. 1×10^4 cells were analyzed using a FACS Calibur instrument (Becton-Dickinson, San Jose, CA). Aggregates were gated out and the percentage of cells in G2/M was calculated using FlowJo software.

Acknowledgments:

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CHAPTER IV

The FANCF/MutL α interaction is required for correction of the ICL-response in FA-J cells

Abstract

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

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 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 FANCD1²². Likewise, FANCI (formally called BACH1/BRIP1) was recently identified as the gene defect in the FA-J patient complementation group^{99,105,106}, 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 FANCI, which also altered its helicase activity in vitro^{5,6}. This connection was furthered by the finding that FANCI mutations confer a two-fold increase in the risk of developing breast cancer⁷.

While other FA genes have not been linked to breast cancer, the network of at least 12 genes (designated *FANCA* to *FANCL*) are critical for maintaining chromosomal integrity¹⁰⁸. Although the molecular function of these proteins is not clear, several gene products, including FANCA, B, C, D, E, F, G, L, and M, form a nuclear core complex (the FA core complex), that is required for monoubiquitination of FANCD2. The FA proteins BRCA2/FANCD1 and FANCI are not required for this event and are considered

downstream of FANCD2 monoubiquitination. Nevertheless, all FA proteins contribute to processing interstrand-cross-links (ICLs)¹⁰⁸. Consequently, in the absence of FA proteins, ICL-treatment leads to reduced viability and an accumulation of cells with a 4N DNA content representing cells in either late S or G2/M. This ICL-induced cell cycle progression defect and sensitivity to ICLs is restored upon re-introduction of the missing FA gene^{54,122-127}. However, the FA-related function or associated partners required for a proper ICL-response is not known.

Consistent with other FA cells, FANCI-null (FA-I) cells have an ICL-induced cell cycle progression defect that can be corrected upon re-introduction of wild-type (WT) FANCI cDNA⁹⁹. Unlike the majority of FA proteins, FANCI has defined domains. Specifically, FANCI binds directly to BRCA1⁵ and FANCI is a DNA helicase⁶. 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 FANCI domains have been limited to chicken DT40 cells where the FANCI /BRCA1 interaction is not conserved¹⁰⁷. If FANCI operates independent of BRCA1 for a particular function, a remaining question will be whether FANCI forms a complex with other proteins independent of BRCA1 to perform that function.

Here, we investigated whether FANCI helicase activity or the FANCI interaction with two distinct proteins was required for restoring FANCI's ICL-response. Specifically, we identified that FANCI interacts with the MLH1 MMR protein, independent of BRCA1. Our findings demonstrate for the first time that the FANCI /MLH1 interaction is as critical as FANCI helicase activity for restoring a normal cell

cycle progression and resistance to ICLs. In contrast, disruption of the FANCD1 /BRCA1 interaction does not interfere with establishment of the ICL-response, suggesting that FANCD1 functions in distinct complexes to facilitate multifaceted DNA repair functions.

Results

FANCD1 functions independently of BRCA1 to correct FA-J null cells

We had previously shown that introduction of WT FANCD1 cDNA into FA-J cells corrects the ICL-induced cell cycle progression defect⁹⁹. However, it is unclear how FANCD1 contributes to the ICL-response to restore the FA pathway especially given that FANCD1's role in the FA pathway appears to be independent of BRCA1, at least in chicken cells¹⁰⁷. To verify and extend this finding, we addressed whether FANCD1 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 FANCD1 construct that is ablated for BRCA1 binding⁹⁵ (Figure 4-1A and B). Both WT and S990A versions of FANCD1 corrected the ICL-induced cell cycle progression defect observed in FA-J cells compared to vector alone (Figure 4-1C). These data support the finding that FANCD1 operates independent of BRCA1 to correct FA-J cells.

FANCD1 is physically linked to the MutL α complex independent of BRCA1

Since FANCD1 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 FANCD1 interacting partners that may function with FANCD1 in this ICL-induced response. A WT

double-tagged FANCI construct was used to create a stable line of HeLa S3 cells. Using a two-step immunoaffinity strategy, the double-tagged FANCI was sequentially immunopurified¹⁴⁷. Interacting proteins co-purifying with the double-tagged WT FANCI were eluted and visualized by silver stain. FANCI migrated at the expected ~140KD size as well as a single larger species that appears to derive from the vector (Figure 4-2A). Individual bands were excised from the gel and analyzed by mass spectrometry (LC-MS/MS). As expected, FANCI 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 MutL α heterodimer¹⁴⁸ (Figure 4-2A). Western blot analyses using specific antibodies confirmed the presence of these proteins (Figure 4-2B). The ~140kD FANCI band was similar in size to the endogenous FANCI and was clearly detected by Flag and FANCI Antibodies (Abs) (Supplemental Figure 4-7) and therefore, exclusively depicted in the FANCI Western blot (Figure 4-2B). To determine whether the MutL α complex associated with the native FANCI protein, MCF7 cell extracts were immunoprecipitated (IPed) with FANCI antibodies (Abs) E67 and E47, and the presence of coprecipitating MLH1, PMS2, and BRCA1 protein was evaluated by Western blot (Figure 4-2C). As a control, we demonstrated that the MutL α complex was not precipitated with preimmune Abs (PI) or with FANCI Abs in 293T cells, which lack expression of the MutL α complex¹⁴⁹. Moreover, FANCI was not precipitated with the MLH1 Abs in FA-J cells, which lack expression of FANCI, unless FANCI was re-introduced (see Figure 4-6A). Furthermore, the interaction between FANCI and the MutL α complex was stable in HeLa cells in the presence or absence of DNA damage

(Figure 4-2D). In a reciprocal IP experiment with anti-MLH1 Ab, FANCI was readily co-precipitated, but BRCA1 was more difficult to detect (Figure 4-2C).

Because MLH1 was reported in a BRCA1 complex^{49,150}, we examined whether BRCA1 mediated the interaction between FANCI and the MutL α complex. Expression of BRCA1 was stably suppressed in MCF7 cells by a shRNA vector, as previously demonstrated⁹⁹. In cells expressing a control shRNA eGFP or the BRCA1 shRNA, FANCI antibodies efficiently co-precipitated the two components of the MutL α complex (Figure 4-3A), suggesting that FANCI binds the MutL α complex independent of BRCA1. In support of this finding, the helicase domain of FANCI was required for MLH1 binding while a C-terminal region of FANCI was required for BRCA1 binding (Figure 4-3B). To further assess the nature of the FANCI/MLH1 interaction, we incubated recombinant FANCI or BRCA1 with MLH1 that had been translated *in vitro*. MLH1 and FANCI were precipitated by their corresponding Abs, and their interactions were analyzed by Western blot. FANCI and MLH1 proteins were co-precipitated with both FANCI and MLH1 IPs, whereas BRCA1 was robustly precipitated only in the FANCI IP (Figure 4-3C). The direct interaction between FANCI and MLH1 was confirmed by ELISA assay using purified recombinant proteins and FANCI bound MLH1 in a protein concentration dependent manner (Figure 4-3D). Furthermore, the interaction of FANCI and MLH1 was demonstrated to be DNA-independent as evidenced by the similar colorimetric signal observed for FANCI/MLH1 interaction in the presence of EtBr or DNaseI (Figure 4-3E). These results suggest that FANCI makes direct contacts with MLH1, independent of PMS2 or BRCA1.

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

Next, we sought to disrupt FANCI binding to MLH1 in order to test the functional significance of the FANCI/MLH1 interaction for FANCI function in the ICL response. To do so, we mapped the domain on FANCI required for MLH1 binding. We generated several FANCI-myc fusion proteins of varying length and expressed them in MCF7 cells (Figure 4-4A and C). FANCI co-precipitated with MLH1 except when FANCI residues 140-145 were absent (Figure 4-4A). To assess the importance of residues 140-145 for binding MLH1 within the context of the full-length FANCI protein, we generated three independent FANCI 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 FANCI 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 4-4B) suggesting that these two lysines were required for MLH1 binding.

We considered that the K141/142A mutation in FANCI could have not only disrupted MLH1 binding, but also FANCI helicase activity. Thus, we generated recombinant versions of WT⁶ and the K141/142A FANCI proteins to assess whether this mutant version was enzymatically active. The recombinant K141/142 FANCI protein was detected as a single Coomassie stained band analyzed by SDS-PAGE that co-migrated with the WT FANCI recombinant protein (Figure 4-5A and D). The DNA unwinding activity of K141/142A FANCI on a forked duplex DNA substrate was compared to unwinding activity of WT FANCI. Both K141/142A FANCI and WT

FANCI were found to be proficient in unwinding whereas K52R FANCI, as previously demonstrated, failed to unwind the forked duplex substrate¹³⁹ (Figure 4-5B).

Furthermore, K141/142A FANCI and WT FANCI unwound the forked duplex substrate in a protein concentration dependent manner achieving 90% of unwound substrate at the highest helicase concentration tested (Figure 4-5C). Thus, the K141/142 mutant only disrupts MLH1 binding, but not FANCI helicase activity *in vitro*.

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

Next, we tested the ability of K141/142A FANCI 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 FANCI constructs, which expressed similarly (Figure 4-6A). Moreover, an MLH1 IP demonstrated that the MLH1/PMS2 complex was intact in FA-J cells and was able to precipitate the reconstituted FANCI (Figure 4-6A). As before MLH1 co-IPed with WT FANCI, but was dramatically reduced in FA-J cells with the K141/142A mutant FANCI (Figure 4-6A). FA-J cells containing vector, WT, K52R, or K141/142A FANCI were treated with melphalan to induce ICLs as described⁹⁹. 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 FANCI-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 FANCI helicase (K52R) failed to correct the 4N accumulation defect (~66%). Likewise, the proportion of K141/142A

FANCI-containing FA-J cells with 4N DNA content (~68%) resembled that of vector-containing FA-J cells, suggesting that introduction of K141/142A FANCI did not correct the cell cycle progression defect in FA-J cells (Figure 4-6B). These data suggest that the FANCI/MLH1 interaction is essential for restoration of the FA pathway in FA-J cells.

To further confirm and examine the importance of FANCI binding to MLH1 for the ICL-induced response, we assessed whether FANCI binding to MLH1 was required for FANCI to correct the ICL-induced sensitivity of FA-J cells. However, the ability of WT FANCI 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 FANCI 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 FANCI reconstituted cells with an IC_{50} of 250mM and ~ 900mM MMC, respectively (Figure 4-6C). In contrast to WT, both the K141/142A, and K52R FANCI reconstituted FA-J cells were sensitive to ICLs with an IC_{50} of less than 250mM MMC. Moreover, the K141/142A FANCI reconstituted FA-J cells were more sensitive than the vector or K52R FANCI reconstituted FA-J cells ($P < 0.01$). As before, the S990A FANCI corrected the ICL-response similar to WT. Although the correction of the ICL sensitivity was greater with the S990A FANCI than with the WT FANCI, at 250mM MMC the values were not significantly different (with P -values between 0.0027-0.22) (Figure 4-6C). These findings clearly demonstrate that both FANCI helicase activity and FANCI binding to MLH1 are required for FANCI to functionally correct the ICL sensitivity of FA-J cells.

Discussion

In this study, we addressed whether FANCD1 helicase activity or different FANCD1 complexes are essential for FANCD1's function in the ICL response. Specifically, we have shown that FANCD1 forms a complex with the MutL α heterodimer, which is composed of the MMR proteins MLH1 and PMS2. FANCD1 directly interacts with MLH1 independent of BRCA1, and this DNA-independent interaction is within the FANCD1 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 FANCD1 and MLH1 is as essential for the ICL-induced response as the FANCD1 helicase activity. Furthermore, our data suggest that formation of a FANCD1/BRCA1 complex is not required for re-establishment of the ICL induced 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 damage¹⁵¹. Furthermore, MMR proteins have been implicated in sensing and repairing ICLs. In particular, the MutL α complex was shown to bind to intrastrand crosslinks produced by cisplatin^{152,153}, and the MutL β complex was shown to be involved with the removal of ICLs produced by psoralen¹⁵⁴. This study now suggests that MLH1 binding to FANCD1 is functionally important for the ICL-induced response, as the FANCD1 K141/142A mutant, defective in MLH1 binding, fails to correct FA-J cells.

Given that the FANCD1/MLH1 interaction is intact in other FA cells, including FA-A, FA-D2, and FA-D1 (Supplemental Figure 4-9), 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 FANCD1 deficiency, MLH1 deficiency does not affect the ATR-mediated FANCD2 monoubiquitination (Supplemental Figure 4-8)^{26, 33,99,107}, suggesting that the FANCD1/MLH1 interaction is not essential for FA-pathway activation.

The question remains as to how FANCD1 or MMR proteins function in the ICL-response. Since FA-J cells arrested DNA synthesis in response to ICL-treatment similar to WT FANCD1 corrected FA-J cells (data not shown), the 4N DNA accumulation may not reflect a failed intra S-phase checkpoint as reported in other FA cells^{53,54}. Instead, the ICL-induced 4N DNA accumulation may represent an inefficient or delayed repair of ICLs⁵². Perhaps essential to the survival of FA cells is the extra time to repair and recover in order to prevent ICL-induced apoptosis. In fact, treatment of FA-J cells with ICL agents does not lead to a dramatic cell death as compared to WT FANCD1 corrected FA-J cells, but rather a sustained 4N DNA accumulation leads to reduction in cell number (data not shown). Thus, the sensitivity of FA-J cells to ICLs may stem from the 4N DNA accumulation combined with delayed re-entry into the cell cycle⁵².

The enhanced ICL sensitivity of FA-J cells expressing the K141/142A FANCD1 mutant compared to vector suggests that loss of FANCD1 binding to MLH1 impacts the ICL-response more than loss of FANCD1 alone. If MLH1 serves to localize or regulate FANCD1 helicase at sites of DNA damage, the K141/142A FANCD1 mutant may be more

disruptive to repair. The MLH1 homologue in *E. coli*, MutL binds the DNA helicase UvrD gene product Helicase II^{155,156} and stimulates its helicase activity^{157,158}. Conceivably, for ICL repair, MMR complexes including MutL α , mobilize or regulate FANCD1 helicase activity to unwind DNA in the vicinity of the DNA damage to facilitate repair processes. Thus, disruption of FANCD1 helicase activity or MLH1 binding will interfere with ICL-induced signaling. We did not detect an effect of the MutL α complex, inhibitory or stimulatory, on FANCD1 catalyzed unwinding of a forked duplex, 5' flap, or Holliday Junction substrate (data not shown). However, it is possible that regulation of FANCD1 helicase activity by MLH1 may require the support of additional MMR proteins. It is also possible that the physical interaction between FANCD1 and MLH1 may serve a non-catalytic role in mediating the ICL-response.

The connection of MMR proteins to the ICL-response has been controversial given the ambiguity of results relating to the relative sensitivity or resistance of MMR deficient cells to agents that induce ICLs^{159,160}. We found that suppression of the MutL α complex in MCF7 cells did not lead to MMC sensitivity (data not shown). Most likely, to uncover the distinct role of the MutL α complex in the ICL-induced response, separation-of-function mutants will be required. Conceivably, MLH1 binding to FANCD1 is required for MLH1's ICL function, but loss of other MLH1 functions mask any defect in its ICL-response. Ultimately, it will be critical to establish whether a FANCD1/MLH1 complex facilitates ICL-repair by promoting HR or other repair functions. While ablation of BRCA1 binding to FANCD1 may not affect the ability of FANCD1 to correct the

defective response of FA-J cells, the timing or mechanism of repair may be altered.

Efforts are underway to address this possibility.

In conclusion, these studies have provided the first evidence for a role of the FANCD1/MutL α complex in the ICL induced response. Further study of the role of FANCD1, BRCA1, and MMR proteins in this process should advance the understanding of how ICL-induced responses are regulated to preserve genomic integrity.

Figure 4-1: **The FANCI/BRCA1 interaction is dispensable for correction of the 4N DNA accumulation defect in FA-J cells.** A) FA-J cells were reconstituted with vector, WT or S990A, and FANCI expression was analyzed in whole cell extracts (WCE) by immunoblot. β -actin serves as a loading control for the WCE samples. B) Immunoprecipitations with FANCI (E67) were analyzed by Western blot with the noted Abs. C) FA-J cells reconstituted with vector, WT or S990A FANCI were either left untreated or treated with melphalan and the percentage of cells with 4N DNA content was analyzed by FACS. 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.

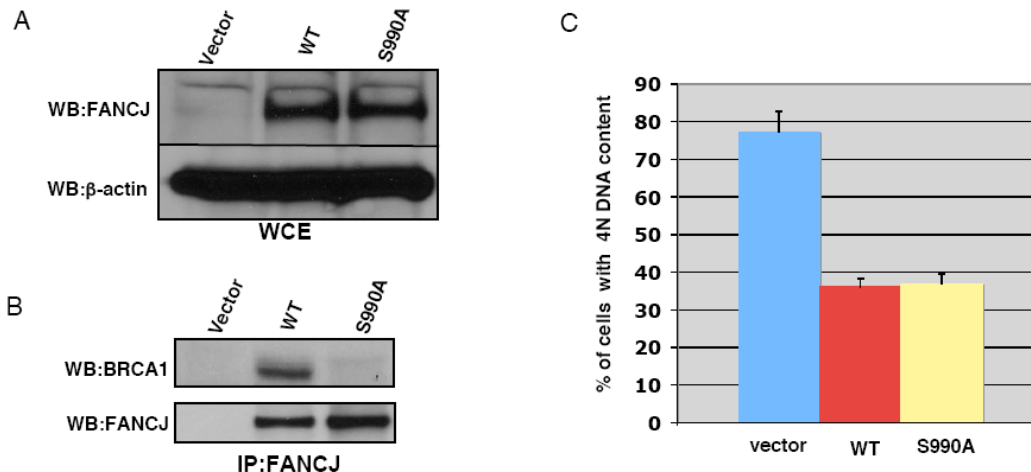


Figure 4-2: FANCI interacts with the MMR proteins MLH1 and PMS2. A) Silver stained gel of the WT FANCI (F) compared to a Vector (V) purified complexes from HeLa S3 cells by consecutive Flag and HA purification steps (Flag/HA). Identified unique bands are indicated and FANCI is observed as two species, the 140kD band is labeled. B) Western blot detection of Flag/HA purified FANCI complexes. C) Immunoprecipitations with either FANCI (E67 or E47) or MLH1 Abs from MCF7 or 293T cells were analyzed by Western blot with the noted Abs. D) HeLa cells were either left untreated or treated with 1mM HU for 24hr or 2.4ug/mL MMC for 1hr. HeLa cell lysates were immunoprecipitated with preimmune (PI) or FANCI Abs followed by Western blot analysis with the noted Abs.

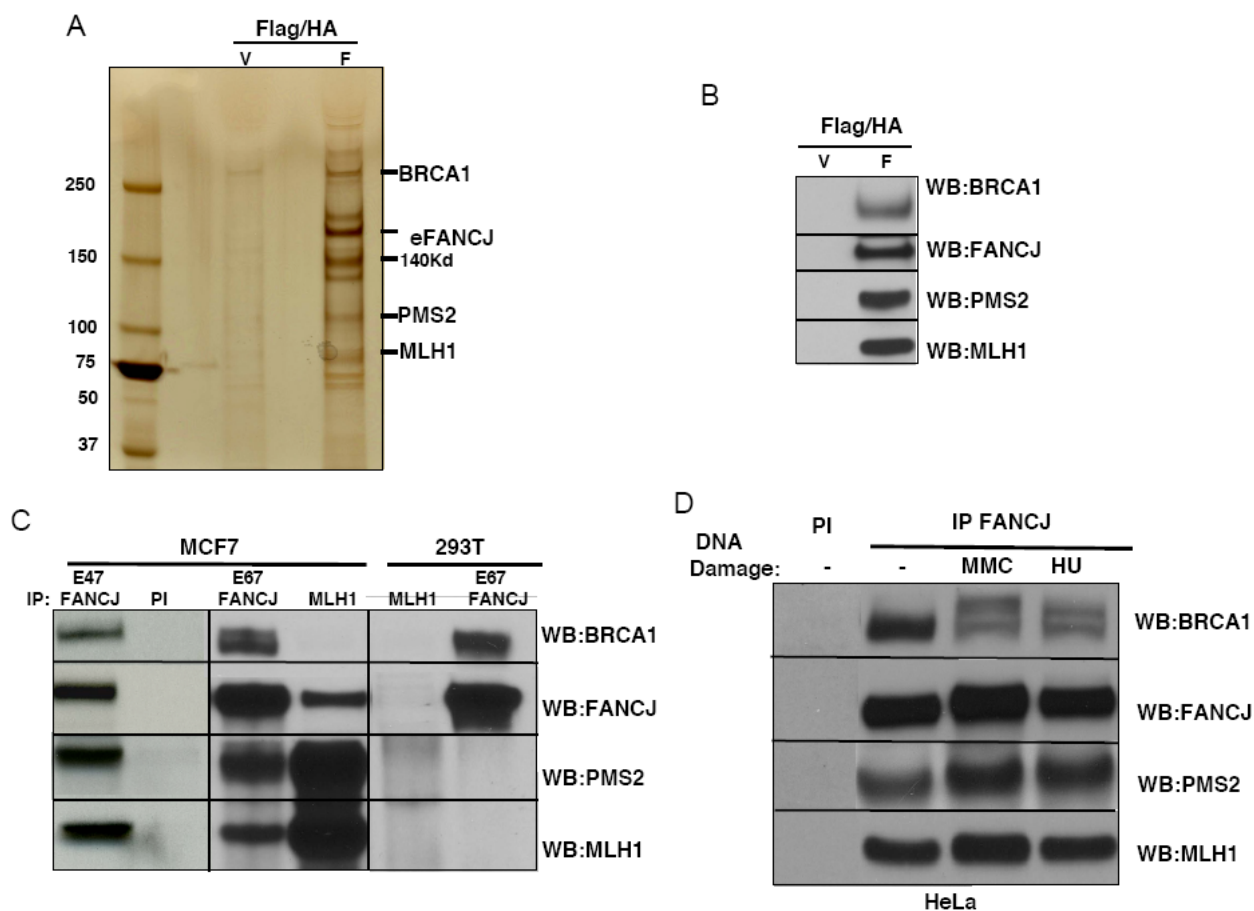


Figure 4-3: FANCD1 helicase domain associates with the MutL α complex independent of BRCA1 and through a direct interaction with MLH1. A) MCF7 cells were stably infected with a lentivirus encoding shRNA for either eGFP or BRCA1. FANCD1 IP was performed followed by Western blot for the indicated proteins. B) MCF7 cells were transiently transfected with pCDNA3 vectors containing no insert (-), full length FANCD1 (FL), helicase domain including amino acid residues 1-882 (HD) or C-terminus including residues 882- 1249 (CT) of FANCD1, then immunoprecipitated with the Myc Ab (9E10). Arrows designate the respective FANCD1 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 FANCD1 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 FANCD1 (0-40 nM) for 1 hr at 30 °C, and bound FANCD1 was detected by ELISA using a rabbit polyclonal Ab against FANCD1 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 FANCD1 alone or in the presence of EtBr (50 μ g/ml) or DNaseI (2 μ g/ml). BSA (3%) was used as a control instead of MLH1 during the coating step.

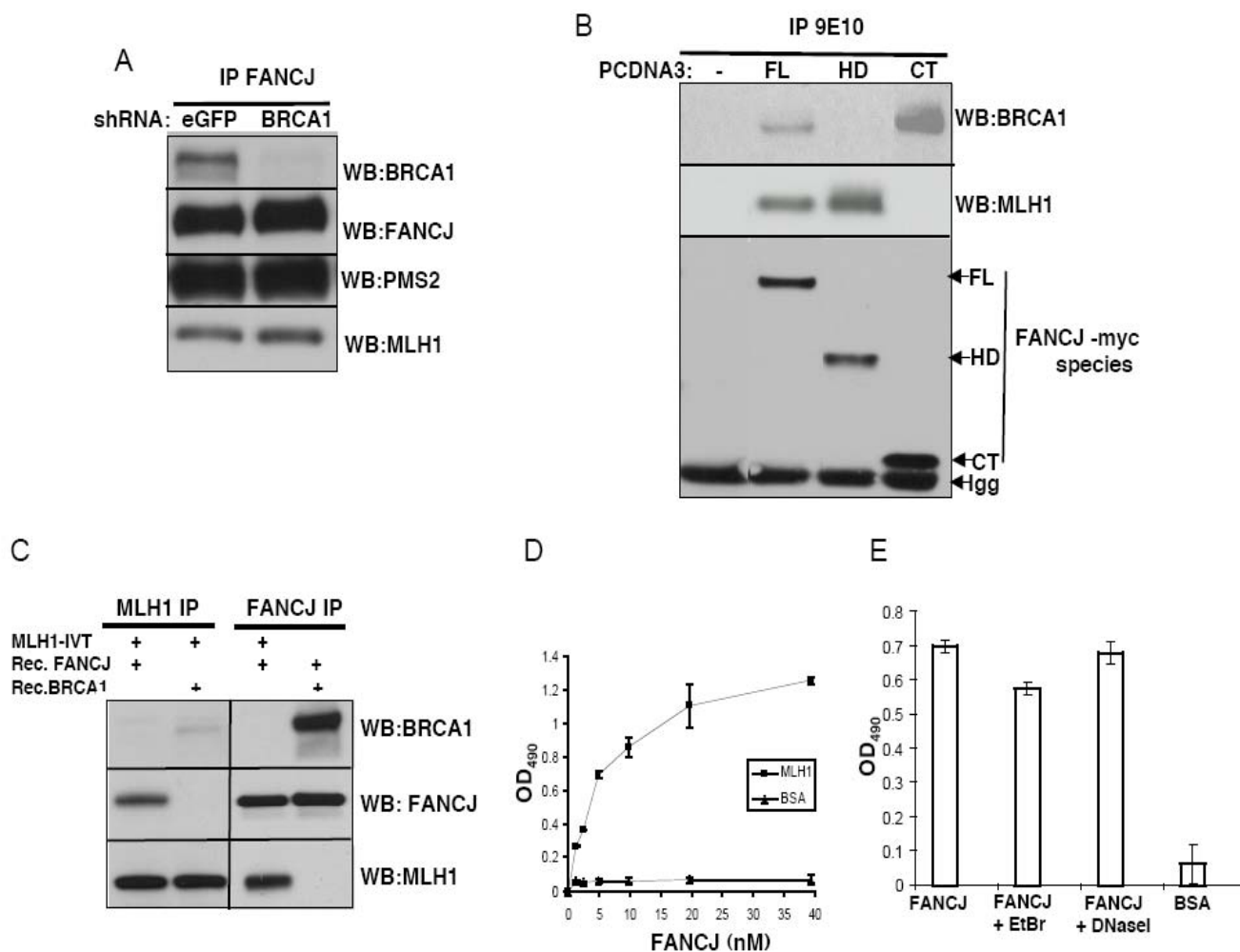


Figure 4-4: **MLH1 binding to FANCI requires lysine residues 141 and 142.** A) Myc (9E10) IP experiments were performed from MCF7 cells that were transfected with vector alone (-), full-length FANCI (FL), and the different FANCI constructs (A-G) shown in C, followed by Western blot with MLH1, and Myc Abs. The asterisk denotes the migration of the different myc-tagged FANCI species. B) Myc IP experiments were performed from MCF7 cells that were transfected with vector alone (-), FL, H, I and J FANCI constructs shown in C, followed by Western blot with the indicated Abs. C) The different FANCI constructs are shown with a positive (+) or negative (-) to indicate binding to MLH1.

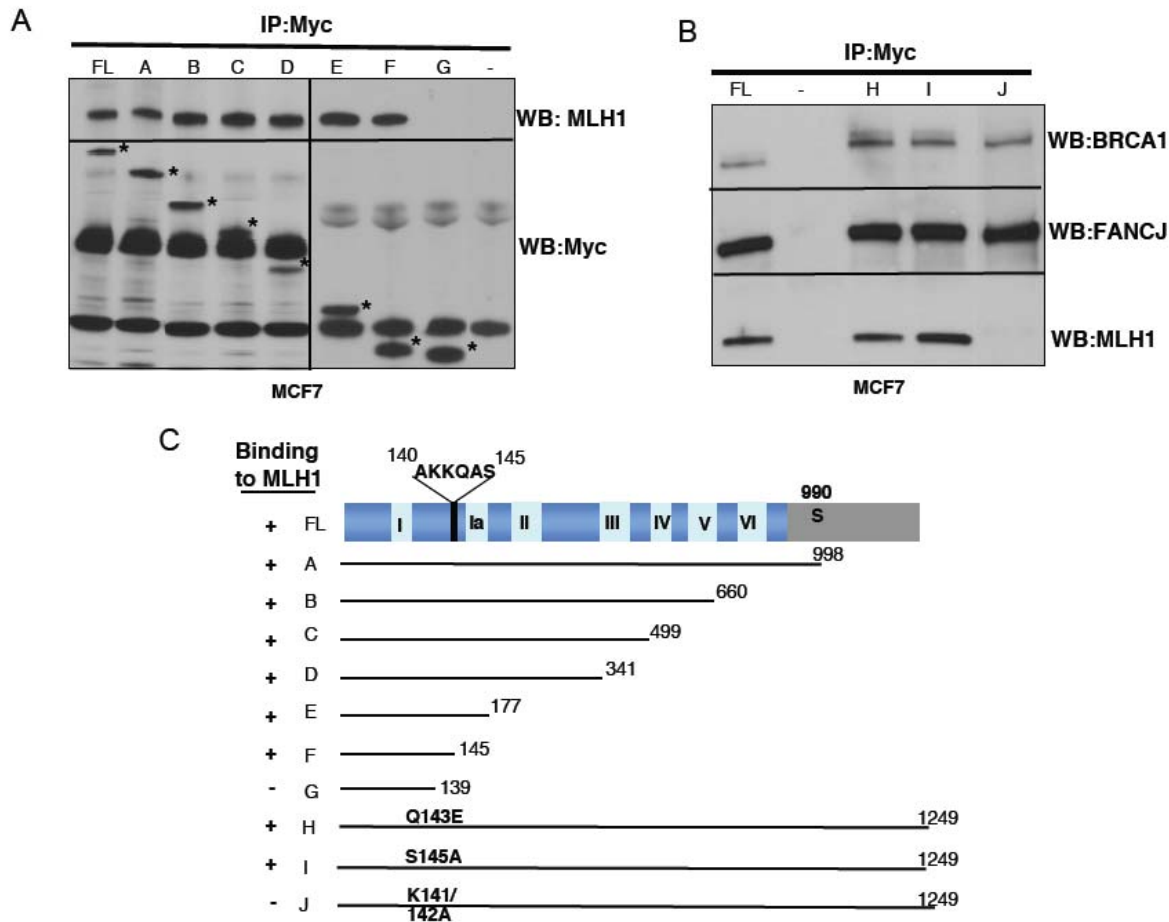


Figure 4-5: The K141/142A FANCI mutant maintains robust helicase activity. A) Recombinant K141/142A FANCI protein was immunoprecipitated with Flag antibodies and eluted using Flag peptide. The purity of the protein sample was visualized by Coomassie stain and arrow denotes both WT and K141/142A FANCI species B) Helicase reactions (20 μ l) were performed by incubating the indicated concentrations of WT, K141/142A, or K52R FANCI as with 0.5 nM forked duplex DNA substrate at 30 $^{\circ}$ 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, FANCI-WT; filled squares, FANCI-K141/142A. D) Recombinant WT, K141/142A, and K52R proteins used in B were separated by SDS-PAGE and analyzed by Western blot using FANCI specific antibodies.

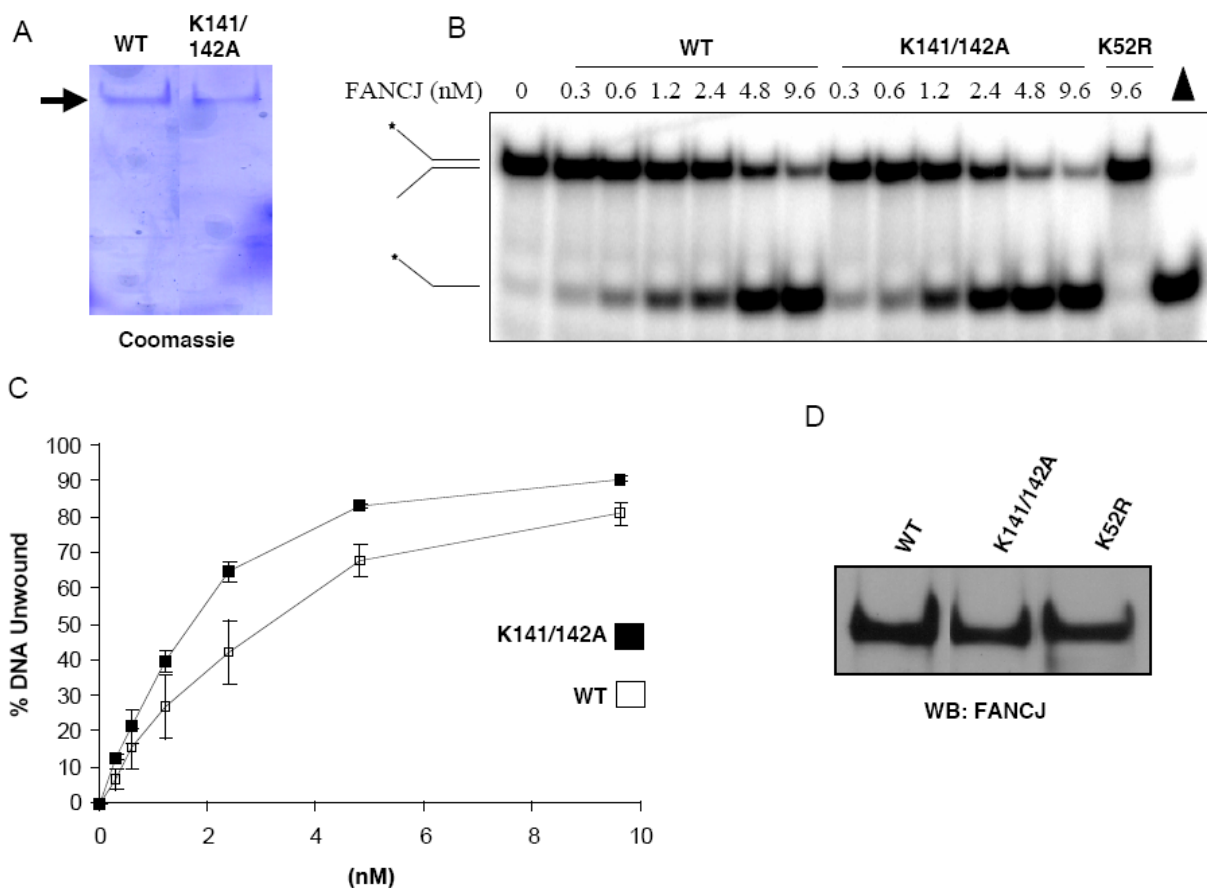
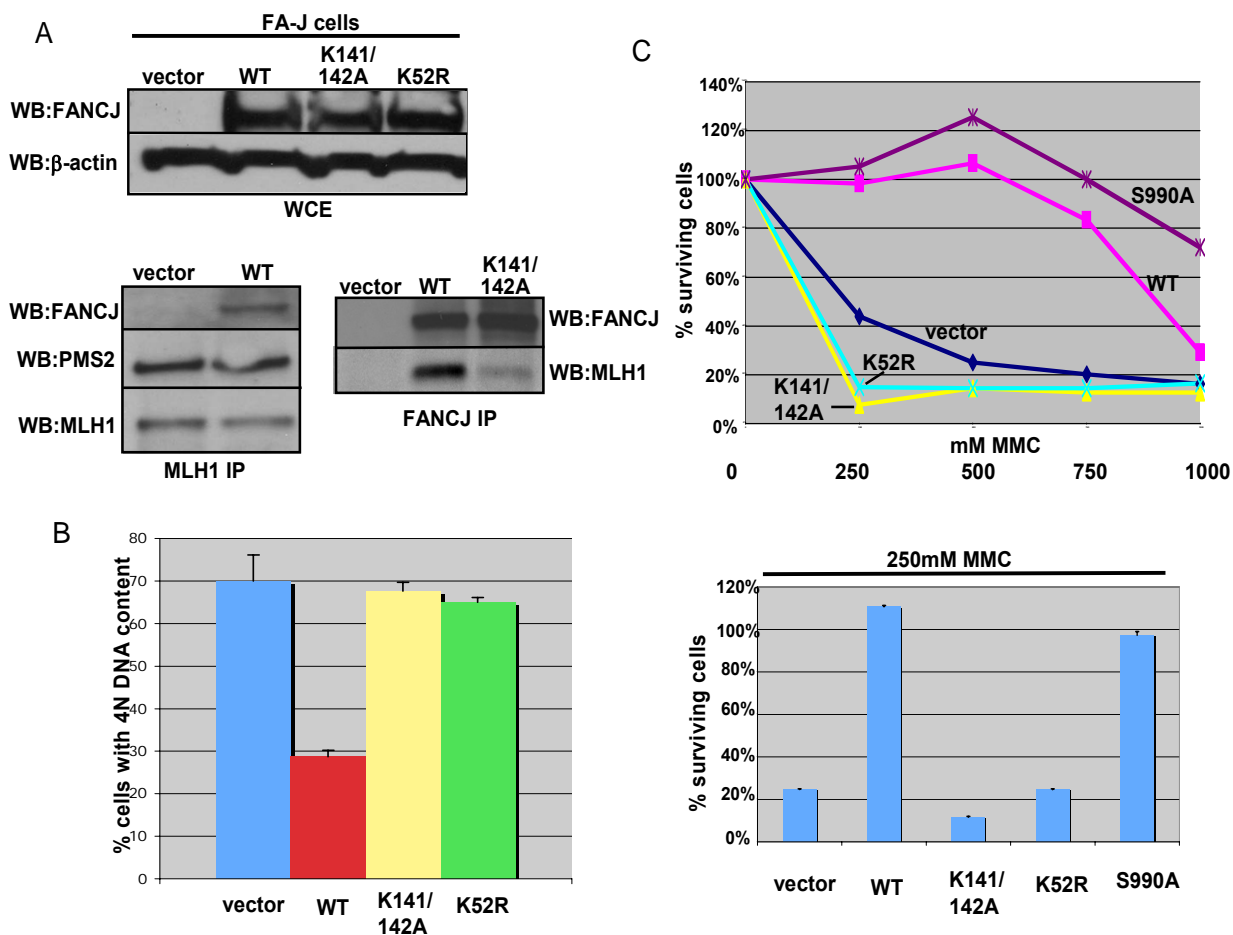
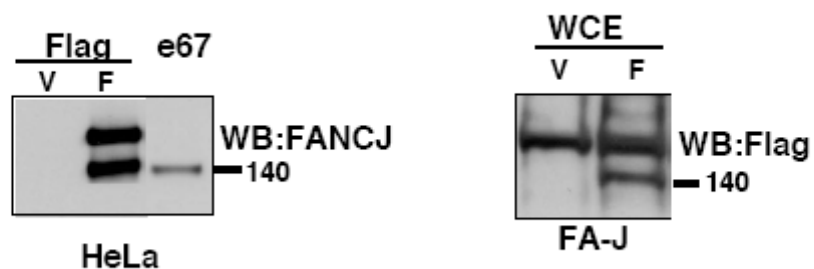


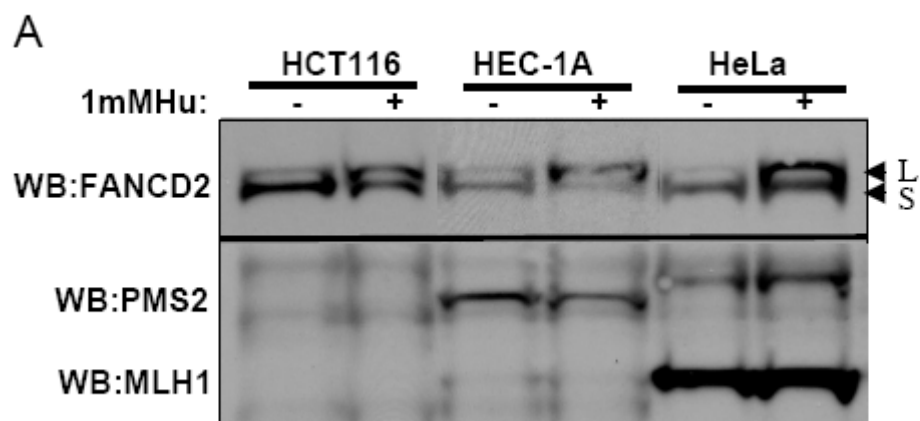
Figure 4-6: MLH1 binding to FANCJ is essential to correct FA-J cells. A) FA-J cells were reconstituted with empty vector, WT, K141/142A, or K52R FANCJ vectors and FANCJ expression was analyzed by whole cell extracts, β -actin serves as a loading control for the WCE samples. Western blot shows the presence of the indicated proteins from both MLH1 IPs and 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) 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 (250mM) was compared for all mutants and error bars represent the standard deviation.



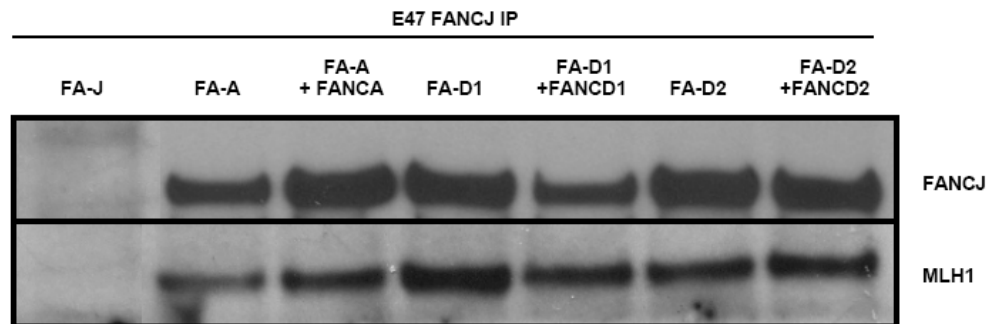
Supplemental Figure S4-7: **The 140Kd FANCI species is recognized by the Flag Ab and migrates similarly to endogenous FANCI.** Left panel: HeLa cells reconstituted with empty vector (V), WT FANCI (F), or uninfected HeLa cells were lysed and immunoprecipitated with Flag or FANCI (e67) Abs and analyzed by Western blot using FANCI Ab. Right panel: FA-J cells reconstituted with empty vector(V), WT FANCI (F), were lysed and analyzed by Western blot using Flag Ab.



Supplemental Figure S4-8: **FANCD2 monoubiquitination is intact in MLH1-deficient cells.** HCT116, HEC-1A, and HeLa cells were either left untreated or treated with 1 mM 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.



Supplemental Figure S4-9: **FANCI/MLH1 interaction is unaltered in other FA cell lines.** FA cells were lysed in 150mM NETN and lysates were normalized. Immunoprecipitations were performed using FANCI Ab (E47) and precipitates were analyzed by Western blot using FANCI and MLH1 specific Abs.



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 described⁹⁹. 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 CO₂. FA-J cells were infected with the pOZ retroviral vector¹⁴⁷ containing no insert, WT, K141/142A, or K52R FANCI inserts, or with the lentiviral vector pLentiV5 (Invitrogen) vector, containing no insert, WT, or S990A FANCI 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 (7µg/mL).

Purification of a FANCI complex

A FANCI complex was purified from nuclear extracts (NE) derived from ~8 X10⁹ HeLa cells stably expressing the double tagged-FANCI by two step immunoaffinity chromatography according to the standard method¹⁴⁷. 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, 10 µg/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 FANCI (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-FANCI (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).

4N DNA content accumulation assay

FA-J stable cell lines 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. 1×10^4 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.

Plasmid construction and in vitro translation

The WT and S990A FANCIJ pLentiviral vectors were a gift of J. Chen⁹⁵. The pCDNA3-myc.his vector (Invitrogen) was digested by NotI/ApaI and different FANCIJ fragments generated by PCR and digested NotI and ApaI were inserted. Primers are available upon request. The WT FANCIJ pOZ-FH vector was generated by PCR cloning. Specifically, 5'-3' XhoI and a 3' NotI 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 FANCIJ-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 FANCI pCDNA3myc.his⁵ and pVL132Flag tagged⁶ 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 (Promega) and MLH1 pCDNA3 vector as a template¹⁶¹ gift of Guido Plotz (Homburg/Saar, Germany).

ELISA studies

Purified recombinant MLH1 protein was diluted to a concentration of 1 ng/ μ l in Carbonate buffer (0.016 M Na₂CO₃, 0.034 M NaHCO₃, pH 9.6) and added to appropriate wells of a 96-well microtiter plate (50 μ l/well), which was incubated at 4 °C. 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 °C with Blocking buffer (phosphate buffered saline, 0.5% Tween 20 and 3% BSA). The procedure was repeated. Purified recombinant FANCI protein was diluted in Blocking buffer, and the indicated concentrations were added to the appropriate wells of the ELISA plate (50 μ l/well), which was incubated for 1 hr at 30 °C. For ethidium bromide (EtBr) or DNaseI

treatment, 50 µg/ml EtBr or DNaseI (2 µg/ml) was included in the incubation with FANCI 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-FANCI antibody (Sigma, B-1310) that was diluted 1: 5,000 in Blocking buffer. Wells were then incubated at 30 °C 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 °C. After washing five times, any FANCI bound to the immobilized MLH1 was detected using OPD substrate (Sigma). The reaction was terminated after 3 min with 3 N H₂SO₄, and absorbance readings were taken at 490 nm.

Recombinant protein and helicase assays

Hi5 cells were infected with pVL132 K141/142A FANCI 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 FANCI proteins were purified as previously described⁶. Briefly, K141/142A FANCI-Flag was immunoprecipitated with 50 µl of FlagM2 conjugated beads for 2 hr at 4°C. 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 FANCI 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 MgCl₂, 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 FANCI helicase. Helicase reactions were initiated by the addition of FANCI and then incubated at 30 °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 described¹³⁹.

Acknowledgments

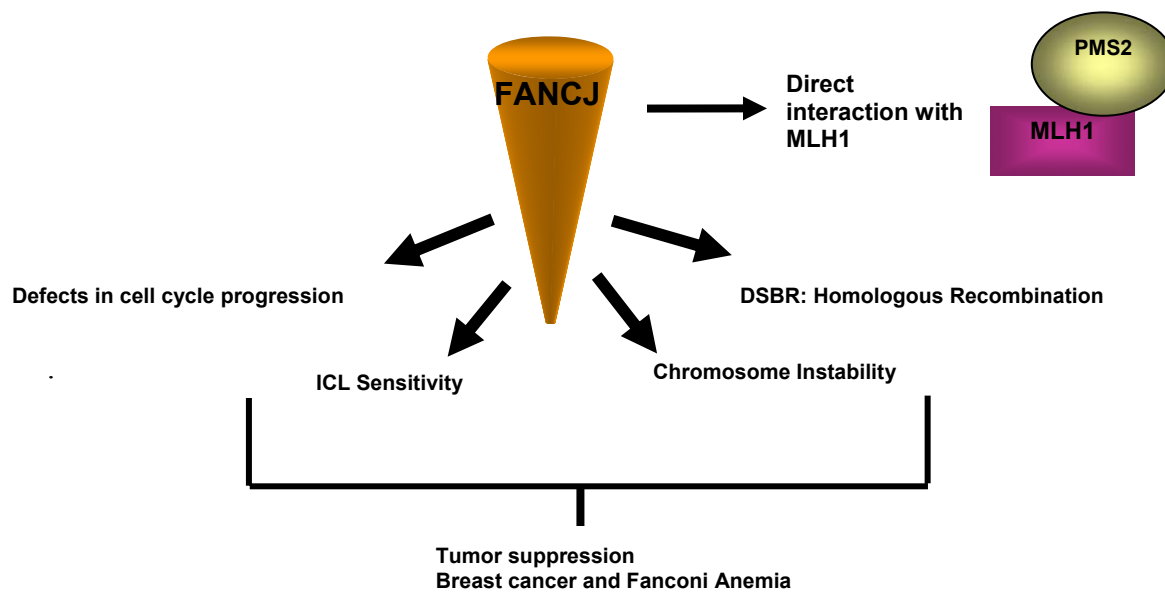
We are grateful to Hans Joenje (UMC, Netherlands) for the FA-J fibroblasts (EUFA30-F), Guido Plotz (Homburg/Saar, Germany) for the MLH1 pCDNA3 vector, and Junjie Chen (Mayo Clinic, MN) for the WT and S990A FANCI pLenti vectors. This work was funded by a grant from the Mary Kay Ash Charitable Foundation and is supported by grant #IRG 93-033 from the American Cancer Society. We are also grateful to Martha Berman and Robert Lipp of the Bari Lipp Foundation for their support. We are grateful to the Fanconi Anemia Research Foundation for FA cells and Abs.

CHAPTER V

FINAL THOUGHTS AND FUTURE DIRECTIONS

Environmental carcinogens and metabolic byproducts pose a constant threat to the genome and highlight the importance of the DNA damage response in maintaining genetic stability. Not surprisingly, mutations in DNA damage response proteins like FANCD1 can result in persistent damage and ultimately lead to the development of cancers. However, this phenotype is not a ubiquitous phenomenon for mutations in all DNA damage response proteins. Thus, I sought to identify how FANCD1 participates in the DNA damage response pathway and why loss, and/or mutations in FANCD1 lead to cellular transformation. From my thesis research, we have demonstrated that FANCD1 is a critical component of the DNA damage response pathway and loss of FANCD1 leads to defects in HR, aberrant cell cycle progression, and genomic instability. Furthermore, we identified germline mutations in FANCD1 in patients with FA and solidified the role of FANCD1 as a tumor suppressor. Lastly, we have demonstrated that the FANCD1/BRCA1 interaction and the FANCD1/MLH1 interaction serve to regulate distinct FANCD1 functions. Disruption of the FANCD1/BRCA1 interaction causes a significant decrease in repair by HR. However, loss of the FANCD1/MLH1 interaction disrupts normal cell cycle progression and sensitizes cells to MMC. Taken together our data suggest FANCD1 provides a novel link between the FA/BRCA and MMR pathways, expanding the role of

Figure 5-1: **FANCI functions.** Functions identified for the FANCI helicase in the DNA damage response and tumor suppression.



FANCI in genomic maintenance and emphasizing its significance in tumor suppression (Figure 5-1).

Our initial studies on FANCI demonstrated that FANCI was a DNA damage response protein, which was phosphorylated following cellular exposure to DNA damaging agents. The effects of DNA damage induced phosphorylation have been documented in several proteins including BRCA1, FANCD2, and the Werner helicase^{162,163}. Following exposure to IR, BRCA1 is phosphorylated at several residues and mutations that abolish phosphorylation at these specific sites cause cellular sensitivity to DNA damage, defects in cell cycle checkpoints, and/or delays in DNA damage repair kinetics. Specifically, the DNA damage-induced phosphorylation of BRCA1 at S988 is critical for maintaining homologous recombinational repair. Serine to Alanine point mutations at the S988 not only disrupt HR, but promote non-homologous end joining¹⁶⁴. Conceivably, DNA damage-induced phosphorylation of FANCI could be central to FANCI function. Thus, it will be important to determine the kinase responsible for the phosphorylation(s) and at what site(s) the FANCI protein is modified. Moreover, it will be critical to understand how this phosphorylation may affect FANCI DNA damage response. We have provided evidence supporting a role for FANCI in HR, thus it is plausible that the phosphorylation of FANCI may contribute to homology directed repair. It is clear that maintaining homologous recombinational repair is essential to preserving the integrity of the genetic sequence. Thus, mutations in FANCI abrogating phosphorylation, may disrupt DNA damage repair, increase mutations, and ultimately, contribute to cancer development.

There is direct evidence linking FA/BRCA proteins to HR. Specifically, in the absence of FA/BRCA proteins, including FANCA, FANCC, FANCD1, FANCD2, FANCI, and BRCA1, homology directed DSB repair is diminished or disrupted. Indirect evidence supporting FA/BRCA recombination function comes from the finding that these proteins are essential for crosslink repair, a process that requires HR. Whether direct or indirect, FA/BRCA proteins respond to DNA damage and are essential for the ICL-repair response. Following ICL damage, the FA/BRCA pathway is activated and FANCD2 is monoubiquitinated by the FA core complex. Activated FANCD2 translocates into chromatin and may help stabilize the replication fork to prevent replication fork collapse and/or illegitimate recombination. Once stabilized, the fork can then be processed by the nucleotide excision repair and/or base excision repair machinery. These repair pathways inevitably lead to the formation of a DSB, which must be repaired by HR to ensure that genetic information is not mutated or deleted. Conceivably, the recruitment of repair factors to the site of the DSB may be initiated by FANCD2 monoubiquitination. BRCA1, FANCD1, and FANCI have all been directly implicated in the HR repair process and have all been linked to an association with FANCD2. These findings suggest that FANCD2 may not only mediate the DNA damage signal, but it may function as a molecular bridge between FA pathway activation and orchestration of the recombination process.

While the core complex may function more indirectly in HR, these proteins should not be overlooked as they likely mediate the repair process. For example, FANCG directly interacts with the RAD51 paralog XRCC3. XRCC3 is a key

component in the recombination repair pathway and facilitates the formation of the RAD51 nucleoprotein filament. FANCG can be coprecipitated with both XRCC3 and BRCA2 and serves as a bridge stabilizing this ternary complex⁴¹. These findings highlight the importance of FANCG not only to FANCD2 monoubiquitination, but also following FANCD2 monoubiquitination during the early stages of recombination. Thus, monoubiquitination of FANCD2 alone may not be sufficient to mediate the ICL response and coordinate repair. The FA core complex is composed of 9 FA subunits and although they are critical for FANCD2 monoubiquitination, they have also demonstrated a role in mediating FANCD2 activity independent of FANCD2 monoubiquitination. Specifically, a FANCD2 monoubiquitin fusion protein expressed in FA-C, FA-G, FA-D2, and FA-L cells failed to complement defects in MMC sensitivity or MMC-induced chromosomal breakage, but complemented these defects in FA-D2 cells. These findings suggested the defects observed in FA-C, FA-G, and FA-L cells are not solely due to loss of FANCD2 monoubiquitination, but underlying defects resulting from the loss of a subunit. In fact, in addition to FANCD2 monoubiquitination, FA-C, FA-G, and FA-L are essential for targeting monoubiquitinated FANCD2 to chromatin and also regulating FANCD2 function in chromatin for effective DNA repair¹⁶⁵. Thus, the FA core complex appears to be critical not only for activation of the FA/BRCA pathway, but also for mediating ICL-induced repair processes.

What remains unclear is if the FA core subunits are required for some aspect of the repair process, why HDR assays have produced conflicting results in some FA null cells. Specifically, loss of FANCA and FANCC have both shown a reduction in HR

frequency by HDR assays, however, FANCC has also demonstrated normal HR repair. These conflicting HR results may be attributed to a more indirect role of these FA subunits in repair. For example, it has been proposed that the FA core subunits may function to stabilize the replication fork at sites of damage, ultimately facilitating homologous recombination. This hypothesis is supported by the finding that the FA core subunits have been observed in both chromatin fractions and also in foci containing FANCD2 following DNA damage. Although the FA core complex is composed of 9 subunits, many of the proteins form smaller subcomplexes. For example, FANCA directly interacts with FANCG and forms a subcomplex with FANCB/FANCL/FANCM. Thus, suppression of FANCA may disrupt FANCD2 monoubiquitination, and assembly of the FANCA/G/B/L/M subcomplex, but leave other subcomplexes in tact. As a result only the remaining subunits are left to stabilize the fork, compromising the integrity of recombination intermediates and framework of the repair machinery. Consequently, only a portion of the replication forks are appropriately processed for recombination causing a partial reduction in HR. Thus, the role of the core complex may be more dynamic than originally thought, extending beyond FA/BRCA pathway activation to homology directed repair.

Although we have demonstrated a clear role for FANCI in HR, it is unclear at which stage in recombination FANCI functions. In collaboration with Robert Brosh, we have shown that in vitro FANCI unwinds D-loop recombination intermediates, yet it is unable to process HJ intermediates. This feature is unique to FANCI, as BLM and WRN DNA repair helicases have shown proficiency in processing HJ structures. Conceivably,

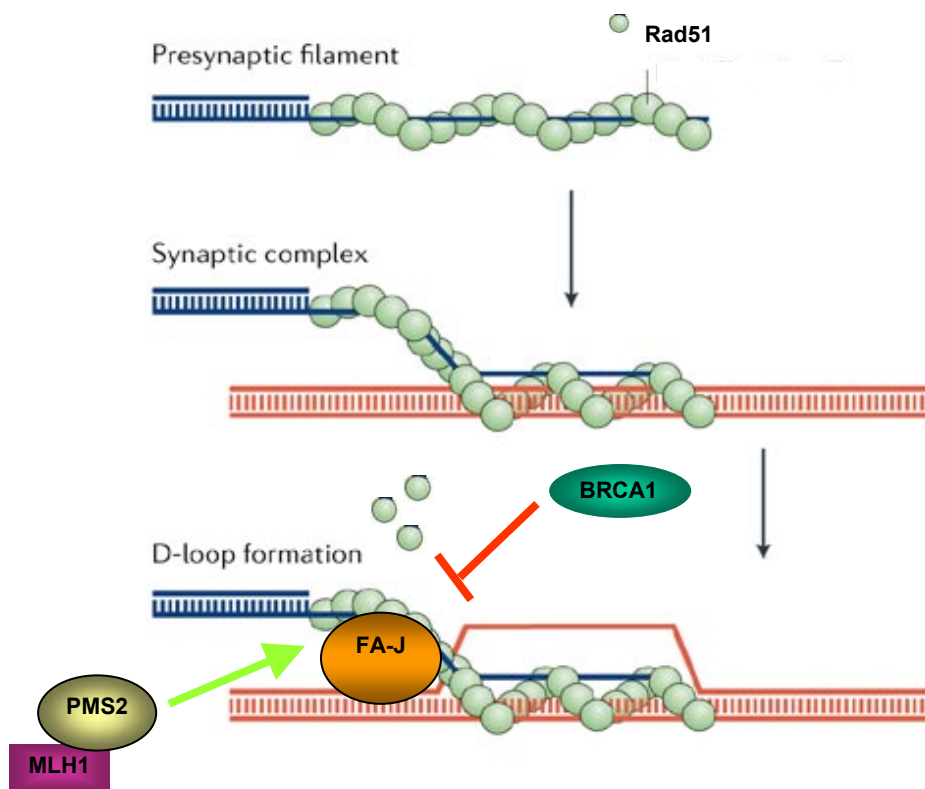
FANCI may bind D-loop structures to regulate homologous pairing of heteroduplex DNA during recombination. The D-loop is the initial structure formed in recombination when the Rad51 nucleoprotein filament invades the duplex DNA in search of homologous repair-template. Thus, Rad51 focal accumulation has been speculated to represent the development of these HR intermediates. Suppression of either BRCA1 or FANCI protein causes a cellular defect in HR, however, one difference observed between BRCA1 and FANCI deficient cells is the visualization of Rad51 foci following DNA damage. Suppression of BRCA1 results in loss of Rad51 foci formation, however, following suppression of FANCI, robust Rad51 foci are readily observed. We speculate that the difference in Rad51 foci formation is a result of unregulated recombination. In unperturbed cells both FANCI and BRCA1 proteins are present and capable of coordinating HR, which we visualize as Rad51 foci. In the absence of BRCA1, the FANCI helicase becomes unregulated and disrupts the formation of recombination intermediates. This disruption leads to inhibition of HR and consequently abolishes Rad51 foci formation. In contrast, in the absence of FANCI, HR is initiated, however, the process becomes unregulated and homologous heteroduplex DNA pairing is replaced by nonhomologous pairings resulting in illegitimate, or error prone, recombination. This hypothesis is further supported by the interaction between FANCI and the MMR protein MLH1. MLH1 may function to regulate FANCI helicase activity in mammalian cells as the *E. coli* homolog of MLH1, MutL, has been shown to regulate the *E. coli* helicase UvrD in vitro. Therefore, we speculate that the interaction between BACH1 and MLH1 is critical for regulating the stringency of recombination between heteroduplex DNA.

Taken together, we have proposed a model in which BRCA1 and MLH1 serve to regulate FANCD1 helicase activity for efficient and accurate HR (Figure 5-2).

Our initial findings suggested that the S990A FANCD1 mutant rescued both the MMC sensitivity and the ICL-induced 4N accumulation in FA-J cells. However, more recently we found that disruption of the FANCD1/BRCA1 interaction disrupted HR in a homology directed repair assay. Due to these potentially conflicting results, we looked more closely at the ability of the S990A FANCD1 mutant to correct FA-J cells.

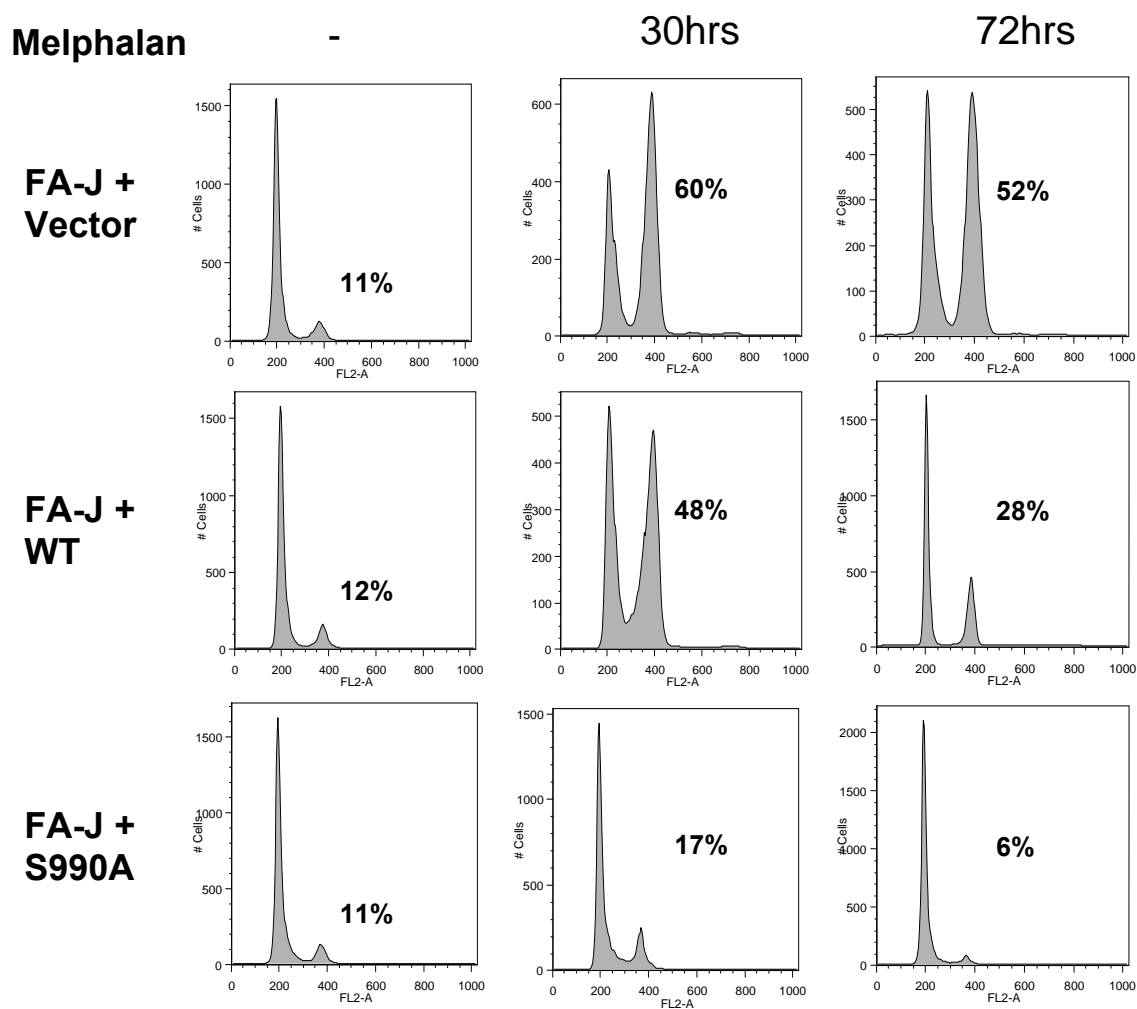
Specifically, we analyzed the ICL-induced 4N accumulation in S990A FANCD1 reconstituted FA-J cells over the course of 96 hours. Although FA cells are characterized by a 4N accumulation at 72hrs following ICL treatment, both vector and WT FANCD1 reconstituted FA-J cells also accumulate with 4N DNA content approximately 30 hrs after ICL treatment. However, the S990A FANCD1 reconstituted FA-J cells did not accumulate with 4N DNA content at 30hrs following ICL treatment. In fact, these cells did not accumulate with a 4N DNA content even at 96 hours following ICL damage. Contrary to our original findings, these data suggest that the S990A FANCD1 mutant does not respond to ICL damage like WT FANCD1 (Figure 5-3).

Figure 5-2: **Model for FANCDJ regulation in DNA repair.** We hypothesize that BRCA1 and the MLH1/PMS2 proteins function to regulate FANCDJ during recombination (see text for details).



Adapted from Sung and Klein *Nature Reviews Molecular Cell Biology* 7, 739–750 (October 2006)

Figure 5-3: **S990A FANCI mutant does not correct defect in FA-J cell ICL response.** FA-J cells were reconstituted with vector, WT FANCI, or S990A FANCI and cell cycle progression was analyzed for 96 hrs following treatment with melphalan. A representative cell cycle profile is shown for untreated or 30hrs and 72hrs following treatment.



To further investigate this difference in ICL response between S990A FANCI and WT FANCI reconstituted cells, we analyzed the formation of both H2AX and RAD51 foci following ICL damage. Both WT FANCI and S990A FANCI reconstituted FA-J cells formed H2AX and RAD51 foci by 12hrs following ICL treatment. In WT FANCI reconstituted cells these foci dispersed to baseline levels by 72hrs following ICL treatment, however, in S990A FANCI reconstituted FA-J cells both H2AX and RAD51 foci returned to baseline levels by 48hrs. Thus, it appears that the ICL response is initiated in both WT FANCI and S990A FANCI reconstituted FA-J cells, however, the S990A FANCI mutant demonstrates different kinetics in response to ICL treatment. Conceivably, the differences in ICL response in the S990A FANCI reconstituted FA-J cells could be explained as differences in types or rates of repair. The early formation of RAD51 foci in both the WT FANCI and S990A FANCI reconstituted FA-J cells suggests that RAD51 mediated repair is initiated effectively. However, the premature dispersal of both H2AX and RAD51 foci in the S990A FANCI reconstituted

FA-J cells suggest that repair is either completed faster, or by a repair mechanism independent of RAD51. This hypothesis is supported by our initial findings that disruption of the FANCI/BRCA1 interaction cause defects in HR. Moreover, S990A FANCI reconstituted FA-J cells are not sensitive to ICL damage suggesting that repair does in fact occur, but that this repair may be non-homologous. It seems likely that disruption of the FANCI/BRCA1 interaction disrupts HR, however, promotes an alternative type of repair such as SSA, NHEJ, or homologous recombination. We intend to quantitate the differences in ICL-induced 4N accumulation and H2AX and RAD51

foci formation observed in the S990A FANCIJ reconstituted FA-J cells to validate our preliminary findings. We have also initiated a collaboration with a recombination expert, Lisa Weismüller, to analyze how disruption of the FANCIJ/BRCA1 interaction affects various recombination substrates (i.e. SSA, NHEJ, and homeologous).

Recombination is highly regulated process, however when it becomes unregulated, non-homologous DNA sequences are permitted during repair and as a result, the original DNA sequence can become mutated. This unregulated recombination leads to genomic instability and can allow cancer cells to rapidly adapt to and bypass replication-blocking lesions generated by DNA damaging agents. Thus, mutations in genes that regulate recombination can predispose cells to cancer and in some instances, make them resistant to chemical therapies that rely on replication blocking lesions for toxicity. For example, loss of the MMR proteins is linked not only to hereditary colon cancer, but also to the acquired resistance of tumor cells to chemotherapy. While the mechanism of chemoresistance afforded by loss of MMR proteins is still largely unknown, it may be linked to unregulated hyper-recombination. In support of this possibility, resistance of MMR-deficient yeast cells is dependent on recombination proteins RAD52 and RAD1¹⁶⁶. If the hyper-recombinogenic phenotype is required for resistance of mammalian cells, inactivation of recombination could re-sensitize MMR-deficient cancer cells to chemotherapy. The goal of our future studies is to test whether inhibition of recombination in MMR deficient cells can restore their sensitivity to DNA damaging agents. Furthermore, if hyper-recombination is universally required for the

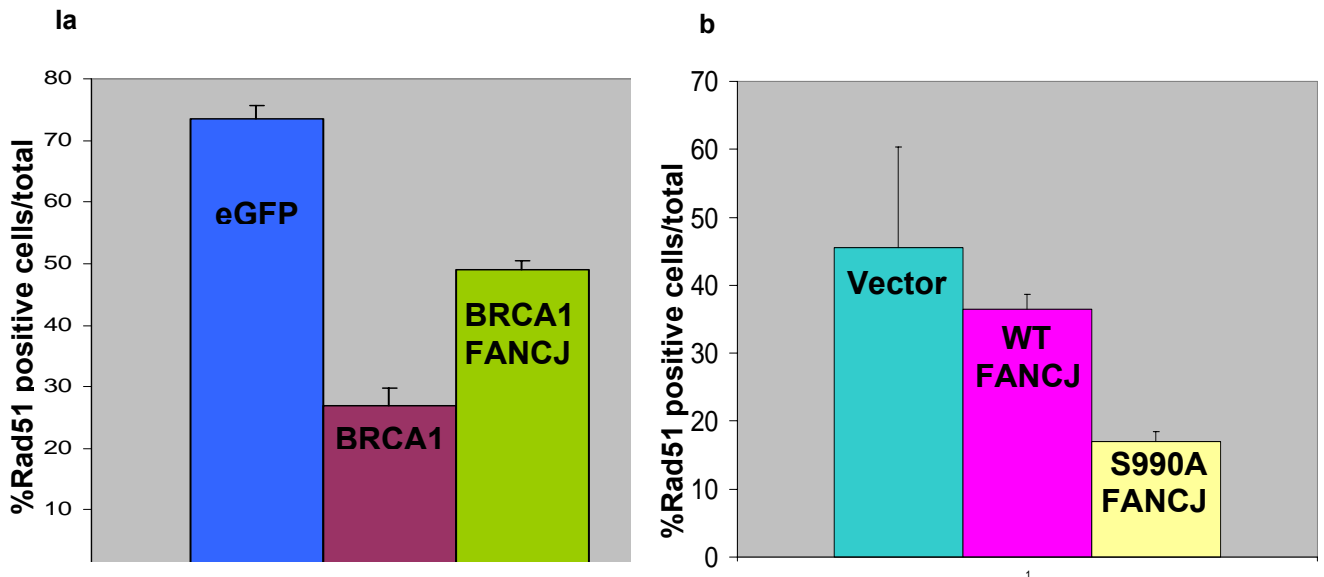
development of chemoresistance, recombination inhibitors could have a major impact on the effective treatment of cancer.

In conclusion, the studies presented in this thesis have had a major impact in our understanding of both the FA and MMR DNA damage response pathways and have encouraged future studies in this area. In the past, basic mechanisms of DNA repair and DNA repair pathways were exploited in cancer cells to treat the cancer. However, these therapies blanketed patients with several treatments in hopes that one would send the cancer into remission. There is a growing interest in developing new cancer therapies tailored to meet the needs of each patient individually and has launched basic research into a new age of specifically targeted anti-cancer drugs. It is our hope that the work presented here will contribute to the development of these treatments and ultimately the fight against cancer.

Appendix I

In our model we propose that BRCA1 and MLH1 serve to regulate FANCD1 during homologous recombination. Thus, we predicted that disruption of either of these interactions would upset the equilibrium of the complex and ultimately, disrupt effective recombination. The obvious difference between BRCA1 and FANCD1 deficient cells is in the accumulation of Rad51 foci following DNA damage. The presence of Rad51 foci visualized by immunofluorescence has been hypothesized to represent the formation of the Rad51 nucleoprotein filament during recombination. We had already demonstrated that both BRCA1 and FANCD1 were required for HR, however, it was unclear why Rad51 foci accumulated in FANCD1, but not BRCA1 deficient cells. Conceivably, BRCA1 regulated FANCD1 helicase activity during recombination. For example, the assembly and disassembly of the Rad51 nucleoprotein filament is critical in the regulation of HR. In *S. cerevisiae* the Srs2 helicase functions to strip Rad51 from the nucleoprotein filament preventing the early stages of HR. Similarly, we hypothesized that FANCD1 functions to strip Rad51 from the nucleoprotein filament following strand invasion releasing Rad51 for subsequent rounds of recombination. Moreover, we predicted that this process was regulated by BRCA1. Suppression of BRCA1 protein expression causes a significant decrease in the accumulation of Rad51 foci. However, if the loss of Rad51 foci is due to unregulated FANCD1 helicase activity, suppressing FANCD1 in these cells would restore the appearance of Rad51 foci. To test this hypothesis we created SKOV3 cells and suppressed EGFP, EGFP/BRCA1, or BRCA1/FANCD1, induced DNA damage, and quantitated the percentage of cells positive for Rad51 foci (> 5 foci) by

immunofluorescence (Figure 1a). We found that co-suppression of BRCA1 and FANCD1 increased the percentage of Rad51 positive cells compared to suppression of BRCA1 alone. To further support these findings we reproduced these results in another cell line. We used FANCD1 null FA-J cells and reconstituted them with empty vector, WT FANCD1, or S990A FANCD1 (no BRCA1 binding). As before, we induced DNA damage and quantitated the percentage of cells positive for Rad51 foci (Figure 1b).

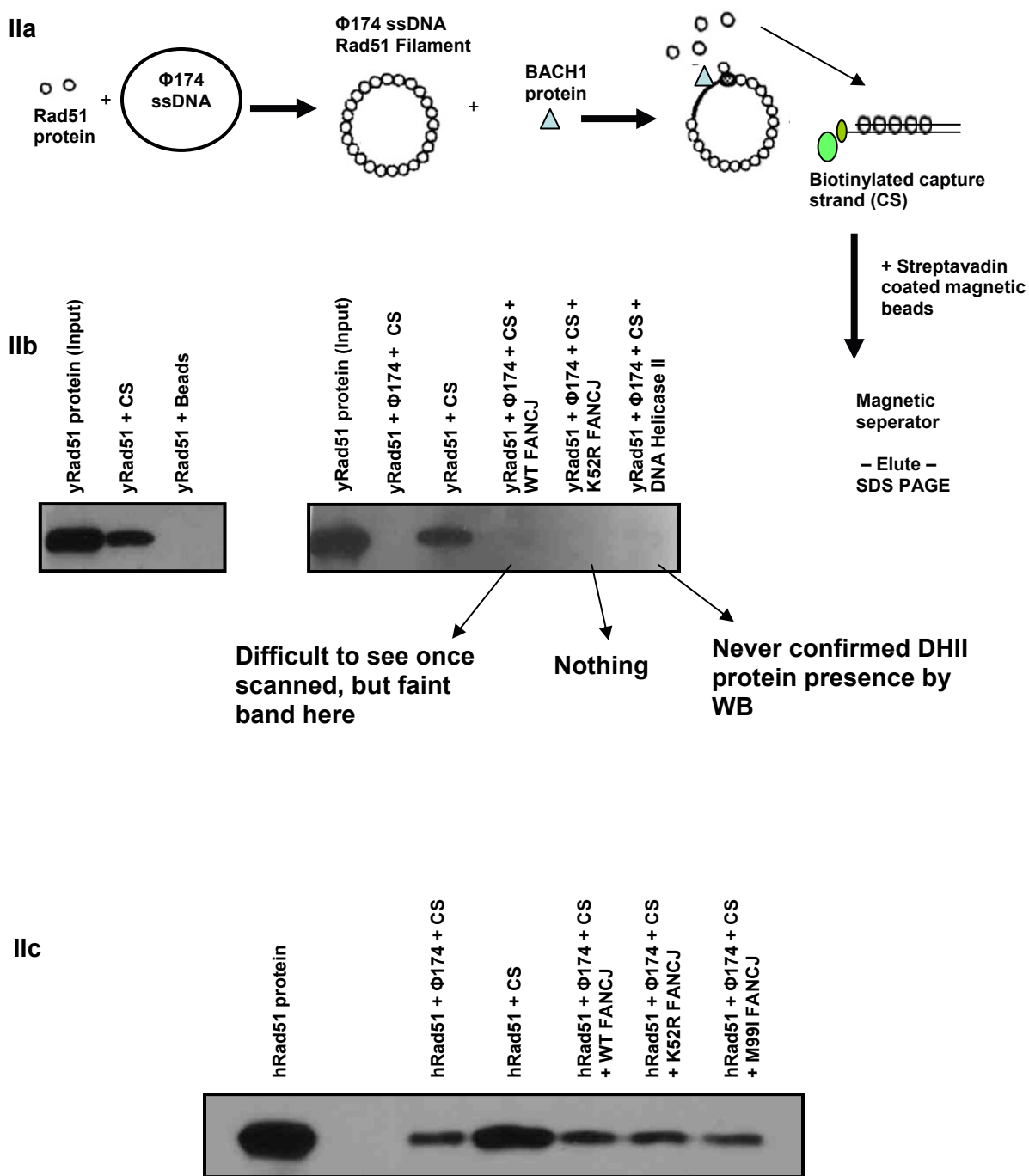


Appendix II

To determine whether FANCI could strip Rad51 directly from ssDNA, we compared the ability of the WT FANCI and the K52R mutant FANCI to disassemble the Rad51 nucleoprotein filament *in vitro*. This experiment was performed as previously described (Fig IIa)¹⁶⁷. Briefly, the 174 ssDNA was incubated with yeast Rad51 (yRad51) protein for 5 minutes at 37°C. In the experimental reactions, WT FANCI, K52R FANCI, or DNA helicase II was added and the reaction was incubated for another 5 minutes at 37°C. During this time, the capture strand was incubated with the streptavidin coated beads and then added to the initial reaction for a final 5 minutes. The reaction was washed over a magnetic separator and the protein was eluted using SDS sample buffer. Finally, the eluate was separated by SDS PAGE and analyzed by Western blot (Figure IIb right side). We could recover greater than 50% of the yRad51 protein input on the capture strand and yRad51 protein did not stick to the streptavidin coated beads alone (Figure IIb left side). The same experiment was repeated using human Rad51 protein (hRad51) and WT FANCI, K52R FANCI, and M299I FANCI (Figure IIc). The M299I mutation in FANCI is a patient mutation which has demonstrated hyperactive helicase activity *in vitro*.

We did not detect an increase in the amount of Rad51 (yeast or human) recovered on the capture strand when the nucleoprotein filament was incubated with FANCI. This suggested that the observed difference in Rad51 foci between BRCA1 and FANCI deficient cells may not be attributed to disassembly of the Rad51 nucleoprotein filament. However, there are several potential caveats to this experiment. First, we used yRad51

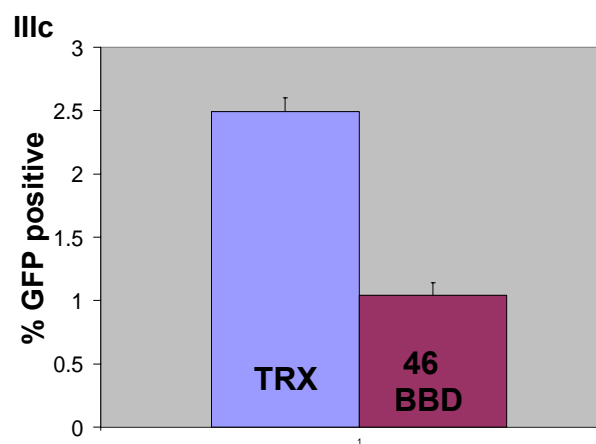
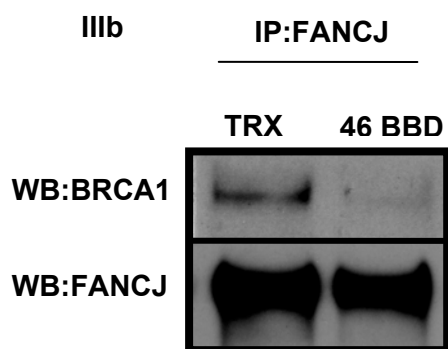
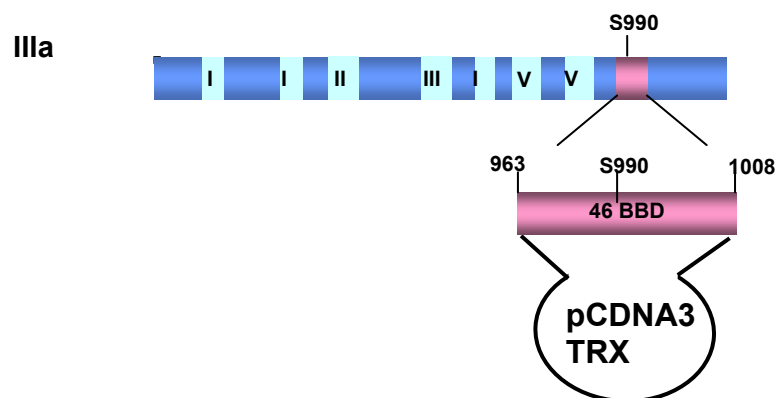
protein in these experiments because the yRad51 protein forms a highly stable interaction with the Φ 174 ssDNA (Figure IIb right side lane 2). However, we could not demonstrate that FANCI functioned to strip the yeast Rad51 protein from ssDNA. In hopes that the FANCI protein required the human Rad51 protein in these experiments, we attempted to form a stable hRad51 nucleoprotein filament. Unfortunately, the hRad51 nucleoprotein filament was not as stable as the yRad51 nucleoprotein filament and we observed an increase in the dissociation of the hRad51 protein from the Φ 174 ssDNA (Figure IIc lane 3). This made it difficult to assess even small changes in Rad51 dissociation between the control and FANCI containing reactions (Figure IIc compare lane 3 and lane 5). Second, it's possible that FANCI requires a DNA damage signal or the association of other protein partners including, MLH1 and/or BRCA1 to displace Rad51 .



RPA was included in all reactions to help stabilize the nucleoprotein filament and stimulate FANCJ helicase activity (Personal communication unpublished data, Robert Brosh)

Appendix III

Although FANCD1 did not appear to disrupt the Rad51 nucleoprotein filament, we continued to investigate whether BRCA1 served to regulate FANCD1 function. If BRCA1 regulates FANCD1 during recombination, disruption of this interaction would result in a decrease in HR by our homology directed repair assay (chapter 3). To disrupt the BRCA1/FANCD1 interaction, we created a 46 amino acid FANCD1 peptide that includes the region of FANCD1 required for its interaction with BRCA1 (46 amino acid BRCA1 Binding Domain - 46BBD) (Figure IIIa). Both the empty TRX vector and the 46BBD were transfected into U2OS cells and incubated for 48hrs. The cells were lysed and the disruption of the FANCD1/BRCA1 complex was determined by FANCD1 immunoprecipitation and analyzed by Western blot (Figure IIIb). Either empty TRX vector or 46BBD FANCD1 peptide was transfected along with and the Sce-1 endonuclease into U2OS cells containing the DR-GFP repair substrate and incubated for 72hrs. The cells were collected and the percentage of GFP positive cells was analyzed by FACS. Error bars represent the standard deviation (Figure IIIc).



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