Role of FANCA in DNA Damage Repair

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ROLE OF FANCA IN DNA DAMAGE REPAIR

By

Anaid Benitez

A DISSERTATION

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ROLE OF FANCA IN DNA DAMAGE REPAIR

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Fanconi anemia (FA) is a severe genetic disorder characterized by bone marrow failure, developmental defects, chromosomal instability, and predisposition to cancer. FA cells are hypersensitive to DNA crosslinking compounds including mitomycin C (MMC), cisplatin, and diepoxybutane, which make defective interstrand crosslink (ICL) repair mechanisms a hallmark for the disease. Of the 17 genes associated with FA, mutations in the complementation group A (FANCA) account for ~64% of all patient cases. Studies explored in this dissertation comprise the biochemical details for the functional role of FANCA in DNA ICL repair and the first biochemical evidence for its possible role in double-strand break (DSB) repair.

In an oligonucleotide-based assay purified FANCA shows enhancement of MUS81-EME1-mediated ICL incision. On the contrary, FANCA exhibits a two-phase incision regulation when DNA is undamaged or the damage affects only one DNA strand. MUS81-EME1 is a DNA endonuclease involved in replication-coupled repair of ICLs. A prevalent hypothetical role of MUS81-EME1 in ICL repair was to unhook the damage by incising the leading strand on the 3’ side of an ICL lesion. The studies presented in Chapter 3 show that purified MUS81-EME1 incises DNA on the 5’ side of a psoralen ICL residing in fork-like structures. Using truncated FANCA proteins, I determined that both the N- and C-regions of the protein are required for the observed FANCA-dependent
MUS81-EME1 incision regulation. Using laser-induced psoralen ICL formation in cells, I found that FANCA colocalizes with and recruits MUS81 to ICL lesions.

Due to the specific ICL recognition activity of FANCA in vitro, I hypothesized that it was likely to catalyze strand separation in order to discriminate damage that crosslinks the DNA duplex from damage that only affects one strand. To test my hypothesis, I examined the effect of FANCA on DNA stability. In an oligonucleotide-based assay purified FANCA shows strong helix destabilization, single-strand annealing and strand exchange activities that are completely dependent on protein:oligonucleotide stoichiometric ratios. While low stoichiometric ratios of FANCA to DNA result in helix destabilization, higher ratios catalyze single-strand annealing and strand exchange. Furthermore, FANCA and RAD51 exhibit synergistic DNA strand annealing activity, suggesting their coordinated function might play a role in fork stability. FANCA also promotes the bidirectional annealing of structures that mimic physiologically relevant intermediates in DSB repair. C- and N-terminal truncation mutants reveal that binding to DNA is not sufficient for the helix destabilization or single-strand annealing activities of FANCA. Patient-derived FANCA mutants Q772X, D598N, R1117G, Q1128E, and F1263Δ exhibit deregulated helix destabilization, strand annealing and strand exchange activities.

It is conceivable that proteins harboring helix destabilization, single-strand annealing, and strand exchange activities work in concert to protect genome integrity and efficiently process replication and recombination intermediate structures. It’s possible that in the presence of an ICL, FANCA may serve to recognize the damage, help anchor the FA core complex to the DNA, recruit structure-specific endonuclease MUS81/EME1 and
enhance its incision activity accordingly. When replication forks are stalled due to sources other than ICLs, FANCA may be recruited to remodel the fork and promote fork-restart through ssDNA annealing of excessively unwound template or fork regression and “chicken foot” formation through single-strand annealing of the nascent DNA. FANCA may also act as a local helix destabilizing protein that promotes fork protection by drawing the nuclease away from the fork, thereby inhibiting it’s incision activity. Collectively, it appears FANCA may have multiple roles in ICL repair, particularly downstream of FA pathway activation during the repair of intermediate DSB structures. It’s possible that FANCA works in concert with RAD51 or RAD52 to promote the repair of DSBs through homology-directed pathways strand invasion and single-strand annealing.
To my loving family and friends.
May we all find the freedom to pursue our dreams
and the courage to live them.
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### ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide(s)</td>
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CHAPTER 1 - INTRODUCTION

Fanconi anemia

Fanconi Anemia (FA) is a rare hereditary disorder caused by biallelic mutations in at least one of 17 genes and is clinically characterized by bone marrow failure, congenital abnormalities, and predisposition to cancer (1,2). Patients with FA may present with a variety of clinical manifestations ranging from short stature and skin pigmentation to radial deficiency and mental retardation. Misdiagnosis or failure to diagnose an FA patient is a common life-threatening problem (3). Close monitoring in highly specialized multi-disciplinary centers is crucial for their survival.

Cells from FA patients show hypersensitivity to DNA crosslinking agents like psoralens, cisplatin and mitomycin C, accumulate chromosomal aberrations and intragenic deletions, and arrest at the G2/M checkpoint (4-7). Clinical diagnosis for FA consists on the evaluation of cellular sensitivity to these DNA crosslinking agents (8-10). Complementation analysis, or FA subtyping, is used to define the complementation group (FANC) responsible for the FA phenotype; in this test patient cells are transduced with FANC cDNAs that complement different FA subtypes (9).

A total of 17 FANC complementation groups have been discovered and proven to function in the maintenance of DNA during cellular replication (11-13). Eight FANC proteins assemble into the FA-core complex including FA complementation group A (FANCA), FANCB/C/E/F/G/L/M, along with FAAP20/24/100 and FAAP10/16 (MHF1/2), a histone-fold protein complex. All members of the FA-core complex are required for complete and successful activation of the FA pathway in vivo. The
coordinated functions of the FA-core complex can be subdivided into one catalytic subunit FANCL/B/FAAP100 and 3 modules that serve in chromatin anchoring FANCM/FAAP24, FANCA/G/FAAP20, and FANCC/E/F (14).

Fanconi anemia complementation group A

The most commonly affected complementation group in FA patients is FANCA, accounting for ~64% of all mutations (15-17). While the gene (1455 aa) was first cloned in 1996 (18), the structure of the encoded 163kDa protein is yet to be resolved and its function is still under examination. FANCA protein can localize to the cytoplasm and nucleus, but its subcellular distribution will vary in accordance to cell cycle phase (19) (Figure 1-1). Although the cell cycle regulation of FANCA has not been extensively studied, its full-length sequence includes a nuclear localization signal on the N-terminus and multiple export sequences that are located throughout the gene (20,21). The active nuclear export of FANCA has been linked to its association with nuclear export protein XPO1, also called CRM1 (20). Several lines of evidence indicate that the function of FANCA may vary in respect to its subcellular localization. Lastly, FANCA is subject to regulation at the mRNA level by alternative splicing mechanisms and at the protein level by phosphorylation, yet the significance of these regulations is still unclear.

In an attempt to decipher the cellular functions of FANCA, multiple proteins have been identified as interacting partners. Notably, FANCA forms part of the FA-core complex and specifically associates with proteins FANCG, FAAP20 and FANCC (22-24). Our lab has previously shown that FANCA has intrinsic affinity to nucleic acids (25). Consistent with this, FANCA was later shown to form part of the FANCA/G/FAAP20 module, which functions as one of three chromatin-anchoring units
of the FA-core complex (14). FANCA has also been shown to interact with breast cancer susceptibility protein BRCA1 (26) and found to be present in BRAFT, a multiprotein nuclear complex containing BLM, RPA, FANCA, FANCG and Topo IIIα (27). These associations are indicative of a role for FANCA in replication fork maintenance and/or DSB repair.

Cells deficient in FANCA protein are 2.3 times more photosensitive than normal cells (28). Currently, the main function of FANCA is thought to align within the context of ICL repair and the FA-core complex. All members of the FA-core complex, including FANCA, are required for FA pathway activation of ICL repair via the mono-ubiquitination of FANCI and FANCD2. Additionally, FANCA deficiency leads to defective unhooking of DNA interstrand crosslinks (ICLs) 24h post-treatment, indicating an important role for FANCA in ICL incision. However, FANCA may have important roles outside of the FA-core complex as it has been implicated in the stabilization and protection of stalled replication forks (29). This indicates FANCA is an important protein in the damage response pathway of ICLs and other fork-stalling sources.

**Interstrand crosslink repair**

FA proteins, FA associated proteins (FAAPs) and structure-specific DNA endonucleases cooperate in the repair process of DNA ICLs, which can be induced exogenously by environmental factors and endogenously as byproducts of normal cellular metabolism (30,31). Crosslinked DNA blocks cellular replication and transcription and its repair is essential for cell survival. Faulty repair and unrepaired lesions can lead to mutations affecting different cell cycle functions and check-points, culminating in cell death or malignant transformation (2).
The FA repair pathway of ICLs is a mixture of independently established DNA repair mechanisms including homologous recombination (HR), nucleotide excision repair (NER), and translesion synthesis (TLS) (2) (Figure 1-2). ICLs encountered during cellular replication lead to the formation of stalled forks; their recognition is directly linked to the recruitment and chromatin association of the FANCM/FAAP24 module and the MHF1/2 complex, which together promote fork remodeling and subsequent activation of the FA signaling pathway. Little work has been published on replication-independent mechanisms of ICL recognition and repair (32,33).

**Pathway activation and regulation**

The FA ICL repair pathway is under regulation by several mechanisms. One mechanism is CHK1 and ATR phosphorylation of FANCA/E/G/D2/I and the MRE11-RAD50-NBS1 (MRN) complex, the latter of which is required for the 5’-end resection step during double strand break (DSB) repair (2,34). Upon ICL recognition and ATR phosphorylation of downstream effectors including FANCI, pathway activation occurs when FANCL, an E3 ubiquitin ligase within the FA-core complex, mono-ubiquitinates the FANCI-D2 complex. This is followed by the recruitment of multiple structure-specific endonucleases and other repair factors to the site of the damage (35-38). Nicking of the crosslink produces intermediate DSBs that get repaired via the HR pathway (Figure 1-2 [3]). Alternatively, other intermediate structures serve as substrates for TLS and NER for successful completion of the ICL repair process (Figure 1-2 [6] and [7]).
**Pathway crosstalk**

Pathway coordination is essential for effective damage removal, replication fork restoration, and cell cycle progression. With rising advances in high-throughput genetic technologies such as next-generation sequencing (NGS), scientists are discovering increasing amount of crosstalk between different DNA damage repair pathways (see (39) for review). Many FA proteins have multiple roles in DNA repair outside of the FA pathway. FANCD1(BRCA2), FANCO(RAD51C), FANCN(PALB2), FANCQ(ERCC4) and FANCJ are all active during HR repair of DSBs. FANCD2, FANCJ, and FANCP(SLX4) have also been shown to interact with proteins involved in mismatch repair (MMR) (31,40,41). As a result, different mutations of the same protein can affect different cellular pathways and present themselves as different phenotypes. A classic example of this is FANCQ(ERCC4), which can present itself when mutant as Xeroderma Pigmentosum (XP), FA, or XFE progeroid syndrome depending on the defective allele (11). In this scenario one mutation impacts the subcellular distribution of the protein leading to XP, while another mutation leads to FA due to impairment in ICL repair without affecting the cellular localization of the protein (11,39). Similarly, there exists crosstalk between the FA and NER pathways depending on sister chromatid availability. FANCP(SLX4), for example, has been shown to mediate both FA and NER pathway events (39). In all, crosstalk between pathways is an emerging field of study that is allowing scientists to better understand the mechanistic details of a variety of diseases.

**Double-strand DNA break repair**

Nicking of crosslinks during ICL repair produces a variety of complex intermediate structures among which are highly toxic double-strand DNA breaks (DSBs). These are
repaired via non-homologous end joining (NHEJ) or homologous recombination (HR) mechanisms. The former can be further subdivided into canonical/classical-NHEJ and alternative-NHEJ, which includes microhomology-mediated end joining (MMEJ) and more recently a microhomology-independent end joining mechanism (42). HR mechanisms include RAD51-mediated strand invasion (SI), single-strand annealing (SSA), synthesis dependent strand annealing (SDSA), and break induced replication (BIR) events (Figure 1-3). Imperfect NHEJ frequently leads to deletions, translocations, and telomere fusion, while HR makes use of the homologue or sister chromatid as a template to copy lost information. Other pathways such as SSA and MMEJ utilize single-strand annealing activity to anneal exposed complementary regions of ssDNA, followed by heterologous 3’ tail cleavage and end ligation (43).

**Pathway regulation**

All four pathways are in dynamic competition with each other and studies have shown that canonical NHEJ suppresses HR, SSA, and MMEJ, although these can still occur in the presence of NHEJ (43). An early decisive factor distinguishing these pathways is the mechanism of end resection and the extent to which it’s allowed to occur. In NHEJ, little to no end resection is required. MMEJ requires minimal end resection since only 1 to 5 bp is sufficient for microhomology-mediated annealing. In contrast, SSA and canonical HR require more extensive end resection to expose longer stretches of ssDNA, which are stabilized by the formation of protein/DNA filaments (43) (Figures 1-2 and 5-1B). SSA and gene conversion by SI are in competition especially when a DSB occurs between repeats. The relative size of the repeat, as well as its proximity to the break will have an effect on the amount of end resection required to expose the region of
homology, which will in turn affect the choice of repair pathway. Greater distance between two repeats or presence of a third repeat endows SSA between two repeats a disadvantage in competition with gene conversion through a collision mechanism effect (44). Though the biologically relevant function of the SSA pathway remains largely elusive, it’s likely an important alternative pathway of DSB repair in cells with defective strand invasion repair considering over two-thirds of the human genome is composed of repetitive elements (45,46).

End resection events are in turn controlled by cell cycle regulators such as cyclin-dependent kinases (CDKs). Cell cycle regulation of DNA processing can be seen following endonuclease-induced DSBs which leave “clean” ends with a 5’ phosphate and 3’ hydroxyl group and are primarily resected during S or G2 phase (43,47). In contrast, DSBs induced by γ-irradiation , which leaves “dirty” ends with phosphoglycolates and terminal nucleotides, can be resected at any time (43,48,49). Additional regulation of end resection and subsequent repair pathway choice stems from a complex interplay of proteins and repair factors. Focus formation studies of the proteins involved in the DNA damage response show Giga-Dalton assemblies that are dynamically involved in the repair process (43). In canonical HR repair of DSBs, 5’ to 3’ end resection of each side of the break exposes 3’ terminated ssDNA that is quickly coated with replication protein A (RPA). With the help of mediator proteins FANCD1(BRCA2) and RAD52, RAD51 is able to displace RPA and catalyzes strand invasion and exchange with intact homologous DNA on the homologue or sister chromatid (50-52). RAD51-ssDNA nucleoprotein filament formation shuttles repair toward gene conversion whilst suppressing the SSA pathway (43). On the contrary, depletion of RAD51 and FANCD1(BRCA2) function
enhances RAD52-dependent SSA events (46,53). In all, competition between pathways is a dynamic process that is dependent on the nature and availability of target DNA and of repair proteins (54).

**HR mediators and SSA effectors**

FANCD1(BRCA2) is an important mediator of RAD51-dependent strand invasion in mammalian cells (51,52); it promotes RAD51-ssDNA filament formation and stabilization by facilitating RPA displacement and blocking ATP hydrolysis, respectively. But FANCD1(BRCA2) is not the main annealing effector in mammalian cells because while it is able to anneal naked ssDNA, RPA-ssDNA filament formation blocks this activity (51). Moreover, complemented FANCD1(BRCA2) cells showed decreased SSA compared with a FANCD1(BRCA2) null background (46). Nevertheless, cells deficient in FANCD1(BRCA2) present with a strong HR phenotype, likely due to its important role in the RAD51-dependent pathway. The key annealing effector in mammalian cells is RAD52.

Since FANCD1(BRCA2) protein is not conserved in *Saccharomyces cerevisiae*; the main mediator of strand invasion through RAD51-ssDNA nucleoprotein filament formation, as well as SSA in yeast is yRAD52 (43,54). Lack of FANCD1(BRCA2) in yeast is supportive of the theory of functional redundancy, which is evident in creatures with bigger genomes, such as humans (51). Similarly, RAD52 is not conserved in *Caenorhabditis elegans*. In its place, a functional homologue of FANCD1(BRCA2), CeBRC-2, retains both SI mediator and SSA effector activities even in the presence of RPA. This coincides with the notion that the SSA effector and SI mediator functions of
CeBRC-2 might also be redundant in mammalian cells between human RAD52 and FANCD1(BRCA2), respectively (Figure 1-4).

Nonetheless, mammalian cells lacking RAD52 function still present with a relatively weak HR phenotype, which raises the possibility that RAD52 alone is not responsible for catalyzing the annealing step in the SSA pathway (55-57). Other FA proteins including FANCA, FANCG and FANCS(BRCA1) have been shown to promote homology-dependent (HR and SSA) repair of DSBs in vivo. In particular, restoration of FANCA and FANCG in patient derived cell lines from the FA-A and FA-G complementation groups, respectively, showed a ~2.4 fold increase in SSA and HR (46). Other studies involving fancg mutant chicken DT40 cells showed severe impairment in HR when compared to wild type cells (58,59). FA patient-derived cell lines defective in FANCA, FANCC, FANCD2, and FANCG exhibit impaired exogenous plasmid DSB repair in a cohesive and blunt end-joining assay and decreased endogenous chromosome DSB repair in a restriction enzyme assay (60). Altogether, this indicates that multiple FA proteins might be important for homology-directed repair events of DSBs in the cell.

Programmed double-strand DNA breaks

V(D)J recombination

V(D)J recombination is another phenomenon with recombination at its core that is observed in the immune system during B cell development. Also known as somatic recombination, V(D)J recombination is a process B and T cells use to generate antigen receptor diversity through random genetic recombination of the variable (V), diverse (D), and joining (J) gene segments (VDJ joining). Each V(D)J event requires a chromosomal DNA DSB and errors in the repair of these lesions can lead to the development of
lymphomas and leukemias (61). The main pathway of repair in V(D)J recombination is NHEJ which results in the deletion or inversions of DNA segments varying in size. Here, end resection remains an equally important early determinant of DSB repair pathway choice. The ends of the DSB are protected from end resection by chromatin-binding proteins that direct the repair to the NHEJ pathway (62). However, FA cells have been shown to exhibit unaffected V(D)J recombination or Ku-dependent NHEJ (60).

**Somatic hypermutation and class-switch recombination**

While V(D)J recombination targets the development of the antigen-binding N-terminal variable region of immunoglobulins (Ig) and T cell receptors (TCR), the C-terminal constant region is encoded for in several downstream exons. Antigen-activation of mature B cells induces two additional cytidine deaminase (AID)–dependent genomic alterations, namely somatic hypermutation (SHM) and class switch recombination (CSR). The process of SHM produces Ig variants with higher antigen affinity through the introduction of targeted but random point mutations in the section of the gene encoding for the variable region of the Ig. Alternatively, CSR retains the same variable region and instead switches constant regions by making use of highly repetitive elements. This results in the generation of an Ig for the same antigen that contributed toward CSR activation but with different effector functions.

The conversion of cytidines into uridines by activation-induced cytidine deaminase (AID) is the initiating factor for both SHM and CSR. The SHM pathway results in mutations through a variety of mechanisms that are beyond the scope of this dissertation. In CSR, base excision repair (BER) and MMR proteins induce DSBs within a given switch (S) region that get directed to the NHEJ and alt-NHEJ repair pathways. Pathway
choice in this scenario is also controlled by end resection, which is under regulation of chromatin-binding protein 53BP1 (63). 53BP1 functions in the protection of DSB ends from end resection enzymes, which leads to an increase in NHEJ. On the contrary, loss of 53BP1 leads to increased end resection of DSB, which in turn favors alt-NHEJ, whilst suppressing NHEJ.

**FANCA in class switch recombination**

Studies involving depletion of FANCA during CSR showed decreased use of short junctional microhomology regions and increased junctional insertions, the latter of which arise from the instability of DSB overhangs and results in the dissociation and re-annealing of DNA ends (64). Additionally, FANCA has been shown to be required for the recruitment of structure-specific endonuclease XPF-ERCC1 (65). This raises the possibility that FANCA is required to stabilize the overhangs created by SSA events and recruit the endonucleases necessary for further processing; consequently FANCA may be an important microhomology stabilization factor during CSR. Consistent with this, *fanca* mRNA expression is increased in the germinal centers of B cells, which exhibit high SHM and CSR (66). Interestingly, *FANCA/-/-* mice exhibit increased intra-switch recombination (ISR) implicating FANCA in the suppression of short-range joining (64). Here, I provide the biochemical basis for the involvement of FANCA in single-strand annealing, possibly implicating FANCA as a fidelity mechanism in CSR.

**Concluding remarks**

In this dissertation, I describe novel biochemical activities of FANCA: DNA helix destabilization, single-strand annealing, strand exchange, and recruitment and targeting of structure-specific endonuclease MUS81-EME1 (67). These previously unrecognized
FANCA functions are consistent with previously published *in vivo* data (46,64,66,68) and support a role of FANCA in repair of DNA breaks by homologous recombination. Additionally, I propose the involvement of FANCA in CSR may be due to its ability to enhance homology-mediated SSA, recruit structure specific endonucleases, and regulate their nucleolytic activity for successful processing of overhangs. Together, these data suggest FANCA may have multiple roles in maintaining genome integrity and the DNA damage response.
Figures

Figure 1-1: The subcellular localization of FANCA varies with respect to cell cycle phase.

Human fibroblast cells RA3087 homozygous for a large genomic deletion in FANCA were transfected with GFP-FANCA and synchronized via double thymidine block and released into S-phase. (Left) Immediately after release. (Right) More than 8 hr after release.
Figure 1-2: Pathways involved in the repair of DNA DSBs.

<table>
<thead>
<tr>
<th>Exogenous sources</th>
<th>Endogenous sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental exposure</td>
<td>Lipid peroxidation - Aldehydes</td>
</tr>
<tr>
<td>Chemotherapeutic agents</td>
<td>Purine Hydrolysis</td>
</tr>
<tr>
<td>Hypoxia - Oxidized abasic sites</td>
<td>BER intermediates</td>
</tr>
<tr>
<td>Nitrate acidification - Nitric oxide, nitrous acid</td>
<td></td>
</tr>
</tbody>
</table>

**Core Complex**

<table>
<thead>
<tr>
<th>FA Genes</th>
<th>FAAPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>FANCA</td>
<td>10 (MHF1)</td>
</tr>
<tr>
<td>FANCB</td>
<td>16 (MHF2)</td>
</tr>
<tr>
<td>FANCC</td>
<td>20</td>
</tr>
<tr>
<td>FANCE</td>
<td>24</td>
</tr>
<tr>
<td>FANCF</td>
<td>100</td>
</tr>
<tr>
<td>FANCG</td>
<td></td>
</tr>
<tr>
<td>FANCL</td>
<td></td>
</tr>
<tr>
<td>FANCM</td>
<td></td>
</tr>
</tbody>
</table>

**Other FA Genes**

- FANCD1 (BRCA2)
- FANCD2
- FANCI
- FANCI (BRCIP1)
- FANCN (PALB2)
- FANCO (RAD51C)
- FANCP (SLX4 or BTBD12)
- FANCP (ERCC4 or XPF)
- FANCS (BRCA1)

**Structure-specific Endonucleases**

- MUS81 / EME1
- FANCO (XPF) / ERCC1
- FANCP (SLX4)
- SLX1
- FAN1

**Homologous Recombination Genes**

- FANCS (BRCA1)
- FANCO (RAD51C)
- FANCI (BRCIP1)
- FANCD1 (BRCA2)
- FANCN (PALB2)
- FANCA

Diagram:

1. FA Core Complex
2. Mono-Ubiquitination (pathway activation)
3. ATR Activation
4. Endonuclease Recruitment
5. Homologous Recombination
6. TLS
7. NER

Details:
- Interstrand crosslink
- De-ubiquitination (pathway inactivation)
- Strand invasion → D-loop formation
- Repair
Figure 1-3: Pathways involved in the repair of DNA DSBs.
Figure 1-4: Spectrum of BRCA2 and RAD52 across various species.

Worms lack RAD52, while yeast lack BRCA2. Mediator activity is represented in green; single-strand annealing activity is represented in yellow.
CHAPTER 2 - MATERIALS AND METHODS

Expression and purification of human FANCA, MUS81-EME1 and RAD51

Complementary DNAs for human MUS81, EME1, FANCA, and RAD51 were obtained by polymerase chain reaction amplification from a universal complementary DNA pool (BioChain Institute, Inc.). The full-length open reading frames were confirmed by sequencing and found to exactly match NCBI Reference Sequences NM_025128, NM_152463, NM_000135, and NM_001164269, respectively. Co-expression of the hexahistidine-tagged EME1 and non-tagged MUS81 and overexpression of non-tagged FANCA were achieved in insect High Five cells using the Bac-to-Bac expression system (Invitrogen, Carlsbad, CA). Mutants for FANCA were produced through a PCR-based site-directed mutagenesis method. Expression of MUS81-EME1, RAD51, FANCA and its mutants was confirmed by western blot analysis using a Pierce ECL kit (Pierce, Rockford, CA). Antibody against MUS81 was purchased from Novus Biologicals (Littleton, CO). A monoclonal antibody against hexahistidine tag (GenScript, Piscataway, NJ) was also used to confirm EME1 expression and subsequent purification. Antibody against FANCA was kindly provided by the Fanconi Anemia Research Fund.

Upon expression of recombinant proteins in insect cells, the cells were homogenized in a protein extraction buffer (20 mM Hepes–KOH, pH 7.5, 0.5 mM MgCl₂, 50 mM NaCl, 0.2 M sucrose, 5 mM b-mercaptoethanol, protease inhibitors (0.5 mM PMSF, 0.3 mg/ml benzamidine hydrochloride, 0.5 mg/ml of pepstatin A, 0.5 mg/ml of leupeptin, 0.5 mg/ml of antipain) by a Dounce homogenizer using 10 strokes on ice. MUS81-EME1 was purified by using a HiTrap chelating column charged with nickel, a Mono S and/or a
Superdex 200 gel filtration column (GE Healthcare, Piscataway, NJ) and by tracking MUS81-EME1 protein through SDS–PAGE and western blot. Wild-type (WT)-FANCA and its mutants were purified using a protocol described previously (25). Protein concentration was determined by the Coomassie (Bradford) Protein Assay Reagent (Pierce, Rockford, CA). Purified proteins were aliquoted and stored at -80°C (Figure 2-1).

Table 2-1: Oligonucleotide name, length, and sequence. Underlined nucleotide represented in red was pre-modified with a covalently linked mono-psoralen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Length (nt)</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>31</td>
<td>TACGTTGTAAACGACGGCCAGTGAATTTCGA</td>
</tr>
<tr>
<td>A2</td>
<td>13</td>
<td>GCTCGGTTACCCGG</td>
</tr>
<tr>
<td>A3</td>
<td>30</td>
<td>AGATCCCTCTAGAGTCACCTGAGTGGCTT</td>
</tr>
<tr>
<td>A4</td>
<td>74</td>
<td>CCTAACAGTACTTGATCAGAGCTCTTGAGAATTTCACCGAGCTCGAATT \ CACTGGCCGTCGTTTTTACAACGT</td>
</tr>
<tr>
<td>A5</td>
<td>73</td>
<td>CCTAACAGTACTTGATCAGAGCTCTTGAGAATTTCACCGAGCTCGAATT \ CACTGGCCGTCGTTTTTACAACGT</td>
</tr>
<tr>
<td>A6</td>
<td>36</td>
<td>CAAGCCACTGCAGGTCAGTCTAGAGATCTCCGGG</td>
</tr>
<tr>
<td>A7</td>
<td>35</td>
<td>AAATTCTCGAAGAGCTCTGATCAAGTACTGTTAGG</td>
</tr>
<tr>
<td>B1</td>
<td>74</td>
<td>TACGTTTGAAAACGACGGCCAGTGAATTTCGAGCTCGATCCCGAGATCC \ TCTAGAGTCCGACTGCTGGCTT</td>
</tr>
<tr>
<td>B2</td>
<td>74</td>
<td>CCTAACAGTACTTGATCAGAGCTCTTGAGAATTTCACCGAGCTCGAATT \ CACTGGCCGTCGTTTTTACAACGT</td>
</tr>
<tr>
<td>B3</td>
<td>74</td>
<td>AAGCCACTGCAGGTCAGTCTAGAGATCTCCGGGTAACCGAGCTCGAATT \ CACTGGCCGTCGTTTTTACAACGT</td>
</tr>
<tr>
<td>B4</td>
<td>37</td>
<td>CCGAGCTCGAATTCTACCTGGCCGTCGTTTTCTACACGT</td>
</tr>
<tr>
<td>B5</td>
<td>37</td>
<td>AAGCCACTGCAGGTCAGTCTAGAGATCTCCGGG</td>
</tr>
<tr>
<td>B6</td>
<td>20</td>
<td>GAGTCGACCTGCAGTGGCTT</td>
</tr>
<tr>
<td>B7</td>
<td>20</td>
<td>AAGCCACTGCAGGTCAGTCTC</td>
</tr>
<tr>
<td>B8</td>
<td>20</td>
<td>TACGTTTGAAAACGACGGCC</td>
</tr>
<tr>
<td>B9</td>
<td>20</td>
<td>GGCGGTCTTTTACAACGT</td>
</tr>
<tr>
<td>B10</td>
<td>55</td>
<td>AGGTCTCGAAGTCACTCTAGTGTTTCTACCCGTCACCCGACGCCACCT \ CCTG</td>
</tr>
</tbody>
</table>
**DNA substrates**

All oligonucleotides were synthesized by Sigma Aldrich. Refer to Table 2-1 for oligo name, length and sequence information. Oligonucleotides A1-A6 were used in the assays described in Chapter 3, while B1-B17 correspond to those in Chapter 4. Oligonucleotides used in the strand exchange assay (B10-12) were adapted from Masuda-Sasa et al. 2006 (69). The 5’ end of A1, B1 and B10 were labeled with $^{32}$P using $[^\gamma-^{32}]P$ ATP and T4-PNK enzyme using the protocol specified by the manufacturer.

**Creation of psoralen ICL substrates utilized in Chapter 3**

To create a site-specific DNA ICL, a short oligo (5’-GCTCGGT$^T$ACCCGG) with an internal psoralen modified T (red, underlined) was synthesized by Midland Certified Reagent Company (Midland, TX). After elongating through ligation with oligonucleotides A1 and A3, annealing with partially complementing oligonucleotide A4 (or A5 1-base shorter for 3’ labeling), exposing to ultraviolet A irradiation and purifying by denaturing gel (Figure 2-2), I obtained an ICL-damaged splayed arm structure.

The splayed arm structure was labeled at the 3’ end on the top leading strand through $^{32}$P-incorporation by Klenow DNA polymerase (Figure 2-3). Labeling of the 5’ ends of
the leading and lagging strands is done before creation of the ICL (Figure 2-3). Through
annealing with different oligonucleotides complementary to the leading (A6) or lagging
strand (A7) or both, 5’ ssDNA branch, 3’ ssDNA branch and static replication fork
structures were created (Figure 2-2).

**Creation of substrates utilized in Chapter 4**

Oligonucleotides were paired up according to Figure 2-4 depending on the assay being
conducted. For assays in which dsDNA structures were required as substrates, the
oligonucleotides involved were pre-annealed at 1:1 molar ratios. Annealing was carried
out in a water bath over ~5h by slowly cooling from 85°C to 20 °C. The quality of
annealing was monitored by native gel electrophoresis. Proper annealing was verified by
the mobility of a corresponding substrate.

**DNA incision assay**

A total of 1.5 nM of labeled DNA substrate was incubated with indicated amounts of
proteins in a 10 µl reaction mixture containing 25 mM Hepes–KOH, pH 7.6, 1 mM DTT,
3 mM MgCl₂, 6.5% glycerol, 120 mg/ml BSA and 100 mM KCl. After incubation for 20
min at 37°C, the reaction was terminated by adding 5 µl proteinase K (25mM EDTA,
0.67% SDS, 150 mg/ml of proteinase K) and 10 min incubation at 37°C, and by adding
15 µl sequencing dye. The reaction mixture was resolved by running a 10% denaturing
sequencing gel. DNA size markers were prepared by labeling a mixture of defined oligos
through [γ-³²P] ATP.
RNA interference, induction of ICL in living cells, and confocal microscopy

To study the interaction of FANCA and MUS81 in human cells, we treated U2OS cells with ON-TARGET Plus SMART Pool siFANCA (Thermo Scientific Dharmacon, cat# L-019283-00) and a non-targeting control small interfering RNA (siRNA) (cat# D-001810-01-05) using the Dharmafect transfection agent for 48h. After western blot verification of knockdown efficiency (Figure 3-11), a GFP-tagged MUS81 construct, pEGFP-N1-MUS81 (vectors were purchased from Clontech), was transfected into the U2OS cells by Lipofectamine 24 h before drug treatment. pEGFP-N1-FANCA was transfected into WT U2OS cells for monitoring status of FANCA. The Olympus FV1000 confocal microscopy system was used (Cat. F10PRDMYR-1, Olympus, UPCI facility) and FV1000 software was used for acquisition of images. To create psoralen ICLs, cells were pretreated with 100 nM of 8-methoxypsoralen (8-MOP) for 10 min right before a 405 nm laser micro-irradiation. The output power of the laser (original 50mW) passed through the lens is 5 mW/scan. Laser light passed through a PLAPON 60X oil lens (super chromatic abe. corr. obj W/1.4NA FV, Cat. FM1-U2B990). Cells were incubated at 37°C on a thermo-plate (MATS-U52RA26 for IX81/71/51/70/50; metal insert, HQ control, Cat. OTH-I0126) in Opti-MEM during observation to avoid pH changes. The images were taken 2 min after laser treatment.

Cell synchronization

Cells were grown to 70% confluency and synchronized using a double thymidine block of 2 mM final thymidine for 18hr (1st block) and 15hr (2nd block). Release into S-phase was done by washing cells with PBS and adding serum containing growth medium.
Helix destabilization, strand annealing, and strand exchange assay

Helix destabilization, strand annealing, and strand exchange activities were all tested using a total of 0.5 nM 5'-32P-labeled DNA substrate and the indicated amounts of protein in a 10 µl reaction of 25 mM Tris-HCl, pH 8.5, 100 mM NaCl, 1 mM EDTA, pH 8. The reaction mixture was incubated at room temperature (22 °C) for 40 min or as indicated in the figure legend and stopped with 1 µl of 10x stop solution (200 mM EDTA, 32% glycerol, 1% SDS, 0.024% Bromophenol Blue), 4x unlabeled competing-oligo and/or 3 µg proteinase K with 10 min incubation at room temperature. Products were separated on a 6 or 10% native PAGE at 100V for 1.5 or 4 h depending on substrate size.

DNA binding assay

DNA binding EMSA was performed as described previously (25) in a 10 µl reaction mixture containing 25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1 mM DTT, 6% glycerol, 1 nM 5'-32P-labeled oligonucleotide substrates, and the indicated amounts of protein. The reactions were incubated at room temperature (22 °C) for 45 min, followed by the addition of 4 µl of 50% (w/v) sucrose. The reaction mixtures were resolved by electrophoresis through a 4% nondenaturing polyacrylamide gel in 40 mM Tris acetate (pH 7.6) and 10 mM EDTA with 6% glycerol using the Owl P9DS electrophoresis system (Thermo Scientific). The setting was 100 V (~1.5 watts/gel) for 40 or 90 min as indicated. DNA substrates and shifted bands were visualized by autoradiography.
**Immunodepletion of FANCA**

This assay was adapted from Sharma et al. 2005 (70). Protein A-agarose beads (40 µl; Roche Applied Science) were equilibrated with reaction buffer and incubated for 1 h at 4 °C with 1 µg rabbit polyclonal anti-FANCA (Abcam) antibody or normal rabbit IgG (Santa Cruz Biotechnology, Inc.) Beads were subsequently washed with reaction buffer and incubated with 30 nM (helix stabilization assay) and 5 nM (helix destabilization assay) FANCA in reaction buffer (40 µl) for 1 h at 4 °C. Beads were collected by centrifugation, and the supernatant fraction was again incubated with antibody-bound beads for 10 min at 4 °C for a second immunodepletion step. Following centrifugation, supernatant fractions were collected and assayed for helix stabilization (30 nM FANCA) and destabilization activity (5 nM FANCA).

**Filter binding assay for ATP binding to FANCA**

FANCA [30 nM] was incubated in the presence and absence of indicated amounts of [α-\(^{32}\)P]-ATP, 5 mM MgCl\(_2\), and/or 1 nM DNA at 30°C for 5 min in a reaction buffer containing 25 mM Tris-HCl (pH 7.6), 70 mM KCl, 5 mM DTT, and 5% glycerol. Samples were then passed through a nitrocellulose membrane (BioRad) and washed several times in ice cold reaction buffer. Portions of nitrocellulose membrane corresponding to each sample were cut out and used to measure trapped radioactivity in a liquid scintillation counter.
Figures

Figure 2-1: Coomassie Brilliant Blue staining of purified proteins.

The purified proteins were stained with Coomassie Brilliant Blue R-250 after SDS-PAGE. Asterisk indicates a hexahistidine tag at the N-terminus of the protein. Protein markers in kilo Daltons are indicated.
Figure 2-2: Assembly of oligonucleotides corresponding to assays in Chapter 3.
Figure 2-3: Dynamics of radiolabeling according to leading or lagging strand, and 5’- or 3’-end.
Figure 2-4: Assembly of oligonucleotides corresponding to assays in Chapter 4.

**SSA Assay**

B1

\[ + \rightarrow B1 : B2 \]

B2

**Unwinding Assay**

B1 : B3

\[ \rightarrow + \]

**Strand Invasion Assay**

5' B1 : B4 3'

\[ + \rightarrow + \]

B6 : B7

B6

5' B1 : B5 3'

\[ + \rightarrow + \]

B8 : B9

B8

**Strand Exchange Assay**

B10

\[ + \rightarrow B10 : B12 \]

B12

B10 : B11

\[ + \rightarrow + \]

B12

B10 : B12

B11

**SSA of DSB Intermediates Assay**

B13 : B4

\[ + \rightarrow 3' Flap \]

B14 : B15

B1 : B4

\[ + \rightarrow \]

B16 : B15

B17 : B15
For the SSA assays, fully homologous oligos B1 and B2 were used as substrates. For the unwinding assays, partially complementary pre-annealed B1:B3 (splayed arm), B1:B4 (3’ overhang), B1:B5 (5’ overhang), and B6:B7 (blunt-end) were used as substrates. For strand exchange assays B10 and B12 were used as control substrates for the 2nd reaction involving pre-annealed B10:B11 and B12. For the SSA of DSB repair intermediates assay pre-annealed B13:B4, B14:B15, B1:B4, B16:B15, B1:B5, and B17:B15 were used as substrates.
CHAPTER 3 - FANCA REGULATES MUS81-EME1 INCISION ACTIVITY

**MUS81-EME1 does not incise on the 3’ side of a psoralen ICL**

Although MUS81-EME1 was previously proposed to unhook DNA ICLs by incising the leading strand on the 3’ side of the damage (71-73), its incision activity on synthetic ICL-damaged fork structures had never been tested. To evaluate how MUS81 incises ICL-damaged DNA, human MUS81 and hexahistidine-tagged EME1 were co-overexpressed in High Five insect cells and purified the MUS81-EME1 complex to near homogeneity (Figure 2-1). Using defined sequences, I prepared a splayed arm structure with a psoralen-ICL located immediately at the junction site of two ssDNA flaps and a dsDNA region (Figure 2-2). The rationale for this design was based on recent observations from Walter’s group (74,75), who reported that ICL incision happens when the ICL damage is within one nucleotide of the nascent strand end.

To test whether MUS81-EME1 incises on the 3’ side of ICL damage as previously hypothesized, I labeled the leading strand on the 3’ end of the ICL-damaged splayed arm by α-32P incorporation (Figure 2-3). By annealing with different oligos on leading and/or lagging strands, 5’ ssDNA branch, 3’ ssDNA branch, and static replication fork structures were created (Figure 2-2). I next incubated the purified MUS81-EME1 with the DNA structures with ICL. If MUS81-EME1 incised the top leading strand at the junction site on the 3’ side of the ICL, a short DNA fragment would be expected. Surprisingly, no incision product below 74 nucleotide could be detected with increasing amount of MUS81-EME1 (Figure 3-1). Instead, a much larger incision product band was observed with 3’ ssDNA branch and static replication fork structures (Figure 3-1, lanes 6-9). These
data indicate that purified MUS81-EME1 incises the ICL damaged DNA and has the same structure specificity for 3’ ssDNA branch and replication fork as previously reported (76-85); however, MUS81-EME1 does not incise the leading strand at the junction site on the 3’ side of the ICL damage as previously proposed.

**MUS81-EME1 incises the leading strand on the 5’ side of the psoralen ICL**

To determine the exact incision site, I next labeled the 5’ ends of the leading and lagging strands separately (Figure 2-3). Again, incubation of MUS81-EME1 with ICL substrates with 5’ end labeling on the lagging strand showed that the endonuclease did not react with splayed arm and 5’ ssDNA branch structures, but it effectively incised ICL-damaged 3’ ssDNA branch and replication fork structures (Figure 3-2, lanes 9-16). The incision was not on the splayed arm side of the lagging strand because only a large incision product was observed (Figure 3-2). As expected, MUS81-EME1 also did not incise undamaged DNA on the lagging strand (Figure 3-3).

Next, MUS81-EME1 was titrated with ICL substrates labeled at the 5’ end on the top leading strand. As shown in Fig. 3-4, incision of the ICL-containing 3’ ssDNA branch and replication fork structures yielded a major band at ~34-35 nucleotide position and some smaller minor bands (Figure 3-4, lanes 9-16). Because the distance between the 5’ end 32P-label and the junction site was 39 nucleotides, the predominant site of MUS81-EME1-mediated incision of ICL was calculated to be ~4-5 nucleotides away from the junction and located on the 5’ side of the ICL. MUS81-EME1 also incised undamaged controls at a similar site with slightly stronger activity (Compare the remaining substrate bands located on the top of lanes 9-16 of Figure 3-4 with those in lanes 9-16 of Figure 3-5). However, no smaller minor bands were observed with the undamaged DNA.
During these tests, I noticed a slight size difference between the major incision product from ICL substrates and the one from undamaged controls (Figure 3-7A). To define the difference, I analyzed the incision products by extensively running the reaction mixtures from ICL damaged and undamaged DNA next to each other with a size marker. Figure 3-6 clearly shows that the major incision product from the ICL damaged DNA is 34 nt in size, 1 nt shorter than the product from the undamaged control. This means MUS81-EME1 incises the psoralen ICL damaged DNA 5 nt away from the fork junction, while it cuts the undamaged DNA 4 nt away (Figure 3-6).

These data establish that MUS81-EME1 unequivocally incises the leading strand on the 5’ side of the psoralen ICL when located at the junction site and the incision sites of MUS81-EME1 are dependent on the type of damage present on the DNA.

**FANCA affects MUS81-EME1-mediated DNA incision in a damage-dependent manner**

Because FANCA interacts with DNA (25) and plays a role in ICL incision (28,65), I reasoned that FANCA may directly interact with other proteins involved in ICL repair such as ICL-unhooking DNA endonucleases like MUS81-EME1 for more efficient ICL incision. To test this hypothesis, purified FANCA was titrated in the presence or absence of the psoralen ICL damage in a defined *in vitro* incision assay with suboptimal amount of MUS81-EME1 (1.5 nM) (Figure 3-7A and B).

In the presence of ICL damage, FANCA dramatically stimulates ICL incision mediated by MUS81-EME1 up to 14-fold (Figure 3-7A-C). Intriguingly, FANCA exerted a 2-phase regulation of MUS81-EME1 activity in the absence of the ICL damage (Figure
3-7A, Undamaged). In Phase I increasing FANCA concentration up to 5 nM enhances MUS81-EME1 incision activity of undamaged DNA, although less efficiently than its effect on ICL damaged DNA (Figure 3-7C, up to 3-fold). In Phase II increasing FANCA concentration from 5 nM to 20 nM inhibits MUS81-EME1 activity on the undamaged DNA. MUS81-EME1 incision activity was abrogated at 20 nM FANCA (Figure 3-7A and B). Using 20 nM of FANCA and 1.5 nM MUS81-EME1, I also performed a time course experiment in order to further examine the extent to which FANCA stimulates or inhibits MUS81-EME1 mediated incision in the presence or absence of ICL (Figure 3-8). The results clearly confirmed that FANCA stimulates MUS81-EME1 incision on the ICL damaged DNA, and under the same condition it inhibits MUS81-EME1 activity in the absence of ICL. These observations demonstrate that FANCA functionally interacts with MUS81-EME1 to distinguish an ICL from undamaged DNA for damage specific incision.

It has been previously reported that MUS81 is involved in ICL incision but spares DNA damage that affects only one DNA strand (86). Inhibition of MUS81-EME1 activity by FANCA in the absence of DNA damage (Figure 3-7A and B) inspired us to hypothesize that FANCA might also be involved in regulation of MUS81-EME1 activity on non-ICL DNA damage. To test this hypothesis, I performed the same FANCA titration using the psoralen thymine mono-adduct designed for the creation of the ICL. The psoralen mono-adduct is identical in sequence and overall structure to the ICL, only it was not exposed to UVA irradiation and consequently not allowed to form an ICL. Surprisingly, like on the undamaged DNA, FANCA stimulated or inhibited MUS81-EME1-mediated incision of the psoralen mono-adduct in a two-phase concentration-
dependent manner (Figure 3-9A, compare the last lane with 20 nM of FANCA to the second lane without FANCA). Furthermore, experiments using a benzo(α)pyrene diolepoxide (BPDE) adducted deoxyguanosine, a damage that affects only one DNA strand (87), also showed that FANCA can stimulate or suppress MUS81-EME1 activity in a two-phase concentration-dependent manner (Figure 3-9B, compare the last two lanes with 15 and 20 nM of FANCA respectively to the second lane without FANCA).

Overall, I demonstrated that FANCA directly participates in ICL-specific incision via the stimulation of MUS81-EME1 activity. Up- and down-regulation of MUS81-EME1 activity by FANCA provides an important mechanism to uncover why it is possible for MUS81 to be involved in the incision of ICLs but not DNA damage that affects one DNA strand (86).

**Both N- and C-terminals of FANCA are required for the regulation of MUS81-EME1**

Because FANCA is a DNA binding protein, I next asked whether the damage-dependent regulation of MUS81-EME1 by FANCA is caused by its affinity to DNA. To test this, two truncation mutants of FANCA, Q772X and C772-1455, were created in the lab. Q772X is a Fanconi anemia disease-causing C-terminal truncation mutant. C772-1455 is the complementing C-terminal fragment of Q772X (Figure 2-1). The DNA binding domain of FANCA is located at the C-terminal C772-1455 fragment (25). I therefore hypothesized that the C-terminal portion of FANCA would be responsible for MUS81-EME1 activity regulation.
Using 20 nM of protein, where wild type (WT)-FANCA stimulates MUS81-EME1-mediated incision of ICL but inhibits incision of undamaged DNA (Figure 3-7A and B), I found that both mutants showed drastic reduction in stimulating MUS81-EME1 in the presence of ICL comparing to the WT protein (Figure 3-10, compare lanes 5-6 with lane 4 in the ICL panel). Additionally, different from WT-FANCA, both the N- and C-terminals of FANCA did not inhibit MUS81-EME1 activity on undamaged DNA (Figure 3-10, compare lanes 5-6 with lane 4 in the undamaged panel).

To further evaluate the functional application of the FANCA mutants, an incision discrimination factor was used to measure the ability of FANCA to regulate MUS81-EME1 mediated incision of ICL damage versus undamaged DNA. Figure 3-10B clearly shows that both mutants in conjunction with MUS81-EME1 lost their ability to discriminate ICL damaged from undamaged DNA during incision. In summary, these data suggest that both N- and C-terminals of FANCA are critical for the regulation.

**FANCA recruits MUS81-EME1 to the ICL damage site**

To examine whether FANCA functionally interacts with MUS81-EME1 *in vivo*, our collaborators at the University of Pittsburgh in Dr. Li Lan’s laboratory created site specific ICL damage in living human cells. It has been reported that cells treated with 8-methoxypsoralen (8-MOP) form ICL damage after light activation (88,89). First, to determine the dynamics of FANCA’s response to ICL damage, GFP-FANCA was expressed in U2OS cells and visualized through confocal microscopy. As expected, GFP-FANCA was expressed in both the nucleus and cytoplasm (Figure 3-11B). The cells were then treated with either 8-MOP, low-energy 405 nm laser beam, or a combination of both. FANCA did not respond to laser treatment indicating minimum damage formation
by the laser alone (Figure 3-11B). Surprisingly, pretreating the cells with 8-MOP and inducing ICL formation through laser beam irradiation resulted in the efficient recruitment of FANCA to the lesion created in the path of the laser only 2 min after ICL induction (Figure 3-11B). I successfully reproduced these results in our institution in collaboration with Dr. Vincent Moy in the department of Physiology & Biophysics.

Second, to investigate whether FANCA interacts with MUS81 on ICL damages, endogenous FANCA level was knocked down by siRNA (Figure 3-11A) and GFP-MUS81 was transfected in the knockdown U2OS cells before treatment with 8-MOP and/or laser (Figure 3-11C). As expected, GFP-MUS81 was only found in the nucleus. Like FANCA, GFP-MUS81 did not respond to laser treatment, further proving that the laser produces minimal damage (Figure 3-11C, 405 nm laser panel). After inducing ICL formation by both 8-MOP and laser, MUS81 was recruited to the ICL damage sites when the cells were mock treated or treated with control siRNA. However, recruitment of MUS81 to the ICL site diminished when FANCA was successfully knocked down (Figure 3-11C, siFANCA panel). This result suggests that recruitment of MUS81 to ICL sites is dependent on FANCA and that FANCA functions upstream of MUS81 in the ICL repair pathway.

**Discussion and future directions**

Heterodimeric DNA endonuclease MUS81-EME1 is known to be involved in the incision of ICLs in mammalian cells (86), and has been proposed to cut the leading strand at a replication fork junction site on the 3’ side of ICL damage (71-73,90). Employing a defined psoralen ICL substrate, I have provided the first biochemical evidence that human MUS81-EME1 does incise ICL damaged fork structures; however, unlike the
previously proposed 3’ side cleavage models, MUS81-EME1 incises the leading strand on the 5’ side of an ICL lesion. This discrepancy was startling at first look. Nevertheless further in-depth analysis revealed that my results are compatible with the previously published incision behavior and substrate specificity of MUS81-EME1. Similar to previous observations using yeast proteins, my results showed that purified human MUS81-EME1 incises 3’ ssDNA branch and replication fork structures 4 nt away from the junction site (76,91). This special activity is likely to lend MUS81-EME1 an ability to incise on the 5’ side of DNA across the ICL damage. Theoretically, cleavage on the 3’ side could happen only if the incision occurs long before the nascent strand reaches the ICL damage because of the incision activity and structure specificity of MUS81-EME1 on undamaged DNA.

Experimental evidence using Xenopus egg extracts and ICL-damaged plasmids showed that incision of ICLs happens after the opposing replication forks converge at the ICL site (74,75). However, in some occasions replication forks may not converge at the ICL site because of chromatin structure, relative distance between origins of replication, and the actual ICL damage. Excellent work done using ICL-damaged plasmids may represent many but not necessarily all situations for replication fork stalling. In the instance where the leading strand DNA synthesis pauses ~24 nucleotides upstream of the ICL, DNA unwinding and lagging strand DNA synthesis may uncouple from leading strand synthesis. The resulting DNA structure from this occasion resembles the ICL damaged 3’ flap structure I used in my study. Nonetheless, studies from McHugh’s group provided strong evidence that DNA exonuclease SNM1A collaborates with endonuclease
XPF-ERCC1 to initiate ICL repair (92). MUS81-EME1 may only act as an alternative mechanism when SNM1A and XPF-ERCC1 fail (72,92).

The most important discovery of the studies presented in this chapter is that FANCA affects MUS81-EME1-mediated DNA incision, positively in the presence of an ICL and negatively in its absence. Enhancement of MUS81-EME1 activity in the presence of the psoralen ICL damage helps to repair the damage more effectively; suppression of MUS81-EME1 in the absence of ICL damage helps in protecting replication forks from being attacked due to other DNA damage affecting only one strand. This in turn may prevent the production of more deleterious damage such as DSBs. Both events are beneficial for the maintenance of replication forks. It is conceivable that the ICL damage is encountered and recognized by components of the replication machinery. The DNA binding activity of FANCA may serve to verify the presence of an ICL stalled replication fork. If an ICL is confirmed, FANCA will recruit and activate MUS81-EME1 for efficient and precise ICL incision (Figures 3-11 and 5-1, ICL). If a non-ICL damage is detected, FANCA will prevent MUS81-EME1 from incising on the stalled replication forks, a mechanism that is yet to be elucidated (Figures 3-7 and 5-1, Non-ICL). This working model reconciles with my observations and explains why MUS81-EME1 promotes ICL unhooking yet avoids non-specific incision of undamaged or non-ICL damaged forks (86). This scenario is similar to the damage recognition and verification steps in NER where XPC recognizes DNA damage and XPA-RPA verifies before incision in order to prevent unnecessary incisions (93). This process definitely requires both the DNA binding activity at the C-terminal as well as an unidentified function at the N-terminal of FANCA (Figure 3-10). Whether FANCA recognizes ICLs and interacts
with ICL repair factors other than MUS81-EME1 for efficient ICL incision needs to be addressed in future studies.

In summary, the findings reported in this chapter provide novel insight into the incision behavior of MUS81-EME1 on ICL damage and establishes that FANCA contributes to the maintenance of replication forks by directly regulating the incision activity of MUS81-EME1 in a damage-dependent manner.
Figures

Figure 3-1: MUS81-EME1 incision on the 3'-labeled ICL-containing DNA structures.

Titration of purified MUS81-EME1 (5 nM - 10 nM) on the 3'-labeled psoralen ICL damaged DNA structures as shown on the top. The schematic appearance of the products after incision were shown on the right. All reactions were performed in 10 µl of 25 mM Hepes, pH 7.6, 100 mM KCl, 3 mM MgCl2, 1 mM DTT, 120 µg/ml BSA, 6.5% Glycerol, and 1 nM of each DNA substrate as indicated. The reactions were performed at 37°C for 20 min and resolved on a 10% denaturing PAGE gel. Letter P with a circle indicates α-32P labeling by Klenow DNA polymerase. Asterisk (74-nt) indicates a decayed and uncrosslinked species.
Figure 3-2: MUS81-EME1 incision on DNA substrates with 5'-end labeling on the lagging strand.

Titration of purified MUS81-EME1 (3 nM, 6 nM, and 12 nM) on Psoralen ICL damaged DNA substrates shown on the top of each gel. The schematic appearance of the products after incision are shown on the right. Letter P with a circle indicates $\gamma^{32}$P labeling by T4 polynucleotide kinase. Asterisk (74-nt) indicates a decayed and uncrosslinked species. Arrows point to the incision sites and corresponding incision products.
Figure 3-3: MUS81-EME1 incision on DNA substrates with 5'-end labeling on the lagging strand.

Titration of purified MUS81-EME1 (3 nM, 6 nM, and 12 nM) on undamaged DNA substrates shown on the top of each gel. The schematic appearance of the products after incision are shown on the right. Letter P with a circle indicates $\gamma^{32}$P labeling by T4 polynucleotide kinase. Asterisk (74-nt) indicates a decayed and uncrosslinked species. Arrows point to the incision sites and corresponding incision products.
Figure 3-4: MUS81-EME1 incision on Psoralen ICL damaged DNA substrates with 5'end labeling on the leading strand.

Titration of purified MUS81-EME1 (3 nM, 6 nM, and 12 nM) on DNA substrates shown on the top of each gel. The schematic appearance of the products after incision were shown on the right. Letter P with a circle indicates γ-\(^{32}\)P labeling by T4 polynucleotide kinase. Asterisk (74-nt) indicates a decayed and uncrosslinked species. Arrows point to the incision sites and corresponding incision products.
Titration of purified MUS81-EME1 (3 nM, 6 nM, and 12 nM) on DNA substrates shown on the top of each gel. The schematic appearance of the products after incision were shown on the right. Letter P with a circle indicates $\gamma^32P$ labeling by T4 polynucleotide kinase. Asterisk (74-nt) indicates a decayed and uncrosslinked species. Arrows point to the incision sites and corresponding incision products.
Figure 3-6: Psoralen ICL alters the MUS81-EME1 incision site.

Lane 1: 32P labeled DNA markers shown in nucleotide on the left. 3 nM of purified MUS81-EME1 was incubated with the undamaged (Lane 2) and ICL damaged 3'-ssDNA branch substrates (Lane 3). The sequence and schematic appearance of the products after incision were shown on the right. 74-mer indicated a decayed and uncrosslinked species. Letter P with a circle indicates 5'-32P labeling. Green coded: incision product from undamaged DNA. Orange coded: incision product from ICL damaged DNA.
Figure 3-7: Effect of FANCA on MUS81-EME1 mediated DNA incision.

(A) Undamaged and Psoralen ICL damaged substrates were incubated with 1.5 nM of MUS81-EME1 respectively and an increasing concentration of FANCA (0, 1.25, 2.5, 5, 10, 15, and 20 nM). DNA markers are shown in nucleotide on the left. Same marker as
described in Fig. 3 was used. (B) Quantitation of three independent experiments. MUS81-EME1 activity was calculated as % of incision products out of the input substrates. The experiment without FANCA was normalized to 0 and used to calibrate all other experiments with the indicated amount of FANCA. Error bars: standard deviation. Dashed line: Phase I and Phase II border. (C) Fold changes of the MUS81-EME1 regulation by FANCA. The experiment without FANCA was arbitrarily normalized to 0 for fold change and used to calibrate all other experiments with the indicated amount of FANCA. Error bars: standard deviation.
Figure 3-8: Time course analysis of MUS81-EME1 mediated incision in the presence or absence of FANCA.

(A) Undamaged 3’ flap structure was incubated with 1.5 nM of MUS81-EME1 in the presence of 20 nM FANCA (0, 1.25, 2.5, 5, 10, 15, and 20 nM) and with increasing incubation time (0, 1, 2, 5, 10, 20, 30 min). The schematic appearance of the products after incision were shown on the right. Letter P with a circle indicates γ-32P labeling. An arrow points to the incision sites and corresponding incision products. 

(B) Psoralen ICL damaged 3’ flap structure was incubated with 1.5 nM of MUS81-EME1 in the presence
of 20 nM FANCA and with increasing incubation time (0, 1, 2, 5, 10, 20, 30 min). The schematic appearance of the products after incision were shown on the right. Letter P with a circle indicates $\gamma^{32}$P labeling. Asterisk indicates a decayed and uncrosslinked species. An arrow points to the incision sites and corresponding incision products. Quantitation of three independent experiments for MUS81-EME1 activity was calculated as % of incision products out of the input substrates and is shown to the right of each gel. Error bars: standard deviation.
Psoralen mono-adducted (A) and a benzo(α)pyrene diolepoxide deoxyguanosine adducted 3’ ssDNA branch structures (B) were incubated with 1.5 nM of MUS81-EME1 and an increasing concentration of FANCA (0, 1.25, 2.5, 5, 10, 15, and 20 nM). The schematic appearance of the products after incision are shown on the right. Letter P with a circle indicates 5’-32P labeling.
Figure 3-10: Effect of FANCA mutants on MUS81-EME1 mediated DNA incision.

A

ICL damage

Relative Incision (%)

Lane 1 2 3 4 5 6

Marker Sub MUS81-EME1 FANCA WT Q772X C772-1455

Undamaged

Relative Incision (%)

Lane 1 2 3 4 5 6

Marker Sub MUS81-EME1 FANCA WT Q772X C772-1455

B

Discrimination Factor (%)

FANCA WT Q772X C772-1455
(A) Psoralen ICL damaged (top panel) and undamaged DNA substrates (bottom panel) were incubated with 1.5 nM of MUS81-EME1 and 20 nM of purified WT and mutant FANCA proteins as indicated. DNA markers are shown on the left. The schematic appearance of the products after incision are shown on the right. Letter P with a circle indicates 5'-32P labeling. Asterisk indicates a decayed and uncrosslinked species. Quantitation of three independent experiments is shown to the left of each gel. Incision efficiency was normalized to WT FANCA (arbitrarily assigned as 100%). Error bars: standard deviation. (B) Ability to discriminate ICL from undamaged DNA by FANCA mutants. The discrimination factor was calculated by dividing the relative incision rate of ICL of a protein by the relative incision rate of undamaged DNA of the same protein. The discrimination factor for FANCA was arbitrarily assigned as 100%.
Figure 3-11: Interaction of FANCA and MUS81 on ICL damage in living cells.

(A) Western blot of FANCA knockdown. U2OS cells were transfected with an ON-TARGET Plus SMART Pool siFANCA, a control siRNA (siCtrl), and dH2O (Mock) through Lipofectamine. 48 hrs later, 50 µg of whole cell protein extract was prepared for Western blot analysis using a FANCA specific antibody. Actin is the loading control. (B) U2OS cells were transfected with GFP-FANCA and treated with 8-MOP and/or a 405 nm laser beam as indicated. Laser path is indicated by yellow arrows. The panel is
representative of 25 examined nuclei of each treatment. 25/25 showed the laser-induced FANCA stripes in the presence of 8-MOP. (C) Mock, control siRNA, and siFANCA treated U2OS cells were transfected with GFP-MUS81 and treated with 8-MOP and/or a 405 nm laser beam as indicated. Laser path is indicated by yellow arrows. The panel is representative of 25 examined nuclei of each treatment. 25/25 showed the laser-induced MUS81 stripes in the presence of 8-MOP for the mock and control treatment. 25/25 of siFANCA nuclei did not show the laser-induced MUS81 stripes in the presence of 8-MOP.
CHAPTER 4 - FANCA AFFECTS DNA HELIX STABILITY

**FANCA is a single-strand annealing factor**

Due to the specific ICL recognition activity of FANCA (Chapter 3 and (94)), I hypothesized that it was likely to catalyze strand separation in order to discriminate interstrand crosslinks from intrastrand crosslinks or mono-adducts. To test my hypothesis, I examined the effect of FANCA on DNA stability. Remarkably, I observed strong single-strand annealing activity, a novel role for FANCA; in a concentration-dependent experiment, FANCA (10-40 nM) promotes annealing of fully homologous DNA 74 nt in length (Figure 4-1A, lanes 4-7). Time-course analysis of 1 to 40 min reveals initial product formation to occur within 5 min of adding 40 nM FANCA to the reaction mixture (Figure 4-1B). No protein-independent formation of annealed DNA was observed throughout the entire time-course experiment. It’s important to note that stopping the reaction with or without protease K renders a substrate of identical migration profile; hence, stopping the reaction with cold unlabeled top strand is an appropriate method of stopping the reaction (Figure 4-2). To control for contaminant proteins in my protein sample preparation, I depleted FANCA from the reaction mixture using anti-FANCA antibody specific to amino acids 1-190 of FANCA (1455 aa). I observed decreased single-strand annealing activity in FANCA depleted samples indicating this activity is intrinsic to FANCA (Figure 4-1C).
**FANCA has bidirectional DNA helix destabilization activity**

Recent insights into helicases have broadened the classification to include annealing helicases; proteins that can perform both unwinding and rewinding of the DNA (95). Because the extent of protein oligomerization is known to affect the unwinding and rewinding activity of certain annealing helicases, I performed a helix destabilization assay using lower FANCA concentrations and found that FANCA (up to 20 nM) destabilized a fork-like structure in a concentration-dependent manner with destabilization activity peaking at 5-10 nM FANCA (Figure 4-3, lanes 6 and 7). This is consistent with the findings that RECQ1 acts as a destabilization factor in smaller oligomeric states and a stabilization factor in larger multimeric complexes (96, 97).

An important aspect of helicases is translocation directionality, which varies according to each particular enzyme (95, 98, 99). Type A helicases translocate in a 3’-to-5’ manner, while type B helicases go in the reverse direction. Both types are vital for many cellular processes including replication, recombination, and damage repair. To examine whether FANCA reserved 3’-to-5’ or 5’-to-3’ directionality, I assayed DNA structures with a 3’-overhang DNA, 5’-overhang, and double blunt-ended dsDNA. Since the ssDNA binding activity of FANCA is considerably higher than that for dsDNA (25), I reasoned FANCA would initially bind to the ssDNA portion of the structure and catalyze its destabilization originating from that end. My results reveal complete bidirectionality in the helix destabilization activity of FANCA (Figure 4-4). For the 3’-overhang DNA substrate, destabilization was low at 0.6 nM FANCA, optimal between 2.5 and 5 nM, and dramatically decreased at concentrations higher than 10 nM (Figure 4-4A-a). Destabilization of the 5’-overhang structures behaved similarly with FANCA activity.
peaking at concentrations between 2.5 and 10 nM (Figure 4-4A-b). This destabilization activity was significantly decreased when presented with a double blunt-ended dsDNA structure (Figure 4-4A-c) of 20 bp in length and completely abolished in the presence of a longer dsDNA substrate of 74 bp (Data not shown). To control for contaminant proteins in my protein sample preparation, I depleted FANCA from the reaction mixture using anti-FANCA antibody. I observed decreased helix destabilization activity in FANCA depleted samples indicating this activity is intrinsic to FANCA and not a contaminant (Figure 4-4B). Together, these data suggest FANCA may be utilizing its helix destabilization properties at stalled replication forks to distinguish diadducts from monoadducts.

**Effect of ATP and Mg\(^{2+}\)**

Helix destabilizing proteins can act in an ATP-dependent or -independent manner. Although the initially observed unwinding activity of FANCA did not require nucleotide triphosphates (NTPs) or divalent cations, I examined their effect using dsDNA with a 3’-overhang DNA. My results show that the helix destabilization activity of FANCA was mildly affected by the presence of 5 mM Mg\(^{2+}\) alone (Figure 4-5A, lanes 9-11). Remarkably, this activity was greatly inhibited in the presence of 2 mM ATP alone (Figure 4-5A, lanes 6-8). The combination of 2 mM ATP and 5 mM Mg\(^{2+}\) did not yield a greater effect than that of ATP alone (Figure 4-5A, lanes 12-14). I hypothesized this inhibitory effect might be a consequence of decreased DNA binding activity of FANCA upon ATP binding, perhaps through a conformational change.

To analyze whether FANCA indeed binds ATP or not, I performed a filter binding assay for ATP binding in the presence and absence of DNA and/or 5 mM Mg\(^{2+}\). My
results show that 30 nM FANCA binds ATP at 48, 22, and 13% efficiency in the presence of 1.5, 3 and 6 pmol ATP, respectively (Figure 4-6). The presence of DNA mildly affects ATP binding by an average change of 1.2 fold. However, Mg$^{2+}$ greatly reduces the ability of FANCA to bind ATP by 4-6 fold in the absence of DNA and 7-13 fold in the presence of DNA. These results lead us to conclude that the inhibitory effect ATP has on FANCA’s helix destabilization activity (Figure 4-5A, lanes 6-8) is predominantly through its binding effect on FANCA directly; adding Mg$^{2+}$ to the reaction mixture relieves the inhibitory effect ATP imposes on the helix destabilization activity of FANCA mainly through the reduction of ATP binding (Figure 4-5A, lanes 12-14).

Because the helix destabilization activity of FANCA was perturbed, I reasoned ATP and Mg$^{2+}$ might also have an effect on the single-strand annealing activity. Additionally, recombination proteins with single-strand annealing activity are stimulated by the presence of ATP (100). To test this I performed the single-strand annealing assay in the presence and absence of 2 mM ATP and/or 5 mM Mg$^{2+}$. My results show that the single-strand annealing activity of FANCA was mildly affected by the presence of ATP alone (Figure 4-5B, lanes 6-8). However, 5 mM Mg$^{2+}$ alone greatly inhibited the annealing activity of FANCA in the absence (Figure 4-5B, lanes 9-11) and presence (Figure 4-5B, lanes 12-14) of ATP, suggesting a role for Mg$^{2+}$ that is independent from ATP.

**FANCA exhibits DNA strand exchange activity**

In canonical HR 5’-to-3’ end resection of each side of the break exposes ssDNA filaments that are quickly coated with replication protein A (RPA). With the help of mediator FANCD1(BRCA2), RAD51 is able to displace RPA and catalyze strand invasion and exchange into intact homologous DNA (50-52). RAD51AP1 acts as a
RAD51 accessory protein and enhances RAD51-mediated HR by cooperating with FANCN(PALB2) to promote d-loop formation. FANCS(BRCA1) forms a complex with FANCD1(BRCA2) and FANCN(PALB2); this complex is also important in RAD51-mediated HR (101-103). Multiple groups have identified a strong correlation of FANCA overexpression with HR proteins including RAD51, FANCS(BRCA1), and RAD51 associated protein 1 (RAD51AP1) in basal breast cancer and retinoblastoma tumors (104-106), as well as in high-risk HPV-infected tissues with increased DNA damage (107). Correspondingly, FANCA null cells exhibit attenuated FANCS(BRCA1) and RAD51 foci formation after MMC treatment (68). More importantly in respect to DSB response, RAD51 foci formation was attenuated in FANCA null cells after CNDAC-induced DSB formation (108). Additionally, FANCA has been found to directly interact with FANCS(BRCA1) independently of DNA damage, indicating a role for FANCA outside of ICL repair (26). Given the dual-role of FANCA in maintaining DNA stability alike other recombination proteins and its high correlation with other DSB repair factors, I hypothesized it might also be involved in recombinogenic events during HR.

Because single-strand annealing and strand exchange are coupled in many DSB repair proteins including RAD51 and RAD52 (109,110) and to further elucidate the role of FANCA in HR-mediated events I tested its strand exchange activity. To this end, I incubated increasing amounts of FANCA with a pre-annealed fork-like substrate in combination with a third unlabeled ssDNA oligonucleotide of 81 nt in length completely complementary to the strand of the fork with an exposed ssDNA 5’ flap. My results show that FANCA promotes strand exchange of what would be an aberrant intermediate structure to form a more kinetically stable product (Figure 4-7A, lanes 14 and 15).
Although the exchanged product (136 nt total) is larger than the initial fork-like substrate (106 nt total), the increased stability of the exchanged product allows for easier and faster migration through the pores of the gel (Figure 4-7A, lane 14). Whether FANCA promotes strand exchange through invasion of dsDNA followed by DNA heteroduplex extension or simply renaturation of ssDNA after destabilization of the initial fork-like substrate is still unclear. The latter mechanism has been coined “apparent strand exchange” since it yields the same products (100).

I further examined the role of FANCA in strand exchange using a 3’-overhang DNA structure, which mimics partially resected dsDNA on the 5’-end, in combination with a short blunt-ended dsDNA with full homology to the uncovered flap of the invading DNA structure. Consistent with my initial helix destabilization findings in Figure 4-4A, my results show that low concentrations of FANCA (0.6-30 nM) destabilize 3’-overhang DNA structures with peak destabilization activity observed at 5 nM (Figure 4-7B, lanes 5-11). Higher concentrations of FANCA (40-50 nM) promote strand exchange with a dsDNA of 20 nt in length in a concentration-dependent manner (Figure 4-7B, lanes 12 and 13). Both activities can be clearly seen in a concentration curve experiment using 0.6 to 50 nM FANCA in a reaction mixture that includes pre-annealed overhang and blunt-ended structures.

First, initial destabilization of a radiolabeled overhang structure, presumably destabilized along with unlabeled blunt-ended structure, can be visualized as a band that runs along the same height as the ssDNA control (Figure 4-7B, lanes 1 and 5-11). This band increases in intensity, then decreases in respect to increasing FANCA concentration. Given the newly discovered characteristics of FANCA as both a helix destabilization and
strand annealing protein, this decrease in destabilization can also be understood to be an increase in stabilization that tips the equilibrium toward the formation of the initial substrates (Figure 4-7B, midpoint between lane 11 and 12, Figure 4-7A, lane 10). Strand exchange with intact dsDNA and further stabilization of a tri-oligonucleotide complex can be visualized as the appearance of a band comigrating at a slower pace with the strand exchange control (Figure 4-7B, lanes 2 and 12-13). To determine the polarity of this activity, I performed the same experiment using a 5’-overhang DNA structure, which mimics partially resected dsDNA substrates on the 3’-end. I observed complete bidirectionality with peak destabilization activity seen at 2.5 nM FANCA (Figure 4-7B, lane 7) and steady increase in stabilization from 30 to 50 nM FANCA (Figure 4-7B, lanes 11-13).

To further examine the role of FANCA in HR events I tested its exchange activity in combination with RAD51 but found that different molar ratio combinations resulted in little to no change in the respective strand exchange activities of their individual counterparts (Figure 4-8). This may be indicative of FANCA having an independent role in HR-mediated strand exchange mechanisms from RAD51.

**FANCA and RAD51 have synergistic helix stabilization activity**

While RAD51 and FANCA are not collectively facilitating the initial strand exchange process, it’s possible they may work in concert for the ensuing single-strand annealing step. To entertain this idea I examined the single-strand annealing activity of FANCA in combination with RAD51 (Figure 4-9). Combinations of RAD51 (40 nM) and FANCA (20 nM) resulted in the synergistic increase (86%) in single-strand annealing of 74 nt-long fully homologous ssDNA compared to that from the individual incubations of
RAD51 or FANCA alone (Figure 4-9, lanes 3-5). Collectively, these results suggest FANCA may play a role in recombination repair, in particular the annealing step required to pair homologous DNA, possibly in concert with RAD51.

**FANCA promotes the pairing of complex DSB repair intermediate structures**

The repair of DSBs gives rise to intermediate structures with 3’-overhanging ssDNA tails. RAD52 serves as the main DNA annealing factor required for the SSA pathway. Yet, cells lacking RAD52 function present with a relatively weak HR phenotype, raising the possibility that RAD52 alone is not responsible for catalyzing the annealing steps in the mammalian DSB repair (55). FA proteins FANCA, FANCG and FANCS(BRCA1) have been shown to promote homology-dependent (HR and SSA) repair of DSBs. In particular, restoration of FANCA and FANCG in patient derived cell lines from the FA-A and FA-G complementation groups showed 2.7 and 2.2 fold increase, respectively, in SSA and HR (46). Other studies involving FANCG mutant chicken DT40 cells showed severe impairment of HR compared to wild-type cells (58,59). Altogether, this indicates that FANCA and FANCG, which embody one of the anchoring units of the FA-core complex, are also important proteins in homology-directed events of DSB repair.

To test whether FANCA is involved in the annealing of more physiologically relevant structures I incubated two 3’-overhang DNA structures with a central homologous sequence stretch flanked by a dsDNA region and a heterologous 3’-tail. These structures were designed to mimic the partially resected dsDNA intermediates that are substrates for the SSA and MMEJ pathways. As seen in Figure 4-11, FANCA promotes the annealing of these structures giving rise to dsDNA products with 3’-overhangs (Figure 4-10A). This intermediate would theoretically serve as substrate for structure specific endonucleases
and ligases for subsequent flap removal and end-ligation. Notably, FANCA also promotes the annealing of resected substrates with 100% homologous tails (Figure 4-10B). To determine the polarity of this activity, I performed the same experiment using partially resected dsDNA substrates on the 3’-end and observed complete bidirectionality (Figure 4-10C). Here too, we can appreciate the concentration-dependent helix destabilization and single-strand annealing activities of FANCA. It’s conceivable that FANCA coordinates its helix destabilization and stabilization activities to achieve more complex strand exchange transactions.

**Several FANCA mutants exhibit defective single-strand annealing and strand exchange activity compared to wild-type**

Currently, there is little information on the structural properties of FANCA despite the variety of mutations found in FA patients. We previously showed that the C-terminal amino acids 772–1455 of FANCA are important for its nucleic-acid binding ability (Figure 4-11) (25). Interestingly, most patient derived FANCA mutations reside within this part of the protein. To determine whether the DNA binding activity of FANCA is important for its helix destabilization, single-strand annealing, or strand exchange activity, I incubated the same C-terminal (Q772X) and N-terminal (C772-1455) truncated FANCA proteins used in our previous studies with the oligonucleotide cocktail specific for each assay. Q772X is a Fanconi anemia disease-causing mutant, while C772-1455 is the complementing C-terminal fragment. My results show that the single-strand annealing activity of FANCA requires both the N-terminal and C-terminal portions of the protein (Figure 4-12A, lanes 4 and 5). This indicates that the DNA binding activity of FANCA alone is not sufficient to catalyze single-strand annealing. Alternatively, either terminals
of the protein are sufficient to promote the destabilization of the 3’-overhang DNA structure (Figure 4-12B, lanes 4 and 5).

To shed light into which region of the protein might be important for helix destabilization, stabilization or strand exchange activity, I tested specific patient-derived FANCA mutations. To this end, the following genetic variants of FANCA were purified: D598N, R951W, R1117G, Q1128E, and F1263Δ. EMSA analysis was used to determine their ability of all variants to bind DNA; all variants retained DNA binding affinity (Figure 4-11). To examine the effect of these variants on DNA stabilization, I performed the same three assays described earlier. F1263Δ was completely deficient in single-strand annealing activity (Figure 4-12A, lane 10); yet D598N, R1117G, and Q1128E exhibited moderately decreased single-strand annealing. In the helix destabilization assay, all variants retained activity with the exception of Q1128E, which showed slightly decreased activity (Figure 4-12B). All mutants behaved similarly in the strand exchange assay as they did in the single-strand annealing assay (Figure 4-12C, lanes 4-11). It’s reasonable that those mutants lacking single-strand annealing activity are unable to catalyze and complete strand exchange.

**Discussion**

We recently showed that FANCA is required for successful recruitment of structure specific endonuclease MUS81-EME1 to crosslinked DNA; molar ratios of 10-20:1 FANCA relative to DNA enhance the incision activity of MUS81-EME1 in the presence of an ICL and inhibit its activity in the absence of an ICL (67). This concentration range coincides with the helix destabilization activity ascribed to FANCA in the studies presented on this chapter. It’s possible that when forks collide in the presence of an ICL,
recruitment of FANCA to the damaged site results in its local accumulation and concentration-dependent regulation of MUS81-EME1 incision activity, mainly due to the inability of FANCA to promote helix destabilization. Ratios below (5:1) or above (20:1) FANCA:DNA were not previously tested in respect to MUS81-EME1 activity.

Nicking of ICLs produces intermediate DSB structures, which are also a common occurrence in cells resulting from endogenous replication fork demise via structure-specific endonucleases or exogenous sources of DNA damage like clastogens, laser- and γ-irradiation. The repair of these lesions is of utmost importance in order to avoid catastrophic cellular consequences. In these studies I show that FANCA exhibits strand exchange activity at 80-100:1 stoichiometric ratio relative to DNA when the invading structure contains a 3′-ssDNA overhang (Figure 4-7B, lanes 12 and 13) and 60-100:1 when the invading structure contains a 5′-ssDNA overhang (Figure 4-7B, lanes 24-26). The higher invasion efficiency of FANCA toward 5′-tailed over 3′-tailed structures is consistent with its higher preference for binding this substrate (25); however, the physiological relevance of this preference is still unclear. I also show that FANCA facilitates strand exchange initiating from the ssDNA 5′-flap of a fork-like structure at 60-80:1 ratios (Figure 4-7A, lanes 14 and 15) and single-strand annealing activity at 40-80:1 ratios (Figure 4-1A).

Nonetheless, it’s important to note that while my experiments show that FANCA exhibits strand exchange and annealing activities, both of which are required steps in HR, I only saw cooperation between FANCA and RAD51 in the annealing assay. my data show that molar ratios of 20:40:1 FANCA:RAD51:DNA allow both proteins to exhibit a synergistic annealing effect on fully homologous ssDNA oligonucleotides (Figure 4-9).
Single-strand annealing is a critical step in second-end capture during HR and SDSA. Other DSB repair pathways aside from HR and NHEJ include SSA and MMEJ, also known as alternative-NHEJ (Figure 1-3); both pathways utilize single-strand annealing activity to anneal exposed complementary regions of ssDNA, followed by 3’ heterologous tail cleavage and end ligation (43). Given the likelihood that RAD52 alone is not the main single-strand annealing factor in the mammalian SSA pathway, the documented role of FANCA in homology-directed repair of DSBs in cells, and the data presented in this chapter, I propose FANCA to be directly involved in the SSA and HR pathways of DSB repair.

**Concluding remarks**

The studies presented in this chapter provide a more complete understanding of how FANCA maintains genomic integrity through its intrinsic helix destabilization, stabilization and strand exchange activities. I propose FANCA may act in the maintenance of stalled replication forks in conjunction with RAD51 and the processing of recombination intermediates by facilitating single-strand annealing of homologous sequences, recruiting structure-specific endonucleases, and regulating their incision activity accordingly.
Figures

Figure 4-1: FANCA exhibits SSA activity at high concentration.

(A) Titration of purified FANCA (10, 20, 30, and 40 nM) on fully complementary ssDNA oligos 74 nt in length. Quantification of SSA activity is represented by the graph on the right. (B) Time course incubations (0, 1, 2, 5, 10, 20, 40 min) of 40 nM FANCA on the fully complementary ssDNA substrates (C) Immunodepletion analysis using anti-FANCA antibody.
Figure 4-2: Examination of the effect of PK stop solution on FANCA-mediated SSA.
Figure 4-3: Helix destabilization assay on a split arm DNA structure.

Titration of purified FANCA (0, 0.6, 1.25, 2.5, 5, 10, and 20nM) on the 5’-labeled split arm DNA structure as illustrated above the gel picture.
Figure 4-4: FANCA has helix destabilizing activity at low concentrations.

(A) Titration of purified FANCA (0, 0.6, 1.25, 2.5, 5, 10, and 20nM) on the a. 3’ single-stranded overhang, b. 5’ single-stranded overhang, and c. double blunt-ended dsDNA structures as illustrated on the left. Schematic appearance of the controls and resulting products is shown to the right of each picture. All reactions were performed in a 10 µl reaction of 25 mM Tris-HCl, pH 8.5, 100 mM NaCl, 1mM EDTA, pH 8. Red asterisks (*) indicate γ-32P labeling by T4 polynucleotide kinase. (B) Immunodepletion analysis using anti-FANCA antibody.
Figure 4-5: Effect of ATP and Magnesium on the helix destabilizing and single-strand annealing activity of FANCA.

(A) Helix destabilization assay. Titration of purified FANCA (1.25, 2.5, and 5 nM) on the 3’ overhang substrate in the presence of 2 mM ATP, 5 nM Mg\(^{2+}\), or both. (B) Single-strand annealing assay. Titration of purified FANCA (10, 20, and 30 nM) in the presence of 2 mM ATP, 5 nM Mg\(^{2+}\), or both. Schematic appearance of the controls and resulting products is shown on the right of each picture. All reactions were performed in a 10µl reaction of 25 mM Tris-HCl, pH 8.5, 100 mM NaCl, 1 mM EDTA, pH 8. Red asterisks (*) indicate γ-\(^{32}\)P labeling by T4 polynucleotide kinase.
30 nM FANCA was incubated in the presence and absence of 1.5, 3 and 6 pmol of [\alpha^{32}P] ATP, 5mM MgCl2, and/or 1 nM DNA.
Figure 4-7: FANCA exhibits strand exchange activity.

(A) Titration of purified FANCA (10, 20, 30, and 40 nM) on ssDNA oligos showing SSA activity (left panel). Titration of purified FANCA (10, 20, 30, and 40 nM) on pre-annealed splayed arm structure showing strand exchange activity (right panel). All reactions were performed in a 10 µl reaction of 25 mM Tris-HCl, pH 8.5, 100 mM NaCl,
1 mM EDTA, pH 8. HD, helix destabilization. (B) Titration of purified FANCA (0, 0.6, 1.25, 2.5, 5, 10, 20, 30, 40, and 50 nM) on a dsDNA structure with partial 5’-end resection of one strand and a short blunt-ended dsDNA with full homology to the uncovered flap of the invading DNA structure (left panel), and a dsDNA structure with partial 3’-end resection of one strand and a short blunt-ended dsDNA with full homology to the uncovered flap of the invading DNA structure (right panel). Quantification of unwinding and strand exchange activity is represented by the graphs below each gel. Unwinding is represented as a negative change in overall % product formation; whereas strand exchange is represented as a positive change.
Figure 4-8: Combined strand exchange activity of RAD51 and FANCA.

Titration of purified RAD51 (5, 10, 20, and 40 nM), purified FANCA (10, 20, and 40 nM), and a combination of 20 nM RAD51 and FANCA (10, 20, and 40 nM) using the strand invasion assay.
Figure 4-9: Combined single strand annealing activity of RAD51 and FANCA.

Purified RAD51 [40 nM] in the presence of 20 nM FANCA on fully complementary ssDNA oligos 74-nt in length. Both RAD51 and FANCA were added to the reaction mixture at the same time. All reactions were performed in a 10 µl reaction of 25 mM Tris-HCl, pH 8.5, 100 mM NaCl, 1 mM EDTA, pH 8.
Figure 4-10: FANCA enables the bi-directional SSA of DSB intermediate structures.

Titration of purified FANCA (0, 10, 20, 30, and 40 nM) on (A) two 3’ single-stranded overhang structures with a central homologous sequence stretch flanked by a dsDNA region and a heterologous 3’ tail (B) two 3’ single-stranded overhang structures with fully homologous exposed sequences (C) two 5’ single-stranded overhang structures with fully homologous exposed sequences. Quantification of unwinding and SSA activity is represented by the graphs below each gel. Unwinding is represented as a negative change (black) in overall % product formation; whereas SSA is represented as a positive change (red). HD, helix destabilization.
**Figure 4-11: Evaluation of the DNA binding ability of FANCA mutants.**

(A) Genetic variants of FANCA: C-terminal truncation (Q772X), N-terminal truncation (C772-1455), D598N, R951W, R1117G, Q1128E, and A1263Del. Q1128E and A1263Del were analyzed for DNA binding activity using an EMSA. (B) Schematic
representation of the FANCA gene indicating the location of important domains and mutations. The nuclear localization signal (NLS) at amino acids 18-34 (67), FANCG-interaction domain amino acids 18-29 (68), BRCA1-interaction domain amino acids 740-1083 (69) and FAAP20-interaction domain amino acids 1095-1200 (70).
Genetic variants of FANCA: C-terminal truncation (Q772X), N-terminal truncation (C772-1455), D598N, R951W, R1117G, Q1128E, and F1263Del were analyzed for (A) helix stabilization (B) helix destabilization (C) and strand exchange activity.
Quantification of each activity is represented by the bar graphs on the right. Statistical significance is represented by the asterisks (** $p \leq 0.01$, * $p \leq 0.05$).
CHAPTER 5 - MODELS AND FUTURE DIRECTIONS

Genomic stability

It is conceivable that proteins harboring helix destabilization, single-strand annealing, and strand exchange activities work in concert to protect genome integrity and efficiently process replication and recombination intermediate structures. Patient-derived cells lacking functional FANCA exhibit degradation of newly synthesized DNA when exposed to the replication stalling agent hydroxyurea, suggesting a role for FANCA in the maintenance of replication fork stability independent of its role in ICL repair (29). We previously reported that FANCA has intrinsic affinity toward nucleic acids (25). FANCA was later proposed to be part of one of the main anchoring modules of the FA-core complex (14). Here, I report that FANCA has helix destabilization activity at 1.2-20:1 stoichiometric ratios relative to DNA, peaking at 10:1 for the fork-like (Figure 4-3) and 3’-overhang DNA (Figure 4-4A-a) structures, and 20:1 for the 5’-overhang DNA structure (Figure 4-4A-b). It’s possible that when replication forks are stalled due to sources other than ICLs, FANCA is recruited to remodel the fork and promote fork-restart via ssDNA annealing of excessively unwound template or fork regression and “chicken foot” formation (Figure 5-1C, left side). In this scenario, local FANCA concentration relative to DNA may never be higher than 20:1.

Previous work has shown that although RAD51 is involved in the repair of DSB intermediates downstream of FANCI-FANCD2 (FA pathway activation during ICL repair), it is still recruited to sites of ICL damage independently of FANCI-FANCD2 activation (111). Also, RAD51 and FANCA have been individually shown to prevent
degradation of stalled replication forks (29). Taken together with my synergistic results involving FANCA and RAD51 in SSA, this suggests a possible requirement for both proteins to work in concert to prevent genomic instability independently of their role in later steps of DNA damage repair. Additionally, FANCA has been found to directly interact with FANCS(BRCA1) independently of DNA damage, indicating a role for FANCA outside of ICL repair (26).

Future directions
It would be beneficial to investigate whether FANCA cooperates with error-prone translesion bypass synthesis (TLS) polymerases given their intrinsic tolerance for monoadducts. For example, monoubiquitinated TLS polymerase REV1 is stabilized by the ubiquitin-binding zinc finger 4 domain of FAAP20, which in turn interacts with FANCA (22,112). One possibility is that FANCA assists REV1 in bypassing monoadducts and unhooked ICLs. Alternatively, FANCA may work in concert with other replication fork restart factors like BLM or WRN given the similarities in their functions.

ICL repair
FANCM has long been considered an ICL damage recognition factor because it can stabilize and remodel stalled replication forks. Thus, it may provide temporal and spatial access for the damage to be repaired (113,114). FANCM also appears to be required for assembly of the FA-core complex onto chromatin and subsequent monoubiquitination of the FANCI-FANCD2 complex (37,71,115-122). Controversially, other reports demonstrated that FANCM is not required for the formation of the eight-subunit FA-core complex and FANCM null cells are only partially defective in damage-induced FANCD2 monoubiquitination (115,123,124). Evidence from FANCM−/− knockout mice further
demonstrated that FANCM may have a stimulatory, but not essential role in monoubiquitinating FANCD2 (125). Furthermore, a direct interacting partner for FANCM-FAAP24 in the FA core complex has not been identified thus far, although FANCM-FAAP24 was originally identified through protein association in a FANCA-specific immunoprecipitation assay (27,117,126). Additionally, FANCM<sup>−/−</sup> cells are sensitive to camptothecin, a topoisomerase inhibitor. Susceptibility to camptothecin is a unique feature identified only for downstream repair factors such as FANCD1(BRCA2) and FANCN/PALB2, but not for components of the FA core complex (123). In summary, these observations suggest that FANCM may act downstream of FANCD2 and therefore the upstream FA-core complex may be recruited to DNA through other mechanisms. One of such mechanisms is likely through the DNA binding activity of FANCA (25).

I propose here a mechanism in which FANCA recognizes the ICL damage, helps anchor the FA-core complex to the DNA, recruits structure-specific endonuclease MUS81/EME1 and enhances its incision activity accordingly (Figure 5-1C, right side). Alternatively, in the presence of non-crosslinked DNA FANCA acts as a local helix destabilizing protein and promotes fork protection by drawing the nuclease away from the fork, thereby inhibiting it’s incision activity (Figure 5-1C, left side). Local accumulation of FANCA past its maximum unwinding ratio of 10:1 (FANCA:DNA) might never occur as unwinding would allow the protein to move away from the fork junction.

**Future directions**

FANCA also interacts and colocalizes with XPF-ERCC1, another important ICL unhooking DNA endonuclease (127-130). Both MUS81-EME1 and XPF-ERCC1 interact...
with FANCP(SLX4), a newly identified Fanconi anemia protein. FANCP(SLX4) in turn serves as a docking protein for other endonucleases including SLX1 (131). The FANCP-SLX4–XPF-ERCC1–MUS81-EME1 tri-endonuclease complex has been demonstrated to orchestrate nuclease actions during ICL incision and subsequent fork reestablishment through homologous recombination (13,30,41,72,90,131-134). It would be important to examine whether FANCA also regulates XPF-ERCC1, SLX1, or FANCP(SLX4) for efficient ICL or DSB repair using our in vitro reconstitution system.

**DSB repair**

Competition between NHEJ, MMEJ, SSA, and HR pathways is a dynamic process that is dependent upon a few important factors such as the extent of end resection, DNA target availability, and protein availability in the nucleus (54). It is reasonable that the cell would choose those repair pathways that reserve higher fidelity, which would require longer sequences of homology to prevent imperfect pairing. This would provide an advantage for SSA and gene conversion over NHEJ events. Similarly, formation of non-crossovers (NCO) would be favored since it allows for the retention of heterozygosity in mitotic cells. Indeed under normal conditions sister chromatid exchange (SCE) formation has been reported to be minimal in cells (135,136). Accordingly, 90% of events in HR are directed to the synthesis-dependent strand annealing (SDSA) pathway which gives rise to NCO only (Figure 5-3). The remaining 10% of events in HR give rise to Holliday Junctions (HJ) which are either dissolved by BLM–topolllα–RMI1–RMI2 (BTR) to give rise to NCO or resolved by SLX1–FANCP(SLX4)–MUS81-EME1 or GEN1 to generate both NCO and crossovers (CO), respectively (137,138). All favored pathways of DSB repair require single-strand annealing activity (Figures 1-2, 5-1, and 5-3, yellow boxes),
providing a possible explanation for the HR phenotype found in FANCA-null cells. Levels of BLM, an annealing helicase involved in the dissolution of HJs, have been shown to peak during S and G2-M phases of the cell cycle and Bloom’s syndrome lymphocytes exhibit increased SCE (139). Intriguingly, FANCA was found to be present in BRAFT, a multiprotein nuclear complex containing BLM, RPA, FANCA, FANCG and Topo IIIα (27). Together, this supports the NCO preference theory. It’s possible FANCA may work in parallel or in concert with BLM to promote the formation of NCO in somatic cells. This could in turn explain the reduced fertility rates found in FA and Bloom’s syndrome patients. Alternatively, it’s also possible that FANCA may work in concert with SLX1–FANCP(SLX4)–MUS81-EME1 to promote the asymmetrical resolution of double HJs, also leading to NCO formation.

ATP seems to be another important determinant of pathway choice since its presence favors RAD51-dependent invasion (HR pathway), but its absence favors strand annealing activity (SSA pathway). For example, the inhibitory effect of RAD51 on RAD52-dependent single-strand annealing requires ATP (54). FANCD1(BRCA2) stimulates RAD51-dependent invasion in an ATP-dependent manner (51). Moreover, Dna2-mediated single-strand annealing activity is inhibited by ATP (69). In my experiments, the single-strand annealing activity of FANCA was unaffected by the presence of ATP. It’s possible FANCA endows RAD52-mediated single-strand annealing an advantage even in the presence of ATP. On the other hand, Mg$^{2+}$ greatly inhibited the single-strand annealing activity of FANCA, possibly through a conformational change on the entire protein. The dramatic decrease in helix destabilization activity of FANCA in the presence
of ATP, but not Mg\(^{2+}\), suggests both activities (helix destabilization and single-strand annealing) are mechanistically distinct.

FANCS(BRCA1), FANCN(PALB2), and FANCD1(BRCA2) form a complex that is important for RAD51-mediated recombination (Figure 5-2, left side). FANCN(PALB2)–FANCD1(BRCA2) interaction is crucial for FANCD1(BRCA2) foci formation, while FANCS(BRCA1)–FANCN(PALB2) interaction is crucial for FANCD1(BRCA2) function (101,102,140). FANCD1(BRCA2) in turn mediates RAD51 displacement of RPA, resulting in strand invasion and exchange into homologous dsDNA (50-52). Depletion of RAD52 in cancer cells lacking FANCS(BRCA1), FANCN(PALB2), or FANCD1(BRCA2) results in synthetic lethality (101-103). This is suggestive of a RAD52-dependent DSB repair pathway, presumably SSA, that is independent of the BRCA pathway. However, RAD52 alone must not be responsible for catalyzing the annealing step in the SSA pathway since mammalian cells lacking RAD52 function with a relatively weak HR phenotype (55-57). Like FANCS(BRCA1), FANCA has been shown to promote SSA biochemically (Chapter 4) and in cell-based recombination assays (46,58-60). Together, this suggests FANCA may work in concert with RAD52 to promote the repair of DSBs through SSA mechanisms.

One possibility is that RAD52 serves as another alternative to the BRCA pathway. FANCN(PALB2) and RAD52 double knockdown presents with attenuated ionizing radiation-induced RAD51 foci formation in comparison to their individual knockdown effects (103,141,142). This suggests that although RAD52 does not seem to be directly involved as a mediator of RAD51-DNA filament formation (perhaps through the modulation of FANCD1(BRCA2) function), it may still play an important role in the
RAD51-dependent pathway of DSB repair in collaboration with other proteins like FANCN(PALB2), particularly upstream of RAD51 foci formation. Given that FANCA null cells exhibited attenuated FANCS(BRCA1) and RAD51 foci formation after MMC treatment (68) and the strong expression correlation between FANCA, FANCS(BRCA1), and RAD51AP1, it’s possible FANCA also plays an important role in the RAD51-dependent pathway of DSB repair.

**Future directions**

The intricate details of mammalian DSB repair are still largely unknown. It’s important to keep in mind that the involvement of both RAD52 and FANCA downstream of RAD51 foci formation in the DSB repair pathway is inherent to their single-strand annealing activity and the requirement for this activity in downstream repair steps. Experimental design is crucial when trying to discern between the upstream and downstream roles for each protein. The establishment of FANCA as a critical player in DSB repair warrants a closer look into its collaboration with other repair factors, particularly RAD51 and RAD52. It would be important to determine which pathway (HR or SSA) FANCA favors on its own and if this role is affected when working in collaboration with other proteins. The ability of FANCA to displace RPA should also be tested; until now only RAD52 is able to do this. While both FANCA and RAD52 are able to promote SSA, it’s possible RAD52 remains as the main mediator of RPA displacement. The annealing efficiencies for both RAD52 and FANCA also need to be determined. Biochemically, the effect of Mg$^{2+}$ and ATP on FANCA structure and how it relates to FANCA function is also yet to be determined.
Despite their synthetically lethal interactions in mammalian cancer cells, RAD52 and FANCD1(BRCA2) are epistatic in chicken DT40 cells (143). These somewhat contradictory results warrant a closer look at these proteins across the evolutionary spectrum.

It remains a mystery why extremely low concentration of FANCA (5:1, FANCA:DNA) stimulates MUS81-EME1 activity in the absence of DNA damage although the stimulatory activity is lower than in the presence of ICL (Figure 3-7). Since MUS81-EME1 is also involved in resolution of Holliday junction, which is a later step in the Fanconi anemia pathway of ICL repair (90,144), and the SSA activity of FANCA and its involvement in fork maintenance make it a good candidate for fork regression and Holliday junction formation, I speculate that FANCA may additionally be involved in the regulation of MUS81-EME1-dependent Holliday junction resolution. It would be interesting to address whether and how FANCA affects MUS81-EME1 mediated incision of Holliday junctions.

**Microhomology-mediated end joining and class-switch recombination**

An important protein involved in the SSA pathway in yeast is RAD59, which interacts with RAD52 and enhances its annealing activity (145). The minimum effective processing segment of sequence homology required for RAD52-RAD59-dependent single-strand annealing was calculated to be ~29-33 bp by linear regression. Below this point, homology dependency becomes non-linear suggesting an alternative pathway is necessary for short homologous sequences (44). Consistent with this, MMEJ events involving microhomologies of 1-5 bp have been reported to be independent of RAD52 (146,147). Intriguingly, FANCA was found to be involved in the stabilization of
microhomologies during class-switch recombination (64). As shown in Figure 4-10A, FANCA enhances the SSA-mediated formation of 3’ overhang structures, which are intermediates in both the SSA and MMEJ pathways and preferred substrates of MUS81-EME1 incision. FANCA has been shown to be required for the recruitment of structure-specific endonucleases MUS81-EME1 and XPF-ERCC1 (65,67). This raises the possibility that FANCA is required to stabilize the overhangs created by SSA events and recruit the endonucleases necessary for further processing; altogether indicating that FANCA may be an important microhomology stabilization factor during CSR. Consistent with this, FANCA mRNA expression is increased in the germinal centers of B cells, which exhibit high SHM and CSR (66). Interestingly, FANCA-/- mice exhibit increased intra-switch recombination (ISR) implicating FANCA in the suppression of short-range joining (64). Given the newly ascribed SSA activity of FANCA, it’s possible it may play a prominent role in SSA and MMEJ pathways and serve as a fidelity mechanism in CSR. I propose a model for the involvement of FANCA in SSA where FANCA acts in a similar manner as RAD52 and promotes the annealing of homologous sequences (Figure 5-3).

**Future directions**

Together, these studies suggest FANCA may play a more prominent role in SSA and MMEJ pathways than RAD52 and warrant future investigation into the minimum homology requirement for FANCA-catalyzed single-strand annealing. Because FANCA has multiple DNA manipulation functions that are similar to that of BLM, it would be interesting to learn whether FANCA, in addition to its strand annealing activity, can also help in the branch migration process during HJ dissolution. It would also be interesting to
see whether the regulatory activity of FANCA on MUS81-EME1 incision reserves any biological consequence in the context of human recombination. Lastly, it’s likely that in the absence of FANCA, HJ processing gets shuttled toward the Gen1-mediated resolution pathway. This provides an explanation for the increased SCE frequency found in patient-derived cells defective in FANCA in response to diepoxybutane and ionizing radiation treatment (148).

**Alternative roles of FANCA - Okazaki fragment maturation**

While replication on the leading strand of the DNA is a continuous process, synthesis on the lagging strand is discontinuous due to the 3’ to 5’ directionality of DNA polymerase and leads to Okazaki fragment formation. Further processing of these fragments in order to form a functional DNA fragment is referred to as Okazaki fragment maturation (OFM). Lagging strand DNA synthesis is initiated by the incorporation of small RNA primers which, after some nucleotide polymerization of the fragment, must then be destabilized to form 5’ flap structures; these structures serve as substrates for structure-specific endonucleases that help remove the flap and allow for subsequent end- ligation (149-151). Two important proteins involved in the nucleolytic removal of RNA/DNA primers during OFM are Dna2 and FEN1. Excessive strand displacement by DNA polymerase δ (pol δ) can result in the formation of long flap structures coated with RPA that are inefficiently processed by FEN1. Such structures are better processed by Dna2, whose incision activity is even enhanced by RPA (69,149,150,152,153). The cooperation of both nucleases is important for successful processing of flaps during lagging strand synthesis (69).
A recent model for Okazaki fragment synthesis and RNA/DNA primer removal is that Dna2, BLM, and WRN helicases are responsible for equilibrating flap intermediate formation by shifting substrate formation between 5’ flap and 3’ flap using their strand exchange, annealing, and unwinding activities (Figure 5-4) (154,155). Here, I show that FANCA also has helix destabilization, stabilization and strand exchange activity. Furthermore, we recently showed that FANCA, like Dna2, BLM, and WRN, not only interacts with FEN1, but also stimulates its nucleolytic activity toward short DNA and RNA flaps (151). Intriguingly, FANCA as part of the multiprotein nuclear complex BRAFT reserves higher binding affinity for ssRNA over ssDNA (~1.9 fold at 8:1 protein:oligonucleotide molar ratios) (25). This is consistent with my findings that FANCA successfully destabilizes duplex DNA in a bidirectional manner at protein:oligonucleotide molar ratios lower than 20:1. I propose the annealing, unwinding, and strand exchange activity of FANCA promotes the remodeling of Okazaki fragments toward the formation of 5’ flap intermediates. This in turn increases substrate availability for FEN1 processing and culminates in the amplified incision of flaps by the nuclease (Figure 5-4).
Figures

Figure 5-1: Hypothetical role of FANCA in ICL and DSB repair.

(A) ICL repair. ICLs encountered during cellular replication lead to the formation of stalled forks. Nicking of the crosslink produces intermediate DSBs (3’-overhang structure seen on top, black) that get repaired via the HR pathway. Alternatively, other
intermediate structures (seen on bottom) serve as substrates for TLS which helps to fill in the gap (nascent DNA, blue). This portion of DNA is homologous to the 3’-overhang DSB structure seen on top and can be used by HR to reestablish a replication fork via a FANCA-dependent (yellow oval) strand exchange and annealing mechanism; (B) Multiple pathways involved in DSB repair. Length of homology required by each pathway is represented by the grey rectangles directly above each pathway. (e.g. HR requires considerably longer regions of homology than MMEJ). Depicted in yellow ovals are the homology-directed steps in which FANCA may play a role in. HR, homologous recombination; SSA, single-strand annealing; MMEJ, microhomology-mediated end joining; NHEJ, non-homologous end joining. (C) FANCA in regulation of MUS81-EME1 activity. In the presence of undamaged or mono-adducted DNA (orange square) FANCA is able to promote fork regression via SSA or locally unwind the dsDNA via helix destabilization and subsequently protect the replication fork by preventing MUS81/EME1 incision. Alternatively, in the presence of an ICL FANCA is unable to remodel the replication fork and instead acts to recruit and enhance the incision activity of MUS81/EME1. As documented before, FANCA also acts as one of the anchoring units of the FA core complex, which activates the FA pathway through mono-ubiquitination of the FANCI/D2 complex. Yellow oval, FANCA.
FANCS(BRCA1), FANCN(PALB2), and FANCD1(BRCA2) form a complex that is important for RAD51-mediated recombination. FANCN(PALB2) and FANCD1(BRCA2) interaction is critical for FANCD1(BRCA2) foci formation, while FANCS(BRCA1) and FANCN(PALB2) interaction is critical for FANCD1(BRCA2) function. FANCD1(BRCA2) in turn mediates RAD51 displacement of RPA, resulting in strand invasion and exchange into homologous DNA. FANCA expression strongly correlates with RAD51 associated protein 1 (RAD51AP1) and FANCS(BRCA1). RAD51AP1 acts as a RAD51 accessory protein and enhances RAD51-mediated HR by cooperating with FANCN(PALB2) to promote d-loop formation. FANCA, FANCS(BRCA1) and RAD52 have been shown to promote homology-dependent SSA repair of DSBs.
Figure 5-3: Proposed model for the involvement of FANCA in DSB repair.
Figure 5-4: Proposed model for FANCA involvement in Okazaki fragment maturation.
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