BIOCHEMICAL ACTIVITIES AND GENETIC FUNCTIONS OF THE *DROSOPHILA MELANOGASTER* FANCM HELICASE IN DNA REPAIR

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ABSTRACT

Noelle-Erin F. Romero: Biochemical activities and genetic functions of the *Drosophila melanogaster* Fancm helicase in DNA repair
(Under the direction of Steve Matson and Jeff Sekelsky)

The DNA damage response in eukaryotes involves multiple, complex, and often redundant pathways that respond to various types of DNA damage that affect one or both strands of DNA. One type of toxic DNA damage that can occur is a double-strand break (DSB). Repair of a DSB can lead to the formation of a recombination product known as a crossover (CO). Crossovers in mitotic cells can be deleterious and lead to chromosomal rearrangements or cell death. In order to limit crossing over during DSB repair, eukaryotes possess mechanisms to ensure crossovers do not occur. In this manner, several helicases function during repair of DSBs to promote accurate repair and prevent the formation of crossovers through homologous recombination.

Among these helicases is the Fanconi anemia group M (FANCM) protein. FANCM is one of 17 Fanconi anemia (FA) proteins and is one of the most broadly? conserved FA proteins. FANCM and its orthologs, Mph1 and Fml1, are DNA junction-specific helicases/translocases that process homologous recombination (HR) intermediates. Additionally, FANCM has been implicated in a number of DNA metabolic processes including activation of the S-phase checkpoint, trasversal of interstrand crosslinks, recruitment of the proteins such as the FA core complex and Blm to sites of DNA damage, and prevention of mitotic crossovers during double-strand break repair.

The helicase activity of FANCM is believed to be important in crossover prevention, but no helicase activity has been detected *in vitro*. I report here a genetic and biochemical study of

Drosophila melanogaster Fancm. I show that purified Fancm is a 3' to 5' ATP-dependent helicase that can disassemble recombination intermediates, but only through limited lengths of duplex DNA. Using transgenic flies expressing full-length or truncated Fancm, each with either a wild-type or mutated helicase domain, I found that there are helicase-independent and C-terminus independent functions in responding to DNA damage and in preventing mitotic crossovers.

For all my homie chromies

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LIST OF ABBREVIATIONS

AMP-PNP	Adenosine-5'-(β,γ-imido) triphosphate
AP	Apurinic Site
ATP	Adenosine-5'- triphosphate
BSA	Bovine serum albumin
bp	Base pair
CO	Crossover
D-loop	Displacement loop
dHJ	Double Holliday junction
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
DSB	Double-strand break
DSBR	Double-strand break repair
EDTA	Ethylenediamine tertaacetic acid
FA	Fanconi anemia
FL	Transgene of full length Fancm
FLKM	Transgene of full length Fancm with a mutation in the Walker A motif
HhH	Helix-hairpin-helix
HJ	Holliday junction
HN2	Mechlorethamine; Nitrogen Mustard

HR	Homologous Recombination
ICL	Interstrand Crosslink
IR	Ionizing Radiation
LB	Luria-Bertani broth
MBP	Maltose binding protein
MMR	Mismatch repair
MMS	Methyl methanesulfonate
NCO	Noncrossover
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
nt	Nucleotide
NTP	Nucleotide triphosphate
OD	Optical density
OH	Overhang
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
PNK	Polynucleotide kinase
rATP	Riboadenosine triphosphate
SCE	Sister chromatid exchange
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SF	Superfamily
ssDNA	Single-stranded deoxyribonucleic acid
TBE	Tris base, boric acid, and EDTA
TE	Tris-HCl (pH8.0) EDTA
TLS	Translesion synthesis
Tris	Tris [hydroxymethyl] aminomethane
tr	Transgene of truncated Fancm
tr^{KM}	Transgene of truncated Fancm with a mutation in the Walker A motif
UTR	Untranslated region
γ-[^{32}P]ATP	Adenosine-5'- triphosphate ^{32}P labeled on the □ phosphate
Δ	Truncation of 840 amino acids
Δ^{KM}	Truncation of Fancm (See Δ) with a point mutation changing the 84 amino acid Lysine (K) to Methionine (M)

CHAPTER 1-INTRODUCTION

Maintaining the structural integrity of DNA is essential to the health and vitality of a cell and organism as it serves as a permanent copy of the cell's genome. Changes in its structure and/or sequence can have severe consequences as it can cause alterations in other cell components, such as proteins and structural RNAs. It is therefore critical that the integrity of DNA be maintained. While the duplex structure of DNA makes it a particularly stable repository of genetic information (1, 2), there are many times in which DNA molecules are more susceptible to damage. This includes periods of time in which the separation of the duplex DNA is necessary, such as replication during cell division (mitosis and meiosis), as well as during transcription of DNA into RNA. During these times the DNA can be altered by the proteins that act upon it or damaged by various factors.

For instance, the incorporation of incorrect bases or deletion of bases during DNA replication can have a profound effect on an organism. Polymerases, proteins responsible for replication, can encounter regions of highly repetitive DNA where the (3, 4) polymerase becomes susceptible to slippage or misreads of the template sequence. This can greatly alter the DNA through insertions, deletions, or duplications of the genome. While not all changes to the DNA are as drastic as genomic deletions or insertions, they can still have severe consequences. A single base change, for example, can alter the protein product encoded by the DNA, for instance, mutation of a conserved residue can change the protein function, change the sequence to an early termination sequence or cause the deletion of a stop codon which can alter the protein product, thus changing the ultimate structure of the protein or generating no protein at all (5).

DNA can also be damaged or modified by chemicals or proteins in their environment, or through spontaneous endogenous metabolic processes. Endogenous damage, like free radicals that occur during normal cellular metabolism, can alter the helical conformation of DNA. The most common types of spontaneous damage to DNA are AP (apurinic) site damage that results through the loss of purine bases

(depurination) that occurs from the cleavage of the bond between the base and the deoxyribose, or through deamination of adenine, cytosine, or guanine (5–7). Exogenous exposure to environmental agents, such as toxins, UV radiation, and pollutants can cause breakage of DNA or toxic lesions. Covalent modifications to the sugar phosphate backbone (backbone modifications) or the nitrogenous bases of DNA (base modifications) can distort or break the helix. The type and frequency of the modification or lesion can vary depending on both the lesion type and the modifying agent. Induced or exogenous damage includes the formation of interstrand crosslinks (ICLs) generated by chemotherapeutic agents, and pyrimidine dimers, in which two adjacent pyrimidines are joined by a ring-like structure, as seen in the case of damage induced by UV radiation. Alkylation, or the addition of a methyl or ethyl group to various positions on the DNA base, is another form of induced damage(5, 8, 9). Modifications, such as oxidative base modifications, can be numerous and can distort the helix.

A number of repair processes exist in order to ensure that detrimental modifications made to DNA are removed to ensure cell vitality and that any damage that occurs to the DNA is corrected efficiently and effectively. Repairing of DNA damage is essential as damage to DNA can block replication or transcription and can result in a high frequency of mutations, which can be detrimental to cell reproduction and viability (4, 5, 10). To maintain the integrity of their genomes, cells have therefore evolved a robust set of mechanisms to repair damaged DNA. These myriad DNA repair mechanisms can be classified as either pre-replicative repair or post-replicative repair depending on where in the cell cycle they take place (Figure 1.1). Pre-replicative repair includes reversal of chemical damage or modification (direct reversal) or removal of the damaged base and synthesis of new DNA (excision repair) (9, 11). These systems act to correct DNA damage before replication allowing DNA synthesis to proceed using an undamaged DNA strand as a template. If these systems fail to remove the damage, alternative mechanisms for dealing with damaged DNA are employed post-replication.

PRE-REPLICATIVE REPAIR

As the name suggests, pre-replicative repair occurs prior to DNA synthesis. Although most damage to DNA is repaired by the removal of the damaged base and subsequent synthesis of new DNA in the excised region, some lesions can be repaired by direct reversal of the damage. Direct reversal

results in the restoration of the original base in an unaltered state without synthesis or remodeling of the DNA. This method of repair is generally viewed as a highly efficient way of dealing with specific types of DNA damage that occur with a high frequency. For instance, UV light is a major source of DNA damage and can induce 6-4 photoproducts and pyrimidine dimers. The formation of pyrimidine dimers distorts the helical structure of the DNA and blocks transcription or replication past the site of damage. The direct reversal of this dimer occurs through a process known as photoreactivation. This process utilizes energy from visible light to break the ring structure that binds the pyrimidines together (12, 13). Although UV irradiation is the cause of almost all skin cancer in humans, photoreactivation is not a repair mechanism found in humans, although a variety of prokaryotic and eukaryotic cells employ photoreactivation as a means of repair (5, 9, 10).

When direct reversal of the DNA lesion is not possible, excision of the damaged base is employed. Unlike direct reversal of DNA damage, excision of damage is a more general means to repair a broad range of alterations to DNA. Various types of excision repair mechanisms exist and are highly important DNA repair strategies in prokaryotic and eukaryotic cells. Excision repair involves the removal of the damage and synthesis of new DNA to fill the resulting gap. Types of excision repair include nucleotide-excision repair (NER), base excision repair (BER), and mismatch repair (MMR).

NER, BER, and MMR, involve the use of specialized nucleases to cleave the phosphodiester backbone to remove the damaged base. A helicase is then responsible for displacing the damaged and soon to be excised strand and the resulting gap is filled in by a DNA polymerase and sealed by a ligase. The MMR, BER and NER repair pathways have been extensively studied in prokaryotes and eukaryotes (for reviews see (14–18).

Mismatch repair involves the binding of the mismatched base and subsequent excision by the *E. coli* MMR proteins, MutS and MutL, and their homologs (14, 15, 19). Mismatched bases are generally recognized and removed by the proofreading activity of the replicating DNA polymerase. However, the DNA polymerase inevitably makes a mistake at a rate of 10^{-6} to 10^{-8} and inserts an incorrect base resulting in a mispair (20, 21). Bases that are missed by the proofreading exonuclease can later be corrected by the MMR system.

Similar to MMR, NER also involves the excision of DNA damage and restoration of the original sequence. In *E. coli,* NER is carried out by the UvrABC complex. UvrA recognizes damaged DNA, recruits UvrB and C, which are then responsible for cleaving on the 3' and 5' side of damage. Using mammalian cell lines and the identified genes that are involved in NER, the NER pathway in humans has been elucidated. Similar to the pathway in *E. coli,* XPA recognizes damaged DNA and recruits the heterodimer XPF/ERCC1 and XPG to the repair complex. XPG and XPF/ERCC1 are endonucleases that cleave DNA on the 3′ and 5′ sides of the damage. XPA also recruits XPB and XPD, which acts as a helicase to unwind the damaged DNA that was excised by XPF/ERCC1. The resulting gap is filled in by DNA polymerase and sealed by DNA ligase (18, 22).

POST-REPLICATIVE REPAIR

If damage persists post-replicative repair mechanisms are used to restore the integrity of the DNA. Pyrimidine dimers left unrepaired, ICLs, and many other types of lesions cannot be copied by DNA polymerases and block movement of the replication fork. One mechanism used to overcome these blockages is recombinational repair. Recombinational repair utilizes the undamaged homologous template to synthesize new, undamaged DNA (22–24). The damaged portion (e.g. a pyrimidine dimer or crosslink), can then subsequently be removed by one of the excision repair mechanisms. During the repair process of lesions that affect both strands, a combination of repair methods may be utilized. Repair of ICLs, explored later in this chapter, uses both excision and recombinational repair processes to restore DNA to its native state. Recombinational repair can also be used when damage occurs to the DNA phosphodiester sugar backbone. Strand modifications include single and double strand breaks, which can result from environmental and metabolic sources (10, 25–28).

Double-strand breaks (DSBs) are among the most biologically hazardous types of DNA damage as a single unrepaired DSB can cause cell death. Moreover, inaccurate repair of a DSB can lead to deletions or chromosomal rearrangements, and thus poses a significant threat to genomic integrity (26, 29–31). Mammalian cells use two mechanisms of recombinational repair to restore DNA after a DSB occurs: homologous recombination (HR) and non-homologous end-joining (NHEJ). Although both these methods are used to repair DNA, they differ in their requirements for template and their fidelity. NHEJ

operates during all phases of the cell cycle and is considered the predominant pathway in mammalian cells for DSB repair while HR is restricted to late-S and G2 phases. NHEJ is considered error-prone and eliminates DSBs through direct ligation of the broken ends (32). HR-directed repair is largely an error-free mechanism as it utilizes the undamaged sister chromatid, or homologous chromosome, as a template (31, 33). The work in this thesis focuses on DSB repair via HR and will not focus on NHEJ as the primary mode for repair of DSBs in *Drosophila melanogaster* is HR.

DOUBLE-STRAND BREAK REPAIR VIA HOMOLOGOUS RECOMBINATION

The fact that identical information is held on the sister chromatid, and a duplicate copy is available in the homologous chromosome, makes repair via homologous recombination an essential pathway to ensure accurate repair of broken DNA. DSBs, which can be induced by radiation and select toxins, are repaired via recombinational mechanisms in which an intact DNA molecule is used. Our current knowledge regarding the mechanism of homologous repair derives largely from studies done in yeast. The process of double strand break repair via homologous recombination was first proposed by Robin Holliday. Holliday and colleagues postulated that, during meiosis, DNA repair gives rise to crossovers (COs), and gene conversions (30, 34)**.** Current models for repair via homologous recombination are based on the model of double-strand break repair (DSBR) originally outlined by Szostak (34). Key to this model are several essential steps (Figure 1.2):

A.) Initiation of recombination by a DSB; B.) resection of the 5' end at the strand break to generate a 3’ single-stranded DNA tail; C.) invasion of the Rad51-coated 3' ssDNA tail into the homologous sequence generating a displacement loop (D-loop); D.) DNA synthesis, primed from the invading 3' end; and finally E.) resolution into one of two classes of recombination product- crossovers (COs) or non-crossovers (NCOs).

These key steps have been well established and supported in meiotic recombination in the budding yeast *Saccharomyces cerevisiae*. Many of the key intermediates formed have been visualized through the use of 2D gel analysis (35–37). Although yeast has been used to establish a model for meiotic recombination, the process is thought to be maintained and followed in other eukaryotes. Although little

evidence exists to support the direct assumption that double-strand break model of meiotic recombination accurately reflects mitotic recombination, core features, such as those outlined above, are believed to also hold true in mitotic DSB repair (38, 39).

While the essential steps of recombination are maintained between both processes, one of the fundamental differences between meiotic and mitotic recombination is regulation and promotion of recombination products. In meiotic dividing cells, recombination products (COs) are actively promoted as they contribute to genetic diversity and ensure proper segregation of chromosomes during cell division (38). Meiotic recombination is indispensable for accurate chromosome segregation and is promoted by generation of a DSB by a meiosis-specific nuclease. The subsequent crossover formation occur at various frequencies depending on the organism, but generally require at least one CO per chromosome, and help facilitate proper chromosomal alignment and subsequent segregation (40). Disruption of this process can lead to a number of aberrations and deleterious effects, such as genomic deletions and improper segregation of chromosomes (nondisjunction) which can lead to aneuploidy (39).

While homologous recombination through programed DSBs is essential in meiotic cells to ensure proper chromosomal segregation and promote genetic diversity, HR in mitotic cells is used to repair spontaneous and induced DSB damage. As mentioned above, repair of ICLs and other base modifications utilize specialized nucleases to generate nicks in the DNA backbone to facilitate removal of the offending lesion. Excision of the damaged base can result in DSBs as nucleases used to excise the damage can nick both strands of the sugar phosphate backbone. The generation of a DSB can lead to the use of HR as a repair mechanism. However, the formation of COs during HR via DSB repair in mitotically dividing cells can be hazardous as they can result in loss of heterozygosity and gross chromosomal rearrangements (39–42). Therefore, prevention of CO pathways through the activation and promotion of NCO pathways is favored in mitotic cells undergoing HR to ensure genomic stability. When HR is used to repair DSBs that occur as a result of induced damage, then repair is biased towards avoiding COs by promoting NCOs via Synthesis Dependent Strand Annealing (SDSA) and double Holliday Junction (dHJ) dissolution.

Repair of DSBs through HR requires multiple repair and recombination proteins. Processing of DSBs involves resection in which the 5' ends on either side of the break are trimmed back to create 3' single-stranded DNA overhangs. This process involves specialized proteins and protein complexes, namely Mre11-Rad50-Nbs1, and Exo1 exonuclease (27, 43). The single-stranded DNA tails are then coated by the single-stranded DNA binding protein RPA to remove secondary structures. RPA is subsequently displaced by Rad51 with the help of Rad51 mediator proteins (44) generating a single-stranded DNA tail coated with Rad51. This Rad51 nucleoprotein filament executes homology-mediated search and invasion of the homologous template. In humans, this template is the sister chromatid, but in some cases and some species, like *Drosophila,* this template can be the homologous chromosome. Following invasion, DNA synthesis is carried out by a DNA polymerase (31) (Figure 1.2).

Following invasion and subsequent DNA synthesis, one of two steps can occur: SDSA or HJ formation. If a Holliday junction is formed, cleavage by structure-specific endonucleases, such as Mus81/Eme1 or Slx1/Slx4, can result in a CO (31, 45). To prevent the formation of COs helicases can act on several DNA intermediates generated during DSB repair via HR. During HR an invading DNA strand from the homologous chromosome forms a D-loop as indicated in Figure 1.2. The invading strand can then be unwound from the template and annealed to the resected end, resulting in a NCO, a process known as synthesis-dependent strand annealing (SDSA) (46). Alternatively, the invading strand can undergo second-end capture, leading to the formation of an entwined structure referred to as a double-Holliday junction (dHJ). The dHJ can be processed by structure-specific endonucleases, possibly giving rise to a CO, or acted upon by a helicase/topoisomerase complex in a process known as dissolution, generating a NCO (23). Thus, helicases are essential in the promotion of NCO products either through promotion of D-loop disassembly through SDSA or the dissolution of the dHJ, thereby preventing the formation of potentially deleterious crossovers during HR-directed repair (23, 25, 31, 39).

FANCONI ANEMIA AND DNA REPAIR

Prevention of mitotic crossovers, either through SDSA or dHJ dissolution, can be achieved through the use of specialized motor proteins known as helicases. DNA helicases are proteins that utilize

the energy of nucleoside triphosphate hydrolysis to transiently convert duplex DNA to single-stranded DNA. One family of conserved DNA helicases/translocases, whose members are involved in HR mediated repair, are relatives of archaeal Hef (Helicase-associated endonuclease for fork-structured DNA) (47–50). The Hef protein from *Pyrococcus furiosus* contains a conserved DEAD-box helicase motif toward the N-terminus and an endonuclease reminiscent of the nucleases ERCC4 endonuclease in its C-terminus (51). Hef processes DNA intermediates that are generated during HR, such as forks and four-way junctions (HJs) (52) either through its helicase activity or nuclease activity. Hef functions as a homodimer in cleaving DNA forks and processing Holliday junctions into splayed arms, indicating roles for this protein during DNA replication and repair (50, 52). These helicases are members of the SF2 helicase superfamily. Relatives of Hef are found as orthologs of the human Fanconi anemia group M (FANCM) protein.

FANCM was first characterized as the yeast gene *mph1* from *Saccharomyces cerevisiae* during a mutator screen to genetically characterizes genes of unknown function (53). This initial screen showed that deletion of *MPH1* led to an increased spontaneous mutation rate. The impact of this protein was not fully examined until both the human ortholog, FANCM, and archael Hef were identified and the biochemical activity was examined (50, 54, 55). FANCM was identified as a 250 kDa component of the FA core complex and identified in an FA patient who carried a bi-allelic mutation. This identification of FANCM led to its classification as a new FA complementation group (55, 56).

There are over 17 FA genes and associated genes classified as FA family members. Mutations in any of the FA complement group of genes are associated with the same disorder, Fanconi anemia. Fanconi anemia is a hereditary disorder characterized by an increased incidence of cancer, developmental abnormalities, and bone marrow failure (55). A classic hallmark of cells from FA patients is a heightened sensitivity to DNA interstrand crosslinking (ICL) agents, including the chemotherapeutic agents cisplatin and mitomycin C (57). The FA proteins are implicated in directing the activities of other repair proteins although the exact role for many of these proteins is still under investigation. For example, the mismatch repair protein MLH1 has been shown to be involved in ICL repair via interaction with FANCJ, an FA protein recruited to sites of damage by the core complex (58, 59). The core FA complex,

consisting of 8 different FA proteins, has also been found to interact with and direct proteins involved in HR, such as the BLM complex and BRCA1/2, to sites of DNA damage (60–63). Although still under investigation, the primary function for FA pathway is thought to be in repairing ICL damage (64, 65). However, the FA proteins are thought to function at various points during the repair of ICLs and can be separated into three groups: The FA core complex, the FANCD2/FANCI (ID) complex, and downstream targets (66). The downstream FA proteins are made up of FANCD1 (BRACA2), FANCJ (BRIP1/BACH1), FACNP (SLX4), and FANCN (PALB2) all of which are associated with recombinational repair (25, 59, 61) (Figure 1.3).

Regulation of the FA pathway is dependent on the ubiquitylation of the ID complex. Upon ubiquitination, the ID complex localizes to chromatin during S phase and in response to DNA damage induced by mitomycin C (MMC), ionizing radiation, and UV exposure (67–69). Monoubiquitylation, and subsequent activation and regulation of the downstream components, occurs through the function of the FA core complex. The FA core complex consists of 8 FA proteins (FANC- A, -B, -C, -E, -F, -G, -L, and -M) as well as FA associated proteins (FAAP24, FAAP100) (57, 70–74). All members of the FA core complex are required for the catalytic subunit, FANCL (75), to function as an E3 ubiquitin ligase (76–79). FANCM, unlike the rest of the core complex, is distinctly different in the involvement of ubiquitylation of the ID complex. Inactivation of FANCM results in an intact core complex and the partial ubiquitylation of FANCD2 (80), leading to the idea that FANCM is partially redundant with another protein, has functions outside of the FA pathway (81, 82), or act as a signaling protein that targets the core complex to DNA (70, 83, 84).

Interstrand crosslinks (ICLs) are a form of cytotoxic DNA damage that consist of covalent linkages between the two strands of dsDNA. The formation of an ICL is typically generated by an alkylating agent, such as nitrogen mustard (HN2) or chemotherapeutics like cisplatin. The covalent links generated by ICLs prevents the separation of DNA strands and therefore represent a potentially toxic block to transcription and replication. Repair of such toxic blocks requires both excision and recombinational repair and the presence of repair proteins from multiple repair pathways, including HR. In repairing ICLs, the current model for repair proposes the generation of a DSB surrounding the site of ICL

(22). The ICL is then untethered by an endonuclease. Recombinational repair can then be used to correct the resulting gap (Figure 1.4).

FANCM AND HOMOLOGOUS RECOMBINATION

Although many of these FA proteins are unique to mammals, and to some extent metazoans, one protein, FANCM, is a constant in eukaryotic organisms. *D. melanogaster,* for instance, lacks the full complement of FA proteins (54), yet the FA proteins that are present in *D. melanogaster* are important for repair of ICLs. FANCM therefore plays a key role in damage repair and has been implicated in recombination. The *S. cerevisiae* FANCM ortholog, Mph1 (54, 66, 85, 86), has been shown to be involved in preventing crossovers (48), and *mph1* mutants show hypersensitivity to DNA damaging agents such as ionizing radiation (IR) andmethyl methanesulfonate (MMS) (53). Biochemical studies using purified Mph1 show that it is a DNA helicase capable of unwinding Rad51-coated D-loops (48, 87), and that it can process DNA intermediates that form later in repair, including HJs (48, 87, 88). Unwinding of HJs and D-loops has also been observed using the *S. pombe* ortholog Fml1 (89). In contrast, no helicase unwinding activity has been detected for human FANCM (55, 90). Together, genetic and biochemical studies suggest roles for FANCM and its orthologs in HR that are dependent upon their ability to use ATP hydrolysis to unwind or remodel DNA structures so as to prevent COs (47, 48, 91–93).

Biochemical and genetic studies on human FANCM and *S. cerevisiae* Mph1 suggest a role for FANCM in HR and regulation of recombination products. *mph1* was originally identified in a mutator screen and cells deficient in Mph1 exhibit a hypersensitivity to genotoxins that product DNA adducts and stall replication, such as MMS and camptothecin (53). Additional evidence for the role of Mph1 in HR comes from DNA damage assays with Mph1 mutant proteins and the HR proteins Rad51, Rad52, and Rad55, showing the Mph1 is epistatic to these proteins (94). Deletion or helicase-dead mutants of Mph1 also affect the rate of spontaneous sister chromatid exchange and DSB-induced crossovers suggesting that Mph1 functions in crossover prevention in a novel way independent of the DNA helicases Srs2 and Sgs1 (the Blm ortholog) (95, 96). Since Sgs1 and Srs2 suppress crossovers via dHJ dissolution, and *in vitro* evidence demonstrates Mph1 dissociating Rad51 made D-loops in an ATP dependent manner (48), it is predicted that Mph1 prevents crossovers during HR by promoting SDSA.

FANCM's proposed role outside of the FA response, and the biochemical evidence that yeast FANCM (Mph1) is involved in HR, suggests that the primary role of FANCM is in promoting CO avoidance by processing DNA intermediates that occur during DSB repair via HR (48, 87, 97). While this does not negate the role for FANCM in ICL repair, it indicates that FANCM may function during various repair pathways and with multiple repair proteins to coordinate repair events. Additional evidence for FANCM functioning in multiple repair pathways comes from structural studies. Various motifs and domains in FANCM have been suggested to have roles in recruiting additional repair proteins. For example, the C-terminus of human FANCM, like its Hef ancestor, has an ERCC4-like endonuclease domain. Although a critical lysine residue within the endonuclease motif found in ERCC4 domain of FANCM is mutated, and no nuclease activity has been detected (55, 70), this domain is involved in protein-protein interactions (84, 98, 99). This domain also houses tandem helix-hairpin-helix $(HhH)_2$ domains that promote both DNA binding and protein dimerization with a second $(HhH)_2$ domain found in FAAP24.

While yeast Mph1 and *Drosophila* FANCM lack the ERCC4 domain, there are additional motifs that promote protein-protein interaction. Yeast Mph1 and human FANCM have several motifs in the C-terminus that facilitate interaction with chromatin, additional FA proteins, and repair complexes (60, 100, 101). In human FANCM, two specific motifs (MM1 and MM2) have been shown to allow for interaction with the FA complex and the Bloom syndrome helicase (BLM) complex, which is involved in DSB repair via HR (60). While these two motifs are not detected in yeastMph1 and *Drosophila* orthologs, there is still the potential for C-terminal interactions with other proteins involved in HR or DNA repair complexes.

A previous genetic study in our lab has shown that *Drosophila* Fancm, like its orthologs, is involved in the prevention of COs (91). My work focuses on the biochemical role of Fancm in CO prevention through unwinding of HR intermediates and the response to DNA damaging agents. To better understand the role of the Fancm helicase activity in directing homologous recombination towards a non-crossover product, I tested the ability of the purified Fancm helicase to act on HR repair intermediates *in vitro*. We generated Fancm ATP hydrolysis mutants *in vivo* to examine the role of the helicase in responding to DNA damage and CO prevention. I also sought to understand the role, if any, of the C-terminus of Fancm in regulating repair events in *Drosophila*. To this end, I generated C-terminal

truncations of Fancm *in vivo* and analyzed how these mutants respond to various DNA damaging agents and their function in CO prevention.

Here I show that purified Fancm can unwind duplex DNA in a 3' to 5' direction in an ATP-dependent manner. Further, I provide evidence that Fancm can disassemble the HR D-loop intermediate. *In vivo* work used to study the role of the helicase activity and the C-terminal domain of Fancm reveals that Fancm lacking either helicase activity or the C-terminus is able to prevent some mitotic crossovers and respond to DNA damage.

FIGURES

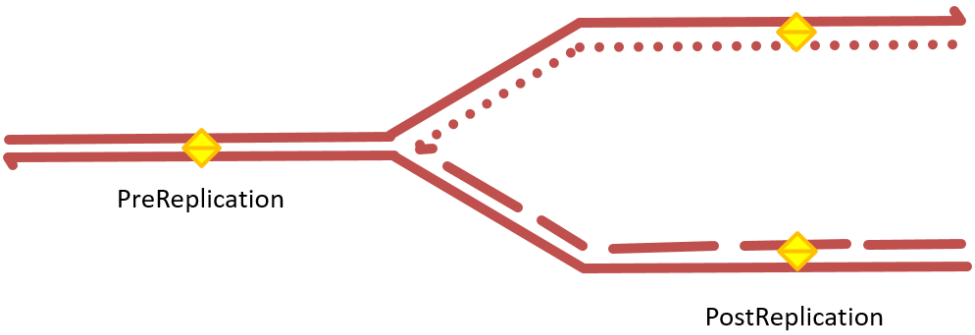

Figure 1.1- *Pre and Post replication repair* - Red lines depict DNA. Dotted lines indicate leading strand synthesis. Long dashed lines depict lagging strand synthesis. Yellow diamonds indicated areas of DNA damage.

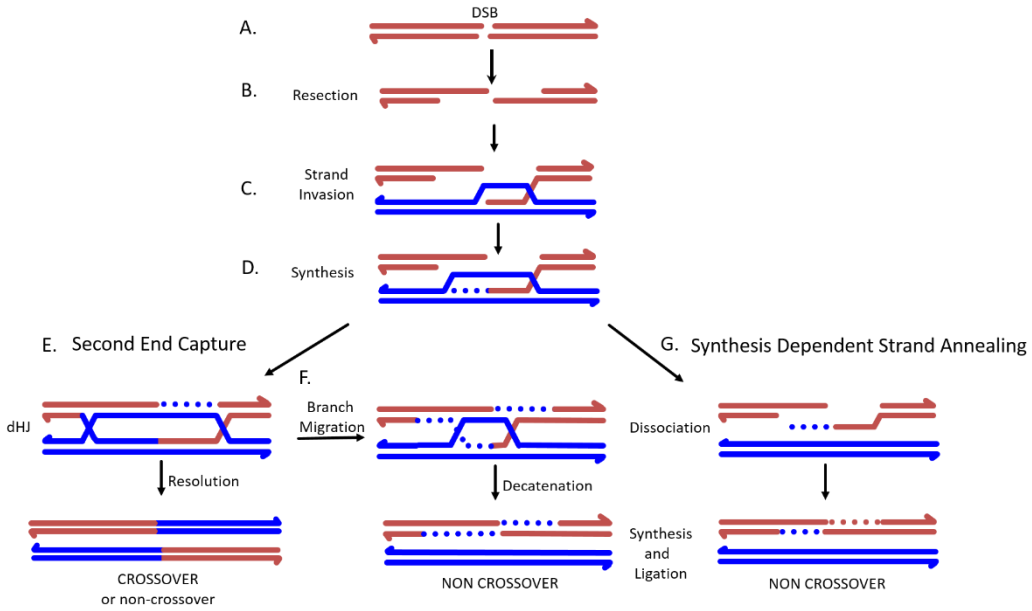

Figure 1.2- *Model for DSB repair Via Homologous Recombination* - A.) Initiation of recombination by a DSB; B.) resection of the 5' end at the strand break to generate a 3' single-stranded DNA tail; C.) Invasion into the homologous chromosome or sister chromatid by one or both of the 3' ssDNA tail coated by the Rad51 recombination protein which generates a displacement loop (D-loop); D.) DNA synthesis, primed from the invading 3' end; E.) The other end of the break can anneal to the D-loop in a process called second-end capture. The formation of a dHJ can be cut by structure specific nucleases resulting in one of two classes of recombination product- crossovers (COs) or non-crossovers (NCOs) or F.) the two HJs are migrated together and then decatenated to produce a NCO. G.) Alternatively, the invading strand could be displaced through SDSA yielding a NCO. Red and blue lines represent DNA. Dotted lines indicated newly synthesized DNA.

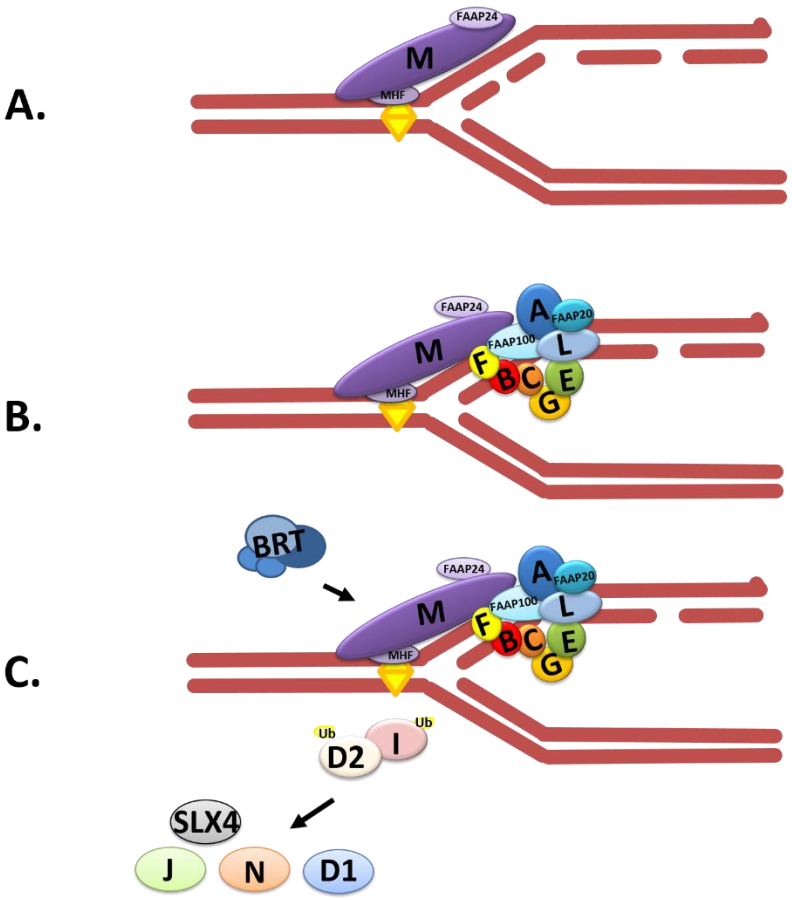

Figure 1.3 - *The Fanconi anemia family repair network* - A.) FANCM/MHF/FAAP24 recognize ICL lesions. B.) Recruitment of the Core Complex, consisting of FANCA, B, C, D, E, F, G, L and accessory components FAAP20 and FAAP100, triggers C.) monoubiquitinate the FANCI and FANCD2 ID complex) which allows for localization of downstream effector proteins, such as FANCN, FANCJ, FANCD1 and SLX4. FANCM can also recruit the BRT complex (Blm/RMI1/2/Topo3α).

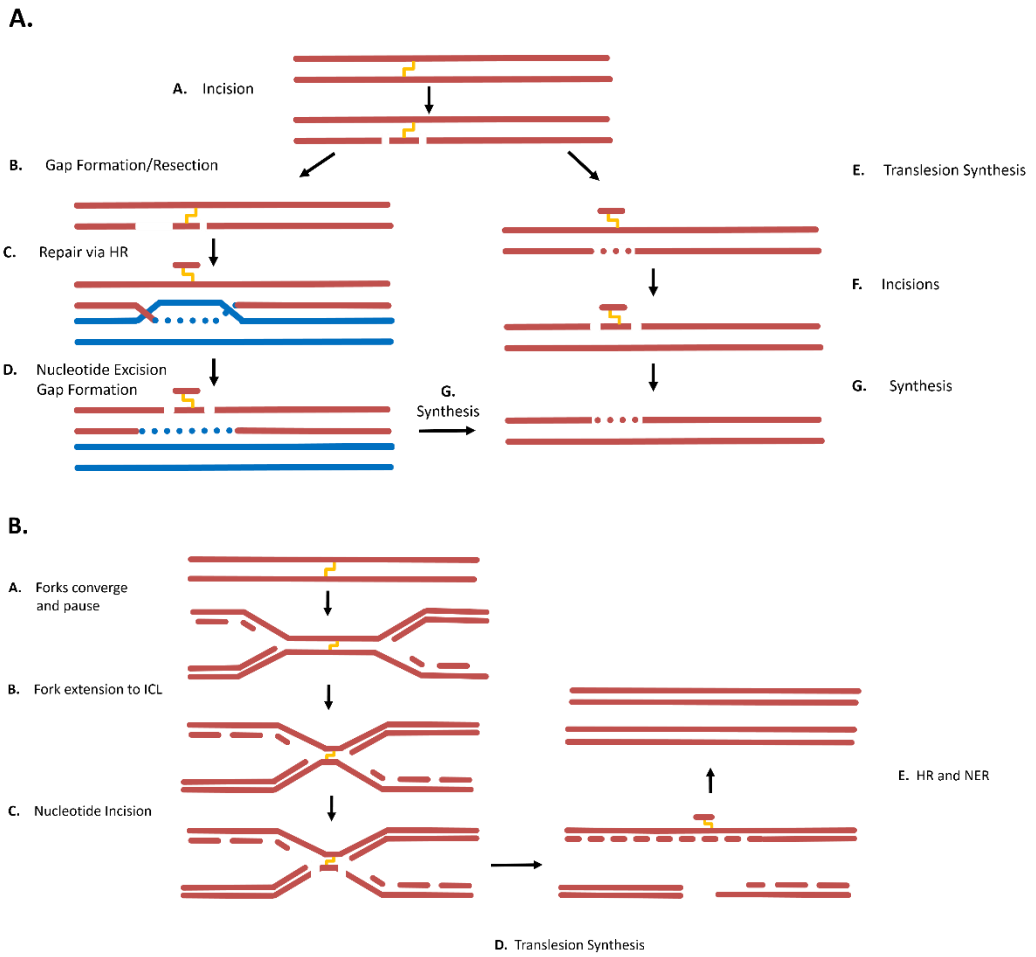

Figure 1.4 - *Model for ICL removal* - **A.** *Prereplication repair of ICLs*- A.) Incisions are made on either side of the crosslink by an endonuclease. B.) A gap is generated by an exonuclease on one side of the crosslink, allowing loading of a single-stranded DNA binding protein, such as Rad51, to load. C.) Rad51 mediated strand invasion promotes homologous recombination and subsequent DNA synthesis on the opposite strand from the crosslink. D.) Incisions are made on either side of the crosslink again, releasing a small piece of crosslinked DNA. G.) DNA synthesis and ligation occurs in the gap generated from the removal of the crosslinked oligonucleotide. E.) Alternatively, translesion synthesis (TLS) is employed. F.) Following TLS, incisions are made on either side of the crosslink, releasing a small piece of crosslinked DNA. G.) DNA synthesis and ligation occurs in the gap generated from the removal of the crosslinked oligonucleotide. **B.** *Postreplication repair of ICLs*- A.) Two replication forks converge near the site of the ICL and replication pauses. B.) The replication forks move toward the ICL. C.) Nuclease(s) catalyze an incision on both sides of the ICL generating DSB. D.) TLS extends the parental strand across from the ICL. E. NER removes the crosslinked oligonucleotide and HR and DNA synthesis repairs the broken DNA. Red and blue lines depict DNA strands. Yellow bar indicates the site of a crosslink. Dotted lines indicted newly synthesized DNA. Models adapted from (102–105).

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CHAPTER 2- BIOCHEMICAL ANALYSIS OF THE *D. MELANOGASTER* FANCM ORTHOLOG

INTRODUCTION

As discussed in Chapter 1, homologous recombination (HR) is critical in genome maintenance and is required for the accurate repair of DNA double-strand breaks (DSBs) as well as a variety of other lesions (1–3). When a DSB occurs, resection of the broken ends by an exonuclease generates a 3' single-stranded DNA tail. Rad51 coated 3' single-stranded DNA tail mediates strand pairing and invasion with the homologous chromosome. The process of strand invasion generates a displacement loop (D-loop). DNA synthesis occurs using the homologous template, allowing for accurate repair of the broken and resected end. The repair process can then diverge into a number of potential pathways resulting in different recombinational products: Noncrossovers (NCOs) with the conservation of the original parental DNA molecules, or the reciprocal exchange of DNA flanking the break site generating a crossover (CO) product (see Figure 1.2) (1, 4).

The formation and processing of D-loop-like structures is a key step in the formation of a noncrossover versus crossover production, as discussed in Chapter 1. In mitotic cells, the promotion of NCOs is preferred and can be achieved through synthesis-dependent strand annealing (SDSA) or double Holliday junction (dHJ) dissolution during DSB repair via HR (5). Important throughout this process are a class of specialized proteins known as DNA helicases/translocases.

DNA helicases are specialized motor proteins that use the energy derived from hydrolysis of nucleotide triphosphates, usually ATP, to bind DNA and break the hydrogen bonds between the two strands of duplex DNA, thus facilitating remodeling of DNA and DNA-protein complexes (6, 7). Helicases comprise the largest class of enzymes and are involved in virtually all biological processes involving nucleic acids (8). Sequence analysis conducted by Koonin and Gorbalenya, as well as

structural/functional analysis by Wigley, is used to classify helicases into one of six helicase superfamilies (SF) (6, 9). Helicases can be either be classified as toroidal, usually hexameric structures, or not. Toroidal enzymes, such as the MCM replicative helicases, belong to SF3 and SF6. SF1 and SF2 house non-ring forming enzymes and will be the focus of the helicases discussed here. Helicases belonging to SF1 and SF2 share a highly similar catalytic core yet perform distinct functions and interact with a broad class of substrates.

A distinct characteristic of SF1 and SF2 helicases is the conserved helicase core. This core contains several conserved sequence motifs (9). Some of the highest level of sequence conservation is within the motifs that coordinate binding and hydrolysis of the nucleoside triphosphate. This includes the Walker A motif, responsible for binding of the nucleoside triphosphate, and therefore essential for the hydrolysis of NTP. Mutations in helicases have been linked to multiple disease states such as cancer, developmental abnormalities, and degenerative diseases (10, 11). Various medical disorders result from defective helicases that impair DNA repair. The Bloom helicase (BLM), for instance, is a member of the RecQ SF2 helicase family. Mutations in BLM result in Bloom syndrome, a disorder characterized by short stature, developmental abnormalities, and a predisposition to cancer. BLM functions within a complex of TOP3A, RMI1, and RMI2 to migrate and dissolve D-loop intermediates that are formed during DSB repair. Mutations in BLM lead to an increased incidence of COs and, subsequently, gross chromosomal rearrangements. The prevention of COs in mitotically dividing cells is essential to ensure genomic stability. Various proteins, including helicases, act to promote the formation of NCOs and prevent COs.

One such helicase involved in the promotion of NCOs is FANCM. FANCM is one member of a class of proteins that, when defective, is linked to the disease Fanconi Anemia (FA) (12). FA is characterized by bone marrow deficiency, developmental abnormalities, and a predisposition to cancer. In addition, cells from FA patients are hypersensitive to DNA crosslinking agents (13). FANCM contains classic motifs related to DEAH box helicases and is known to be a component of the FA core complex responsible for catalyzing the monoubiqutination of FANCD2/I, an important step in the repair of interstrand crosslinks (ICLs) (13).

Although the primary repair response for which FANCM is known is in the repair of ICLs via recruitment the FA core components (13–15), there may be additional aspects of DNA repair in which it is involved. For instance, mutations in the Walker A box increase cell sensitivity to crosslinking agents but did not greatly affect the ubiquitination of FANCD2/I (13). This difference indicates that the ATPase activity of FANCM is not necessary for recruitment of additional components, but that the motor activity of the protein may be required during later steps of repair.

One possibility for the involvement of FANCM in repair of ICLs coordinating SDSA during the repair of DSBs generated when excising the ICL (Figure 1.4). During the repair of an ICL, a DSB may be generated (16, 17). As discussed earlier, the DSB repair pathway generates a D-loop which can be processed through SDSA, producing a noncrossover product, favored during mitotic recombination. The *S. cerevisiae* FANCM ortholog, Mph1 (18–21), has been shown to be involved in preventing crossovers (22), and *mph1* mutants show hypersensitivity to DNA damaging agents such as ionizing radiation (IR) and methyl methanesulfonate (MMS) (23). Biochemical studies using purified Mph1 show that it is a DNA helicase capable of unwinding Rad51-coated D-loops (22, 24), and that it can process DNA intermediates that form later in the repair pathway, including HJs (22, 24, 25). Unwinding of HJs and D-loops has also been observed using the *S. pombe* ortholog Fml1 (26). In contrast, no helicase unwinding activity has been detected for human FANCM (27, 28). Together, genetic and biochemical studies suggest roles for FANCM and its orthologs in HR that are dependent upon their ability to use ATP hydrolysis to unwind or remodel DNA structures so as to prevent CO products (22, 29–32).

A previous genetic study in our laboratory has shown that *Drosophila* Fancm, like its orthologs, is involved in the prevention of COs (29). This study tested the role of Fancm in CO prevention and response to DNA damaging agents. To better understand the role of the putative Fancm helicase activity in directing homologous recombination towards a non-crossover product, we tested the ability of the purified Fancm helicase to act on HR repair intermediates *in vitro*. Here we show that purified Fancm can unwind duplex DNA in a 3' to 5' direction in an ATP-dependent manner. Further, we provide evidence that Fancm can disassemble the HR D-loop intermediate.

MATERIALS AND METHODS

Expression and purification of *Drosophila* FANCM

Truncated FANCM, lacking 840 C-terminal residues (FANCMΔ), was cloned into pLIC-HisMBP using InFusion cloning (Clontech), with primers FAM1 and FAM2 (Table 2.1) and cDNA (DGRC). The K84M (FANCMΔ^{KM}) mutation was introduced into FANCMΔ using QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) with the pLIC-HisMBP-FANCMΔ construct as the template and the KMQC primer (Table 2.1). The protein expression plasmid was maintained in *E. coli* BL21DE3/pLysS and protein expression was induced by auto induction (33, 34). Briefly, bacterial cultures were grown in three liters of ZYM5052 autoinduction media (34) at 25°C for 24 hours. Cells were harvested by centrifugation, washed with 20 mL of STE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM NaCl), harvested again by centrifugation and stored as a cell pellet at −80°C until use.

Drosophila FANCMΔ and FANCMΔ^{KM} were purified to near homogeneity (Figure 2.1) using Ni-NTA resin (Qiagen) and Amylose resin (New England Biolabs) to take advantage of the two affinity tags present on the fusion protein. Cells were lysed in buffer L (500 mM NaCl, 50 mM Tris-HCl (pH 7.0), 10% glycerol) with 100 mM PMSF, EDTA-free protease inhibitor cocktail, 0.1% triton X-100 and 1 mg/mL lysozyme by incubation at 4^{o}C for 45 minutes and then sonicated to reduce viscosity in 10 second bursts. Cleared lysate was collected by centrifugation, incubated with 3 mL Ni-NTA resin, and 12 column volumes of Buffer L were flowed through the column. Protein was eluted using 300 mM imidazole in buffer L and protein was detected using a Bradford assay (Biorad). Peak fractions were concentrated and the buffer was exchanged with buffer M (200 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA) using Amicon Ultra, Ultracel 50K centrifugal filters (Millipore). The protein was then bound to a 1.5 mL Amylose column, washed with 10 column volumes of buffer M, and the protein was eluted in buffer M with 50 mM maltose and 10 mM dextrose. Protein was detected by Bradford assay and dialyzed against storage buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.0), 10% glycerol, 0.1 mM EDTA) and stored at -20°C. Protein purity was evaluated using SDS-PAGE.

DNA Substrates

Synthetic oligonucleotides (Table 2.1) used for DNA substrate preparation were PAGE purified by the supplier (IDT). Radioactively labeled substrates were prepared by incubating 10 pmols oligonucleotide with 3 μM [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) at 37°C for 50 minutes followed by a 20 minute incubation at 70°C to inactivate the enzyme. Labeled oligonucleotide was then annealed to its complement oligonucleotide in a ratio of 1:1.3 labeled:unlabeled oligonucleotide for fork substrates or 1:1.3:1.3 labeled:unlabeled oligonucleotide for D-loop substrates. Annealing occurred in buffer A (50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$) by heating at 95°C for 5 minutes and slowly cooling to room temperature. Hybridized DNA substrates were separated from unannealed oligonucleotide and free [γ^{32}P]ATP using a Sephadex G-50 column (Pharmacia).

ATPase assays

ATPase reactions were conducted using 212 nM of either FANCMΔ or FANCMΔ^{KM}. Reaction mixtures (20 μL) contained buffer C (25 mM Tris-HCl (pH7.5), 20 mM NaCl, 5 mM 2-mercaptoethanol, 10 μg/mL bovine serum albumin), M13mp18 ssDNA titrated from 0 to 120 nM (nucleotide phosphate) and 3 mM $MgCl_2$. All reagents except ATP were mixed and allowed to incubate on ice. 3 mM ATP with trace amounts (~60 nCi/μL) of [γ-^{32}P] ATP was added to initiate the reaction and incubation was at 37°C for 5 minutes. Aliquots (5 μL) were removed, and stop solution (5 μL) was added to a final concentration of 17 mM EDTA, 3.4 mM ATP, and 3.4 mM ADP. Of this mixture, 2 μL were spotted onto a cellulose matrix TLC-PET plate (Sigma) and developed in a 0.8 M LiCl/1M Formic acid solution. Plates were allowed to dry, exposed on a phosphor storage screen, and imaged using a Phosphorimager (Amersham Biosciences). All images were quantified using ImageQuant software.

Helicase Assays

Steady-state helicase unwinding reaction mixtures (20 μL) contained 0.1 nM radiolabeled DNA substrate (Table 2.1), 25 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 20 mM NaCl, 5 mM 2-mercaptoethanol (βME) and 10 μg/mL bovine serum albumin. Protein was titrated from a concentration of 0.5 nM to 212 nM. Reactions were initiated by the addition of 3 mM ATP, incubated at 37^{o}C for 15 minutes and stopped with the addition of 10 μL of helicase stop solution (37.5% glycerol, 50 mM EDTA, 0.3% SDS, 0.5x TBE

and 0.1% BPB.) All reactions were resolved on 7.5% non-denaturing polyacrylamide gels containing 0.5X TBE and 0.1% SDS, at room temperature for 2 hours at 180 v. Gels were transferred to Whatman paper, allowed to soak for 30 minutes in drying buffer (40% methanol, 10% acetic acid, 3% glygerol), and dried for 6 hours using a gel dryer. Dried gels were exposed on a phosphor storage screen and imaged using a Phosphorimager (Amersham Biosciences). All images were quantified using ImageQuant software.

Fluorescence Anisotropy

Reaction mixtures (50 µL) contained 10 nM fluorescently 5 labeled 6-FAM DNA substrate (Table 2.1), 25 mM Tris-HCl (pH7.5), 3 mM $MgCl_2$, 20 mM NaCl, 5 mM βME and 10 µg/mL bovine serum albumin. The fluorescence anisotropy was measured as a function of Fancm concentration from 1 nM to 212 nM. Reactions were incubated at 25°C for 5 minutes. Fluorescence anisotropy was measured using a Jobin Yvon Horiba Fluorolog-3 fluorometer with a Wavelength Electronics temperature control box. Labeled dsDNA substrates were excited at 495 nm and emission was measured at 520 nm. Fluorescence anisotropy was calculated using the software provided by the instrument.

RESULTS AND DISCUSSION

Fancm is a ssDNA-dependent ATPase

Previous genetic studies of *Drosophila Fancm* indicated a modest role for Fancm in SDSA and in preventing mitotic crossovers (29). This study used a gap repair assay in which SDSA can be distinguished from other types of repair, such as non-homologous end joining. In this assay a gap is generated by excision of a P element on the male X chromosome. This element carries an allele of white, apricot, that results in an orange eye color instead of the red wild-type color. Excision generates a 14 kb gap that is repaired using the sister chromatid with an intact P element as a template. Restoration of the white gene is the product if two-ended SDSA occurs. Aberrant SDSA disrupts the apricot allele and results in a white eye phenotype. In this way, involvement in SDSA can be measured. When Kuo et al. measured SDSA events (i.e. red eyes) Fancm mutants decreased SDSA by 50% compared to wild-type controls, indicating a reduced ability to complete repair by SDSA.

This modest role of Fancm in SDSA led us to investigate the role for the Fancm helicase in directing homologous recombination towards a non-crossover product through D-loop displacement.

Human FANCM and its orthologs in yeast have been shown to dissociate D-loops (22, 26, 31, 35). To further understand the role(s) of Fancm in DNA repair, we investigated the biochemical properties of purified Fancm.

We were unable to express and purify full-length Fancm so we overexpressed a truncated form, FancmΔ, and a form of this truncated protein with a mutation in the Walker A motif, FancmΔ^{KM}, as His6x-MBP tagged proteins in *E. coli* and purified each to near homogeneity (Figure 2.1). The Walker A motif is a conserved motif characterizitic of SF2 helicases and binds the triphosphate tail of ATP and consequently plays a role in ATP hydrolysis (36, 37). This truncation was generated to encompass the helicase domain and is based off of purified truncations of the fission yeast ortholog, Fml1 (26). The plasmid for construction was made so that expression of the protein would include the amino-terminal 649 amino acids of Fancm (Figure 2.2).

We confirmed the ATPase activity of purified FancmΔ and measured several biochemical parameters to characterize this activity. FancmΔ was found to have 5X greater ATPase activity at 37°C than 25°C (Figure 2.3). The ATPase activity also increased with time (Figure 2.4) and NaCl concentration (Figure 2.5), although ATPase activity declined at NaCl concentrations above 100 nM. For the purposes of this study, we chose conditions under which the ATPase activity was in a linear range. To this end, activity of Fancm was measured at 37°C for 5 minutes in 35 mM NaCl.

There was no detectable ATP hydrolysis in the absence of DNA whereas the ATPase activity of the purified protein was higher in the presence of circular M13 ssDNA compared to that of dsDNA, confirming that the protein is a DNA-dependent ATPase (Figure 2.6). In addition, we measured the K_{eff} (2.8 μM) and the V_{max} (65.3 pmols) for ssDNA and dsDNA, K_{eff} (5.7 μM) V_{max} (40.1 pmols), under these conditions, further confirming that ssDNA stimulates ATPase activity more strongly than dsDNA. As expected, the FancmΔ^{KM} mutant lacked ATPase activity (Figure 2.6). Taken together, these results indicate that Fancm is a DNA-dependent ATPase and this activity is dependent on the lysine residue found in the canonical helicase motif I. ATPase activity stimulated by ssDNA as well as dsDNA has also been reported for human FANCM and yeast Fml1, while Mph1 only exhibits ssDNA-dependent ATPase activity (24, 27, 38). Fluorescence anisotropy was used to determine if differences in ATPase stimulation

were a result of DNA binding (Figure 2.7 A). No significant differences in binding of Fancm to ssDNA as compared to dsDNA were detected.

Fancm is a 3′ to 5′ DNA helicase

To determine if *Drosophila* Fancm is active as a helicase, unwinding assays were performed using partial duplex DNA substrates under steady-state conditions. Purified protein was incubated with DNA substrate and the reaction was initiated by the addition of ATP. The wild-type (FancmΔ) helicase completely unwound a 15 bp partial duplex substrate with a 25 bp 3′-ssDNA tail (15/40) (Figure 2.8 A, lane 3). There was no detectable unwinding of the substrate at an equal concentration of mutant protein FancmΔ^{KM} (Figure 2.8 A, lane 4). When the same reaction was conducted with a 15 bp partial duplex with 25 bp 5′-ssDNA tail (-15/40), the wild-type helicase failed to unwind the substrate (Figure 2.8 B). This represents a directional bias for unwinding and classifies Fancm as a 3′ to 5′ helicase, consistent with previous work on the yeast ortholog Mph1 (24). In addition, these data support the conclusion that Fancm cannot unwind blunt-ended duplex DNA as no unwinding of the -15/40 substrate was detected even at longer incubation times.

As shown in Figure 2.8 C**,** no unwinding of the 15/40 substrate was detected when either ATP or $MgCl_2$ were omitted from the reaction. Moreover, unwinding was undetectable when the non-hydrolyzable ATP analogue AMP-PNP was substituted for ATP. Taken together, these data indicate that unwinding by the Fancm helicase is dependent upon the ability of the protein to hydrolyze ATP and the FancmΔ^{KM} mutant is a 'helicase-dead' protein.

Fancm catalyzes limited unwinding reaction

Further testing of the helicase activity of purified Fancm revealed a limit in unwinding longer regions of duplex DNA. A substantial decrease in unwinding activity was observed using a 20 bp partial duplex DNA substrate with a 20 bp 3′-ssDNA tail (20/40). Only 60% of the DNA substrate was unwound by the wild-type helicase at a concentration of protein that unwound all of the 15/40 partial duplex substrate (Figure 2.9 A). To exclude the possibility that the reduced length of the free 3′-tail was responsible for this result, we generated a 20 bp partial duplex substrate with a 25 bp 3′-ssDNA tail

(20/45). As seen with the 20/40 substrate, Fancm was only able to unwind 60% of the 20/45 substrate (Figure 2.9 A).

We also measured unwinding activity using two splayed-arm substrates, one with a 3ˈ-single stranded region of 25 bp, and one with a 3ˈ-singled stranded region of 20 bp; both substrates had a 15 bp duplex region. In each case the substrates were completely unwound, indicating that neither the length of the 3ˈ-tail nor the complexity of the substrate affects unwinding. An additional splayed arm substrate with a 25 bp duplex region and 25 nt 5ˈand 3ˈ-ssDNA arms was also tested (Figure 2.9 B), with no detectable unwinding. Although Fancm was able to unwind a 20 bp partial duplex, the increase from 20 bp to 25 bp reduced unwinding to undetectable levels under these conditions. It is possible that Fancm is able to unwind greater than 20 bp partial duplexes under different conditions as discussed later in this chapter.

Based on *in vivo* data (29), we hypothesized that Fancm may be involved in SDSA with a role in displacing D-loops. Previous studies have shown that the yeast ortholog, Mph1, can unwind the D-loop structures generated during recombination (22). To test the ability of Fancm to unwind complex DNA structures we constructed substrates resembling a recombination D-loop intermediate. We incubated Fancm with a 40 nt bubble-like structure with 25 bp of duplex on either end. As expected from previous studies, Fancm does not unwind the bubble (Figure 2.10), as the duplex region on either end of the bubble is longer than 20 bps.

We next tested if Fancm can unwind a D-loop by incubating Fancm with the bubble structure containing an ‘invading’ homologous strand in which the duplex region was limited to 15 bp. To determine whether the position of the invading strand had an effect on unwinding, the invading strand was positioned at the ‘front’, ‘middle’ and ‘end’ of the homologous template strand within the bubble (Figure 2.11 A). Fancm catalyzed robust unwinding of substrates with the invading strand positioned in the “middle” and at the “end” of the bubble. However, Fancm unwound the substrate with the invading strand positioned at the “front” with much lower efficiency.

The decrease in substrate unwound as the position of the duplex region is moved is most likely an inability of Fancm to access the duplex region rather than the length of the duplex. The reduced unwinding of the substrate with the invading strand in the ‘front’ position is likely due to the lack of ssDNA

region to which the helicase can bind to initiate unwinding. The “middle” and “end” substrate both have regions that mimic the partial duplex with a ssDNA 3' tail. However, the “front” position substrate does not have a partial duplex with a ssDNA 3' tail, but instead has a 5' ssDNA tail. As shown above, Fancm does not catalyze unwinding of a substrate with a 5' -ssDNA tail (See Figure 2.8 B). However, in this more complex substrate there is an open ssDNA region on the opposite strand of the bubble. Fancm most likely unwinds enough of the duplex arm, generating a 3' tail and thereby catalyzing reduced unwinding of the invading strand. When a 5'-ssDNA tail was added to more closely mimic an “invading strand”, no difference in unwinding was detected (Figure 2.11 B).

To test if the initial rate of the reaction or the duration of the reaction affected unwinding, the rate of unwinding for each substrate was determined using 10 nM and 150 nM protein at various time points for the 15/40 and 20/40 DNA substrates (Figure 2.12 A and B). At 10 nm protein concentration (Figure 2.12 A), FancmΔ was able to fully unwind the 15/40 substrate over the course of the experiment. Under the same conditions FancmΔ was only able to partially unwind the 20/40 substrate. The same was observed for reactions using 150 nm protein (Figure 2.12 B). While the length of time did increase the amount of 20/40 substrate unwound -- 60% at 15 minutes to 78% at 40 minutes -- FancmΔ was unable to fully unwind the 20/40 substrate. There are many factors that might influence Fancm’s ability to catalyze unwinding of longer duplex regions. The structure of the protein, protein interactions, and even posttranslational modifications could influence unwinding of DNA by Fancm.

To determine if the differences seen in unwinding were a result of DNA binding to the substrates, and if there was a difference in binding efficiency between FancmΔ and FancmΔ^{KM}, we used fluorescence anisotropy to measure the formation of DNA-protein complexes. As seen in Figure 2.7 A and B, FancmΔ and FancmΔ^{KM} are capable of binding DNA. However large deviations from the mean make it difficult to assess whether there is preference in DNA binding. At this time, it is not clear whether preference of Fancm for certain structures reflects the differences in unwinding. One potential area of development for studies of Fancm is to measure the binding of Fancm to the structures mentioned in this study, along with structures with longer duplex regions. If Fancm is indeed able to bind structures with long duplex regions

with the same efficiency, then the inability to unwind greater than 20 bp of duplex DNA could reflect some mechanistic response of Fancm to limit unwinding.

The data presented here indicate that Fancm as a 3′to 5′ DNA helicase able to unwind up to 20 bp of partial duplex DNA substrates in an ATP-dependent manner. In addition, the enzyme is able to dissociate short duplex regions in more complex D-loop like structures. The failure of the protein to unwind longer duplex regions may be the result of in vitro conditions or lack of an important accessory protein. Efforts to detect unwinding of longer duplex regions under other conditions (*e.g.,* different salt concentration) or in the presence of a ssDNA binding protein were unsuccessful.

Mph1 and Fml1 have both been shown to be active helicases unwinding up to 100 bp of duplex DNA (24). On the other hand, human FANCM has been shown to migrate D-loops and HJs, but no unwinding activity has been reported (24, 26–28, 35, 39). The data presented here suggest that *Drosophila* Fancm is similar to both the yeast and human orthologs but unique. Unlike the human ortholog, it is an active helicase, yet we could not detect unwinding of longer duplex regions like the yeast orthologs. Although we were not able to detect DNA unwinding by the protein of duplex regions greater than 20 bp, there may be other factors that can contribute to an increase in helicase activity. The unwinding activity of Mph1 was stimulated upon the addition of RPA, a heterotrimeric ssDNA binding factor (24), and it's possible that other proteins may also stimulate the unwinding activity of Fancm. We cannot rule out the possibility that the C-terminal region of Fancm may regulate the helicase activity of the protein and that greater tracts of DNA can be unwound by full length Fancm.

FIGURES

Table 2.1-List of oligonucleotides used in this study.

NAME	SEQUENCE
FAM1	AGTGGATAcCGGATCATGGATGTGAATTGGATGGACG
FAM2	GCTCGAATTCGGATCTCATCAGCTCATTTGGTAGGGTTTTATTC
KMQC	GGGAATGACCTTCATCGCCGCGGTGGTTATG
40	GCTAGCAGTAGCCAGCATCGAACGTACGATCGGTAACGTA
45	GCTAGCAGTAGCCAGCATCGAACGTACGATCGGTAACGTAATGCA
15	CTGGCTACTGCTAGC
20	CGATGCTGGCTACTGCTAGC
-15	TACGTTACCGATCGT
SA25	GACGCTGCCGAATTCTGGCGTTAGGAGATACCGATAAGCTTCGGCTTAAA
SA25a	ATCGATGTCTCTAGACAGCACGAGCCCTAACGCCAGAATTCGGCAGCGTC
A1	CATTGCATATTTAAAACATGTTGGAAGGCTCGATGCATGCTGATAGCCTACTAGTGCTGC TGGCTTTCAAATGACCTCTTATCAAGTGAC
A2	GTCACTTGATAAGAGGTCATTTGAATTCATGGCTTAGAGCTTAATTGCTGAATCTGGTGC TGGGATCCAACATGTTTTAAATATGCAATG
Front	TCCCAGCACCAGATT
Middle	CAGCAATTAAGCTCT
End	GCTCTAAGCCATGAA
SAL	TTGATAAGAGGTCATCTGGCTACTGCTAGC
SAS	TACGTTACCGATCGTTTGATAAGAGGTCAT
56F	GACGCTGCCGAATTCTGGCGTTAGGAGATACCGATAAGCTTCGGCTTAA
DS	TTAAGCCGAAGCTTATCGGTATCTCCTAACGCCAGAATTCGGCAGCGTC
3OH	CCTAACGCCAGAATTCGGCAGCGT
5OH	TTAAGCCGAAGCTTATCGGTATCT

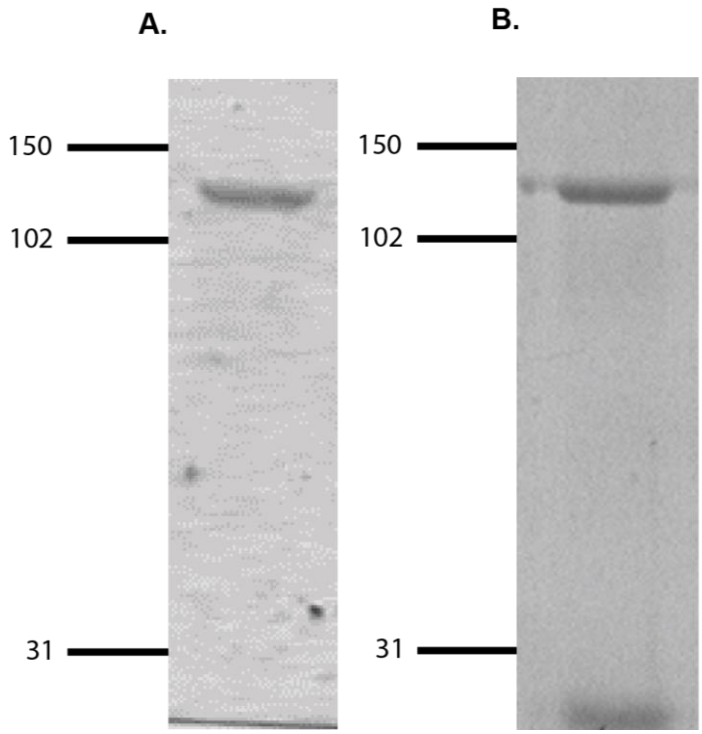

Figure 2.1 - *Purification of Fancm∆ and Fancm∆KM* - Fancm∆ and Fancm∆KM were purified as described in “Materials and Methods”. A.) Fancm∆; B.) Fancm∆KM. 100 ng were loaded for each protein.

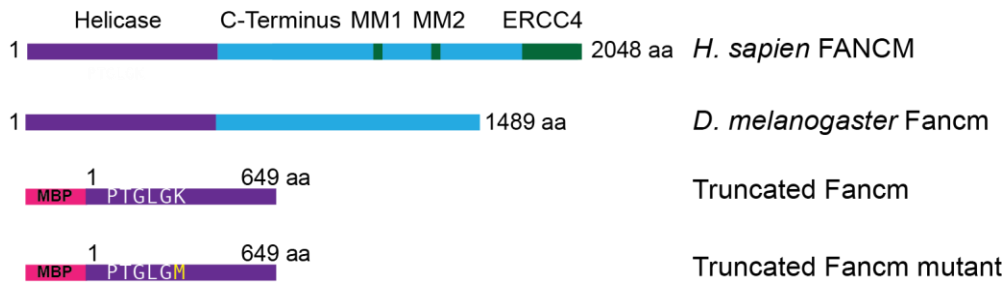

Figure 2.2 - *Schematic of Fancm* - Domains and motifs present in human FANCM are marked. Conserved domains or motifs in *Drosophila melanogaster* are noted. Truncated forms depicted are with an N-terminal MBP tag.

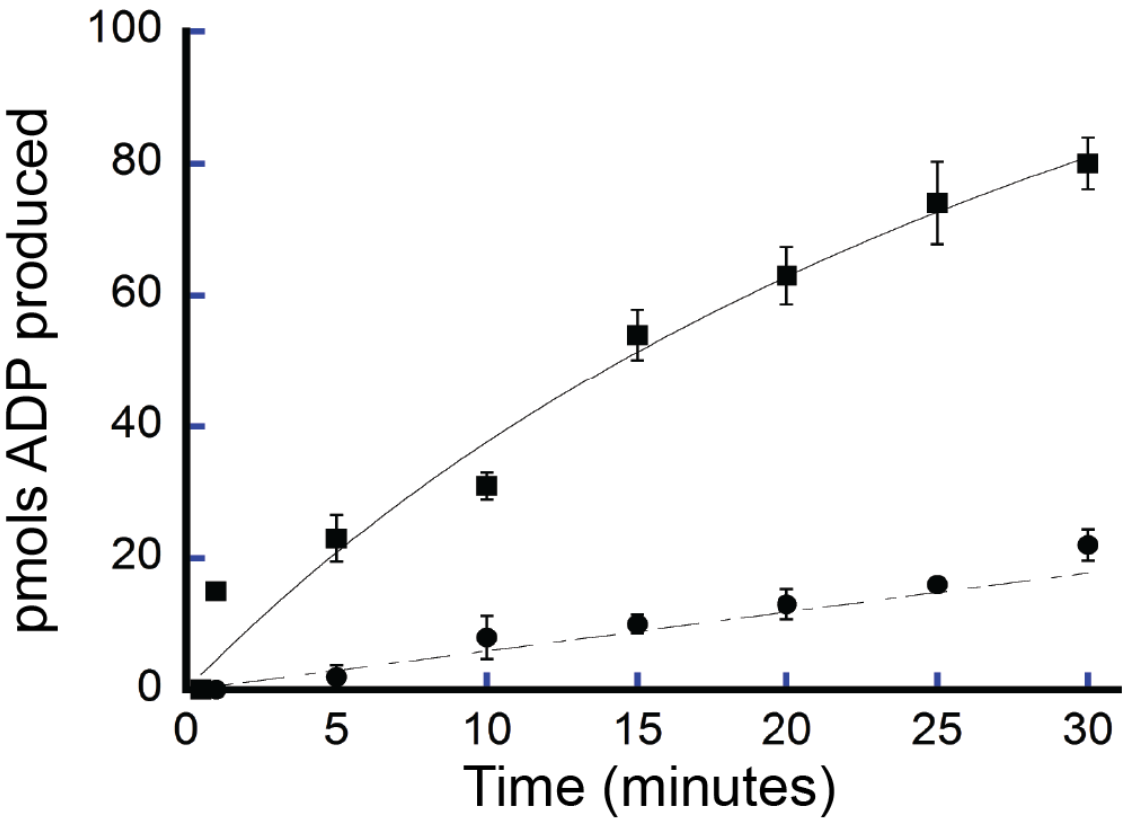

Figure 2.3 - *ATP hydrolysis by Fancm as a function of temperature* - Fancm ATPase activity was examined as a function of temperature using 212 nM FancmΔ and M13mp18 ssDNA as the DNA co-factor. All reactions were incubated at 37° (■) or 25° (●) for the time indicated. The average values from at least three independent experiments were plotted. Error bars represent standard error about the mean.

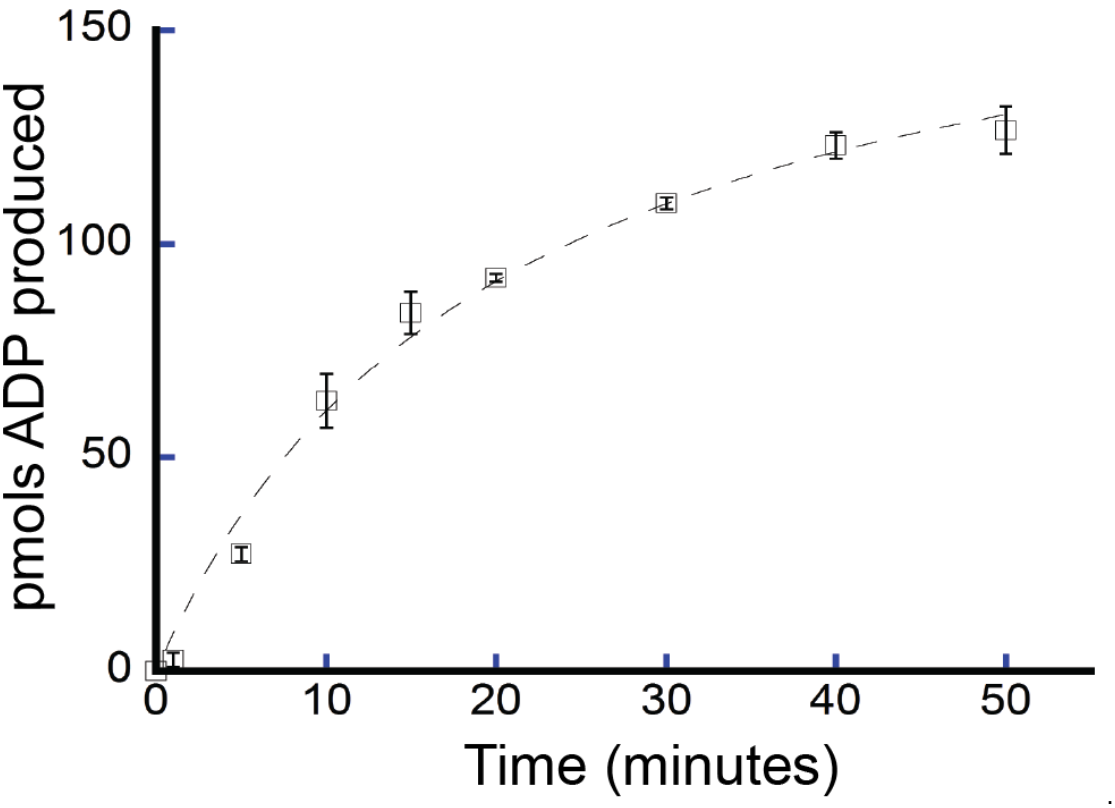

Figure 2.4 - *ATP hydrolysis by Fancm as a function of time* - Fancm ATPase activity was examined as a function of time using 212 nM Fancm∆ and M13mp18 ssDNA as the DNA co-factor. All reactions were incubated at 37° for the time indicated. The average values from at least three independent experiments were plotted. Error bars represent standard error about the mean.

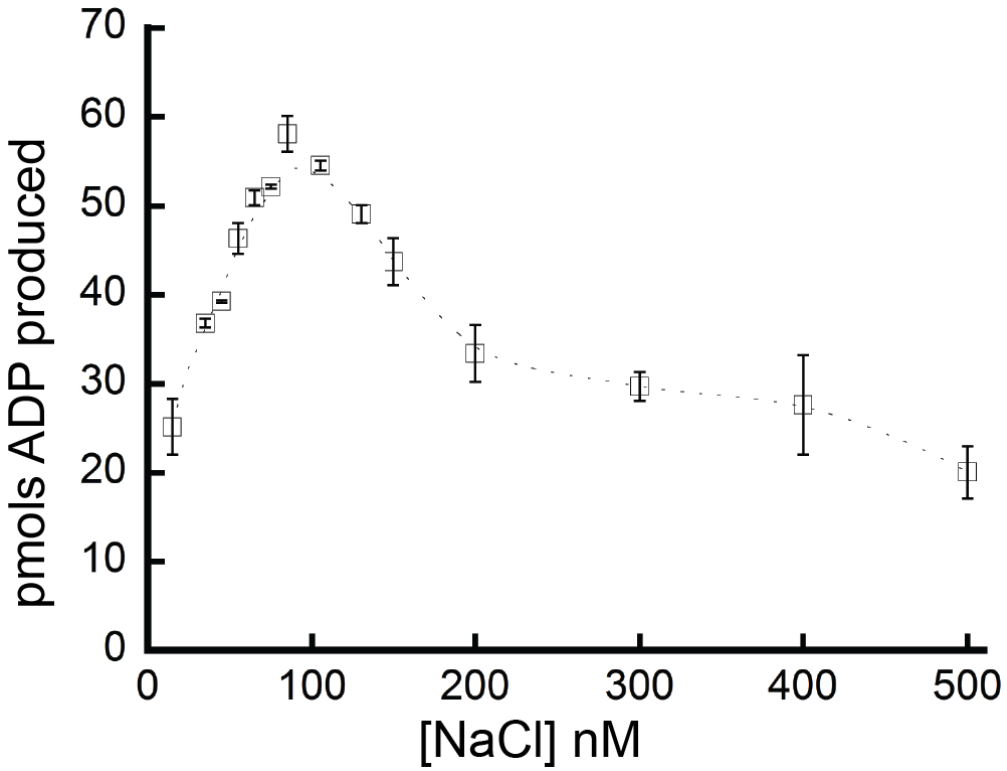

Figure 2.5 - *ATP hydrolysis by Fancm as a function of NaCl concentration - (nM)*. Fancm ATPase activity was examined as a function of salt concentration using 212 nM Fancm∆ and M13mp18 ssDNA as the DNA co-factor. All reactions were incubated at 37° for 5 mins. The average values from at least three independent experiments were plotted. Error bars represent standard error about the mean.

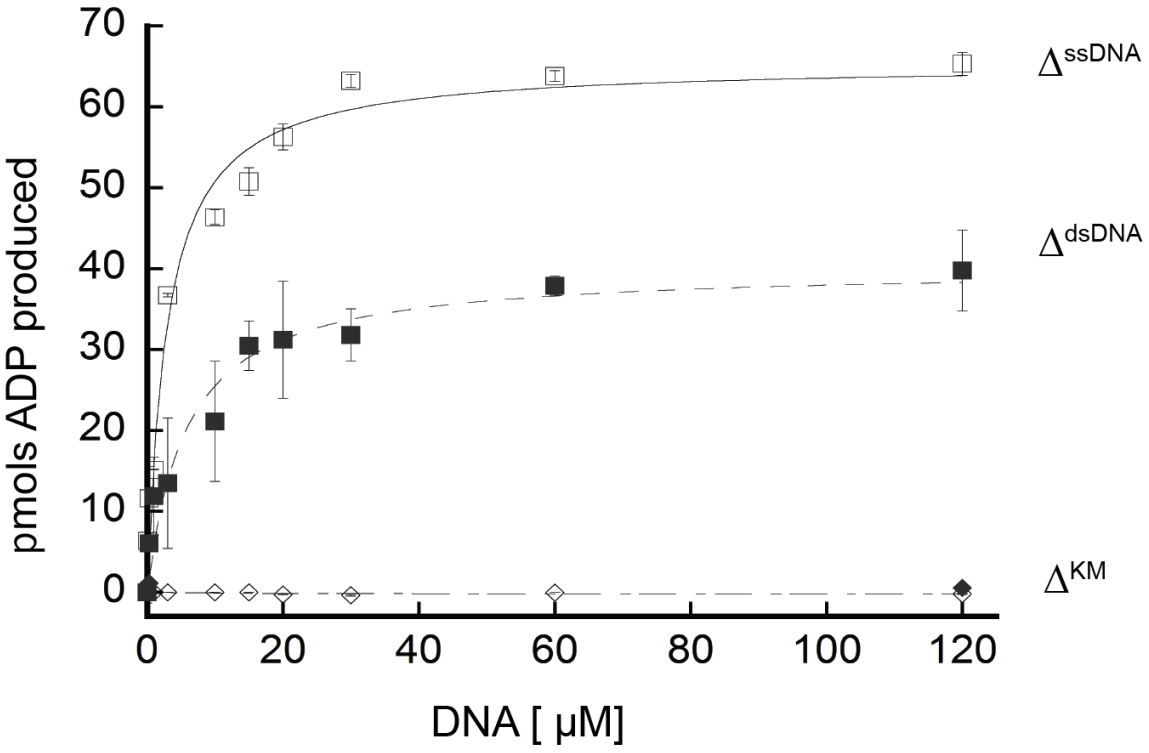

Figure 2.6 - *ATP hydrolysis by Fancm* - Fancm ATPase activity was examined as a function of DNA concentration using either M13mp18 ssDNA (□◊) or dsDNA (■♦) as the DNA co-factor. All reactions were incubated at 37° for 5 minutes. □ ■ 212 nM FancmΔ (Δ) on ssDNA; ◊ ♦ 212 nM FancmΔ^{KM} (Δ^{KM}). The average values from at least three independent experiments were plotted. Error bars represent standard error about the mean (ssDNA) or standard deviation about the mean (dsDNA).

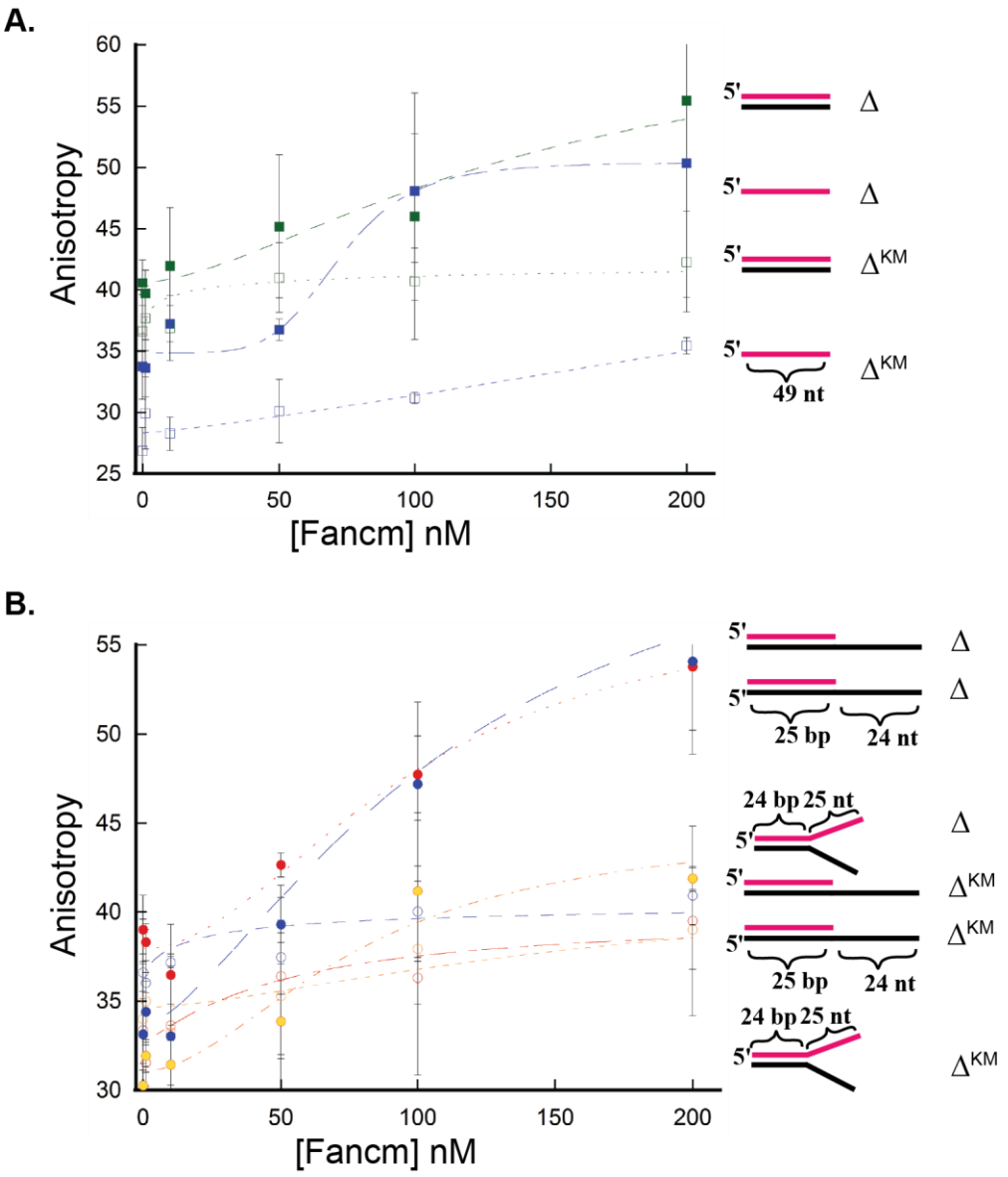

Figure 2.7 - *Fancm binding of DNA substrates as measured by fluorescence anisotropy* - Binding reactions were performed as described under "Materials and Methods". The indicated concentrations of Fancm were incubated with 10 nM of the indicated substrate. Colored strand on each substrate represents the 5' fluorescent strand. Quantitative data from at least 3 experiments were plotted as the average for each protein concentration. Error bars represent the standard deviation about the mean. Oligonucleotides used to make these substrates can be found in Table 2.1. A.) Comparison of substrate bound between FancmΔ on dsDNA (■) (56F/DS), ssDNA (■)(56F); and FancmΔ^{KM} on dsDNA (□) (56F/DS), ssDNA (□)(56F); B.) Comparison of substrate bound between FancmΔ on a 25 bp duplex region with a 24 nt 5' overhang (●)(56F/5OH); 25 bp duplex region with a 3' 24 nt 3' overhang (●) (56F/3OH); and a 24 bp duplex region with 25 nt 3' and 5' ssDNA arms (●); and FancmΔ^{KM} on a 25 bp duplex region with a 24 nt 5' overhang (○)(56F/5OH); 25 bp duplex region with a 3' 24 nt 3' overhang (○) (56F/3OH); and a 24 bp duplex region with 25 nt 3' and 5' ssDNA arms (○);

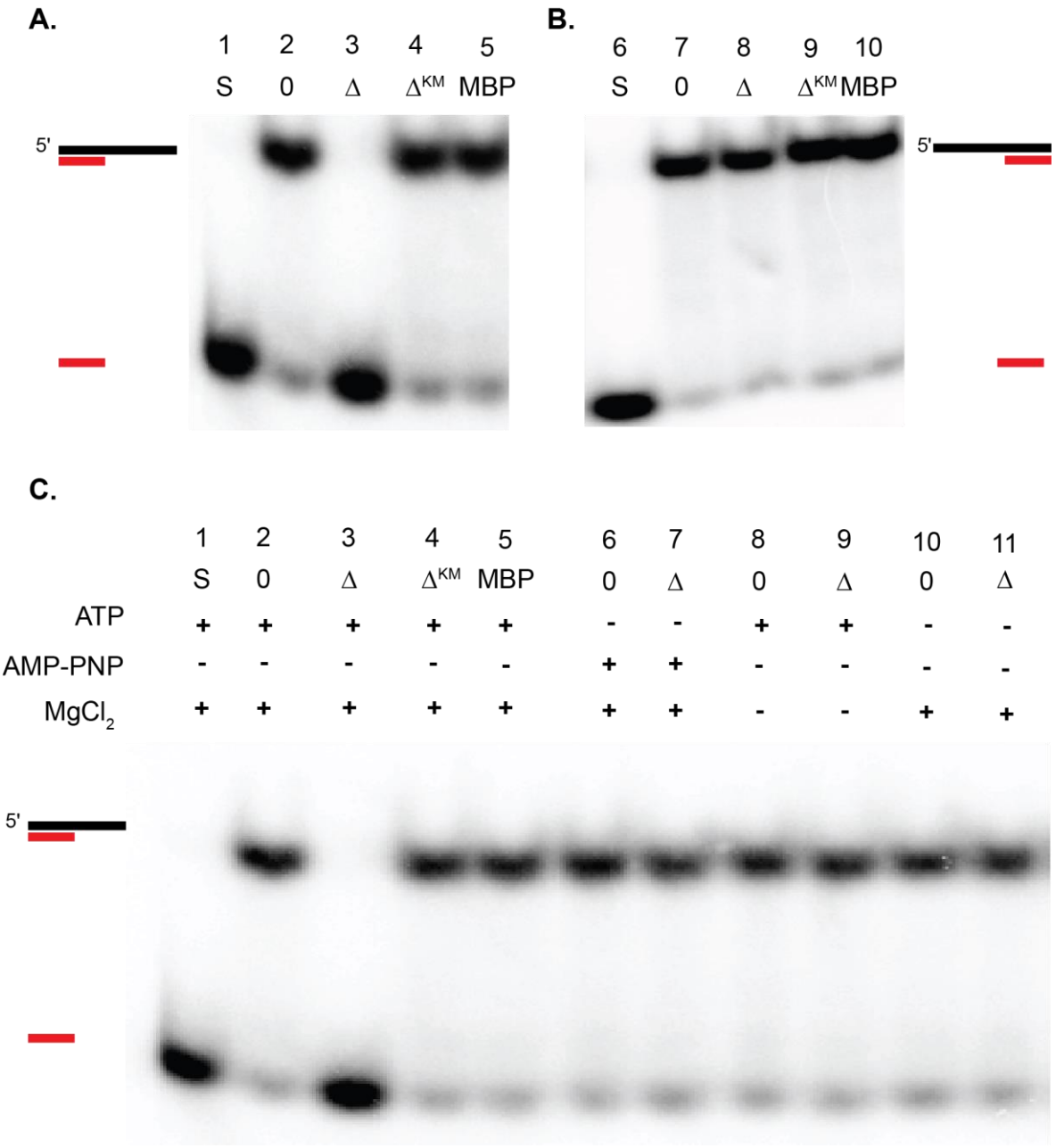

Figure 2.8 - Fancm unwinds duplex DNA in an ATP dependent manner - A.) Protein (212 nM) was incubated with a 5' radiolabeled 15 bp partial duplex with a 25 nucleotide 3' overhang (15/40). B.) Fancm is a 3'-5' DNA helicase. Protein (212 nM) was incubated with a 5' radiolabeled 15 bp partial duplex with a 25 nucleotide 5' overhang (-15/40). Lane 1 and 6 (S) are boiled loading controls indicating ssDNA. Lanes 2 and 7 (0) are no protein controls. FancmΔ (lane 3 and 8, Δ); FancmΔKM (lane 4 and 9, ΔKM); maltose binding protein (MBP) (lane 5 and 10, MBP). C.) Protein (212 nM) was incubated with a 5' radiolabeled 15 bp partial duplex with a 25 nucleotide 3' overhang (15/40). Lane 1 is a boiled loading control indicating ssDNA (S); Lanes 2, 6, 8, and 10 are no protein controls (0); Lanes 3, 7, 9, and 11, are FancmΔ (Δ); Lane 4 is FancmΔKM (ΔKM); Lane 5, maltose binding protein (MBP) was used a negative control. Reactions were performed at 37° for 15 minutes in the presence (+) or absence (-) of ATP, AMP-PNP, and MgCl2. Colored strand represents radiolabeled strand. Substrate oligonucleotides are in Table 2.1.

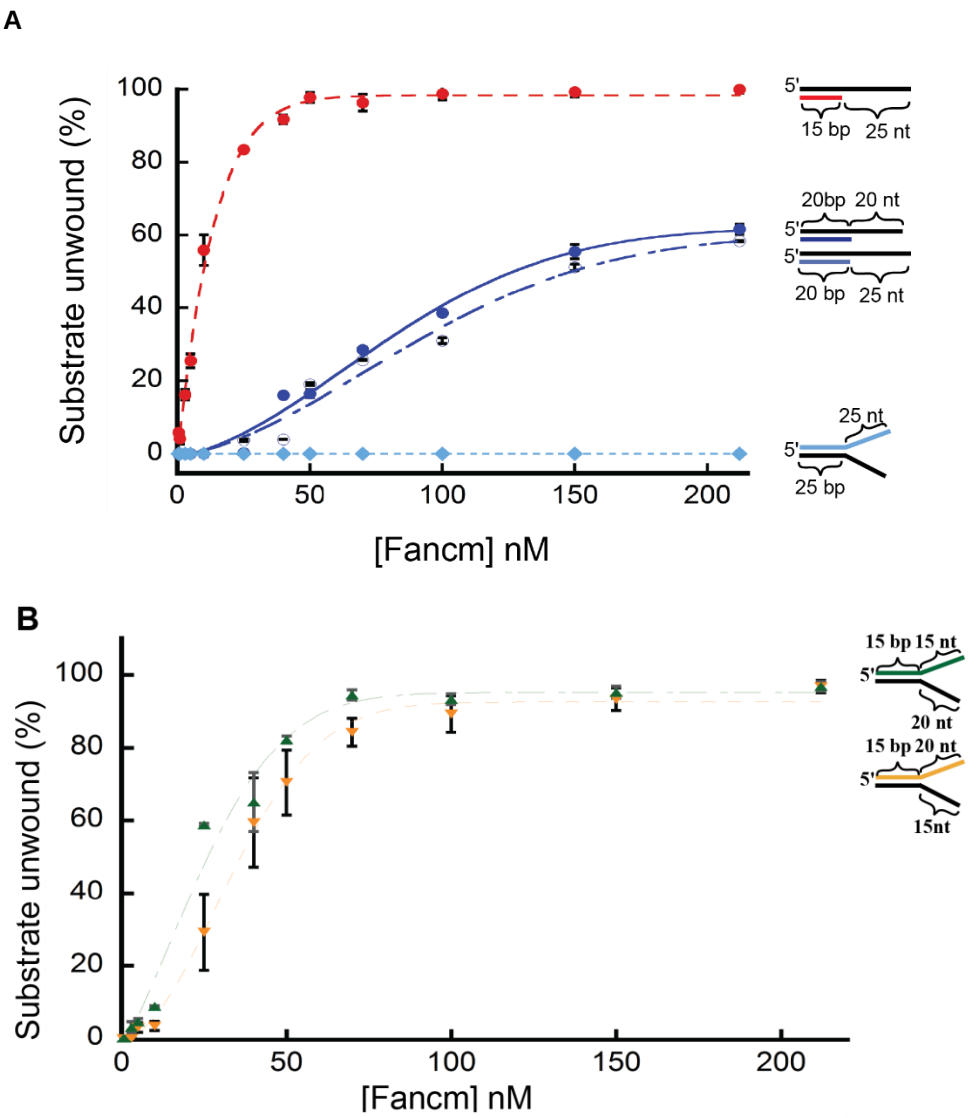

Figure 2.9 **-** *Unwinding of partial duplex DNA substrates by Fancm* - Helicase reactions were performed as described under "Materials and Methods". The indicated concentrations of Fancm were incubated with 0.1 nM of the indicated substrate for 15 minutes. Colored strand on each substrate represents radiolabeled 5' strand. Quantitative data from at least 3 experiments were plotted as the average for each protein concentration. Error bars represent the standard error about the mean. Oligonucleotides used to make these substrates can be found in Table 2.1. A.) Comparison of the fraction of substrate unwound with partial duplex substrates of different duplex lengths. ● 15 bp duplex region with a 25 nt overhang. ● 20 bp duplex region with a 20 nt overhang; ○, 20 bp duplex region with a 25 nt overhang; ♦ 25 bp duplex region with 25 nt single stranded arms. B.) Unwinding of splayed arms by Fancm ▼ 15 bp duplex region with 25 nt single stranded 3'arm and a 15 nt single stranded 5' arm (SAL/40); ▲15 bp duplex region with 15 nt single stranded 3'arm and a 20 nt single stranded 5' arm (SAS/40).

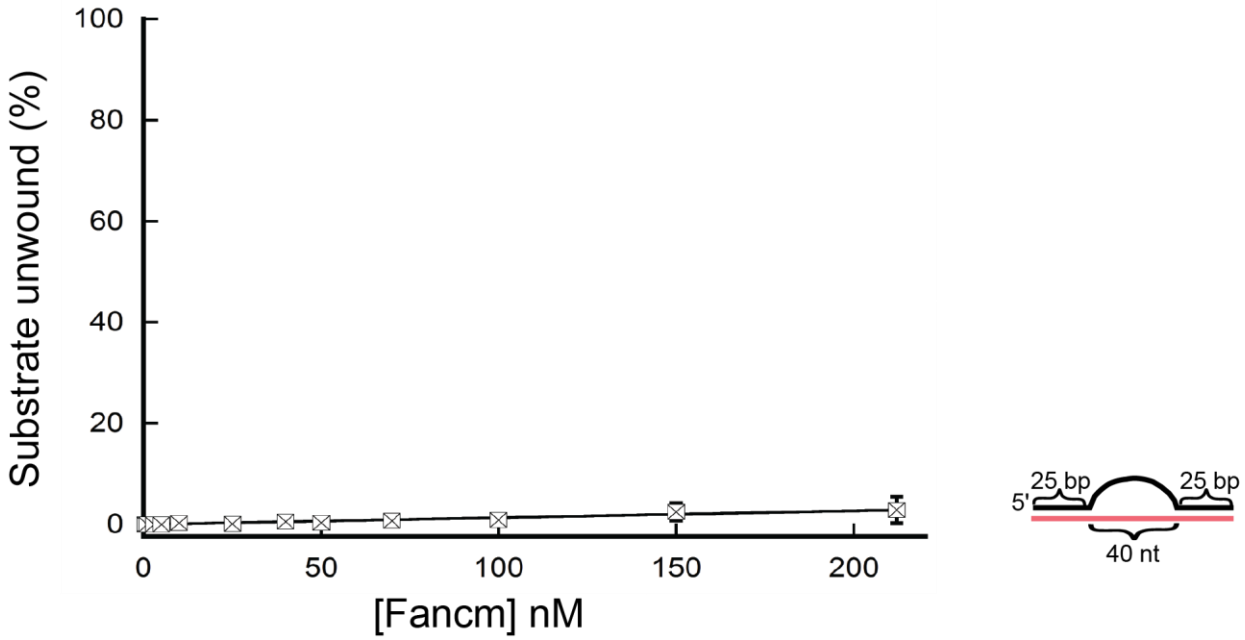

Figure 2.10 - *Unwinding of bubble-like structure by Fancm* - Bubble structure was made using a two 90 nt oligonucleotides with 25 bp of complementary ends with a 40 nt non-complementary middle (A1/A2). Colored strand on each substrate represents radiolabeled 5' strand. Quantitative data from at least 3 experiments were plotted as the average for each protein concentration. Error bars represent the standard error about the mean. Oligonucleotides used to make these substrates can be found in Table 2.1.

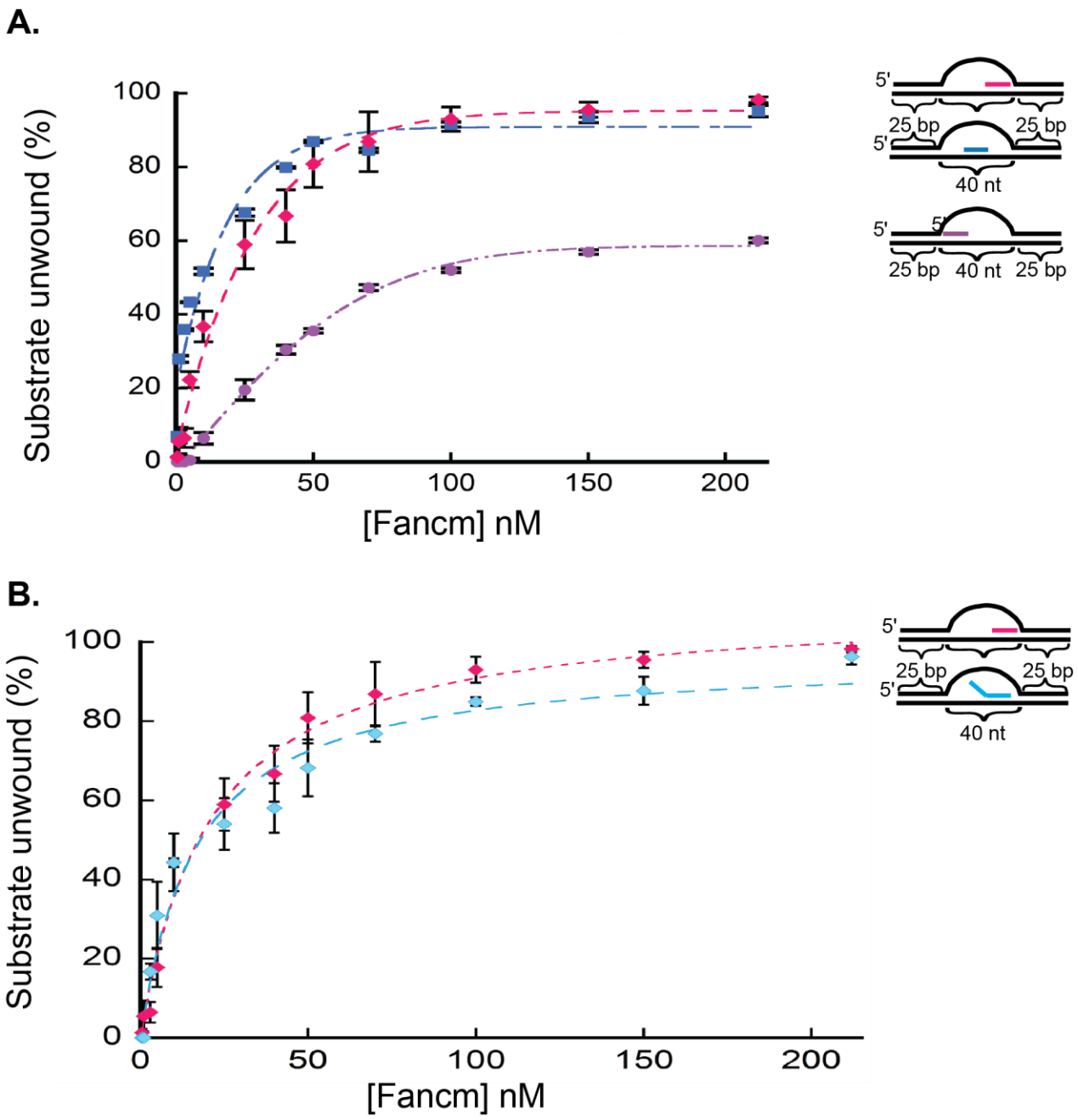

Figure 2.11 - *Unwinding of D-loop intermediate substrates by Fancm* - ● Front; ■ Middle; ♦ End. Bubble structures were made using a two 90 nt oligonucleotides with 25 bp of complementary ends with a 40 nt non-complementary middle (A1/A2). B.) Unwinding of D-loop intermediate substrates by Fancm. Bubble structures were made using a two 90 nt oligonucleotides with 25 bp of complementary ends with a 40 nt non-complementary middle. ♦ End; ♦ End with a 15 nt 5' -ssDNA tail. Colored strand on each substrate represents radiolabeled 5' strand. Quantitative data from at least 3 experiments were plotted as the average for each protein concentration. Error bars represent the standard error about the mean. Oligonucleotides used to make these substrates can be found in Table 2.1.

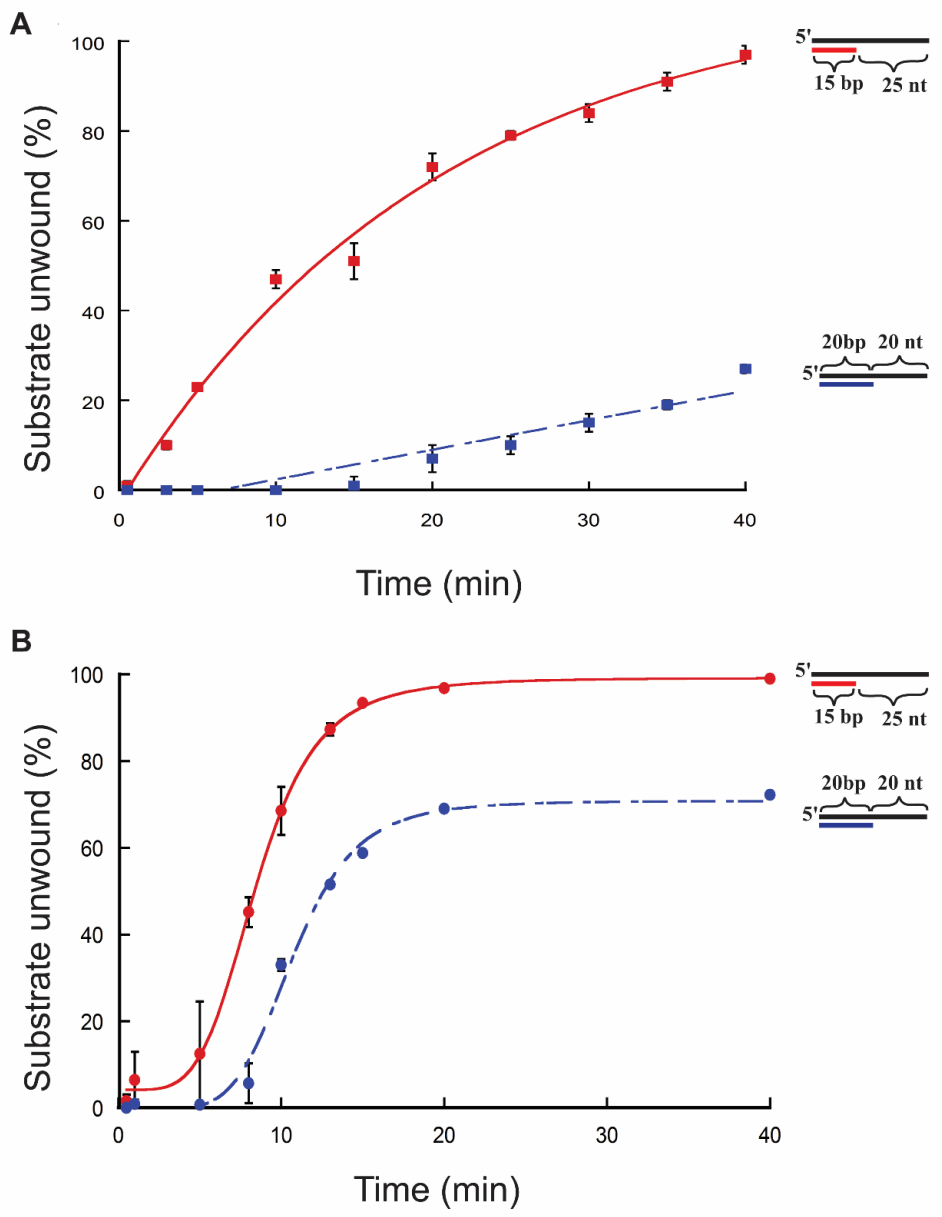

Figure 2.12 - Timecourse unwinding of partial duplex DNA substrates by Fancm - Helicase reactions were performed as described under “Materials and Methods”. The indicated concentrations of Fancm were incubated with 0.1 nM of the indicated substrate for indicated time. Colored strand on each substrate represents radiolabeled 5’ strand. Quantitative data from at least 3 experiments were plotted as the average for each protein concentration. Error bars represent the standard error about the mean. Oligonucleotides used to make these substrates can be found in Table 2.1. A.) Comparison of the fraction of substrate unwound with 10 nm FancmΔ on partial duplex substrates at the indicated time points of different duplex lengths. ■ 15 bp duplex region with a 25 nt overhang (15/40); ■ 20 bp duplex region with a 20 nt overhang (20/40). B.) Comparison of the fraction of substrate unwound with 150 nm FancmΔ on partial duplex substrates at the indicated time points of different duplex lengths. ●15 bp duplex region with a 25 nt overhang (15/40); ● 20 bp duplex region with a 20 nt overhang (20/40).

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CHAPTER 3- GENETIC ANALYSIS OF *D. MELANOGASTER FANCM*

INTRODUCTION

DNA damage repair is essential to maintain both genome stability and integrity. As discussed in Chapter 1 and 2, helicases play an important role in facilitating repair. One network for repair is the Fanconi anemia (FA) family of repair proteins. Gene products in the FA repair pathway are involved in signaling and DNA-processing. Various DNA damage repair response networks are associated with the FA repair pathway. For instance, BRCA1, ATR, and BLM have all been shown to interact with one or more members of the FA response pathway (1–5). Key in the FA network is the FANCM helicase. FANCM is a conserved helicase with ATP-dependent DNA translocase activity. As a component of the FA repair pathway, FANCM has been shown to mediate the interaction of FA proteins with DNA (6, 7).

The structure of FANCM lends support to the idea that the protein has a role in facilitating protein-protein interaction. In addition to the conserved DEAH helicase domain, the C-terminus of FANCM contains several motifs that facilitate protein-protein interaction and an ERCC4-like endonuclease domain (8, 9), classifying FANCM as an XPF-ERCC1 family protein. The ERCC4-like domain, while catalytically inactive, helps facilitate interaction with FAAP24, an interaction that helps stabilize FANCM on chromatin and assists in targeting FANCM to single-stranded DNA (ssDNA) and Y-shaped DNA structures (10–14). Although FANCM contains both a helicase and ERCC4-like domain, neither helicase activity nor endonuclease activity has been detected for FANCM or the FANCM-FAAP24 heterodimer (10, 15). The lack of DNA-processing activities traditionally associated with a DEAH helicase domain or ERCC4-like nuclease domain suggest FANCM functions differently compared to other members of the XPF-ERCC1 family.

The involvement of FANCM in protein-protein interactions is well documented and fairly extensive. In addition to forming a heterodimer with FAAP24, FANCM also interacts with the FANCM-associated histone-fold proteins 1 and 2 complex (MHF1-MHF2) (16, 17). Both FAAP24 and the MHF

complex interact with FANCM via the C-terminus to establish two stable complexes that bind different DNA structures. The binding and stabilization of FANCM on DNA is integral in facilitating repair (10, 16, 18). The C-terminus also houses two specific motifs (MM1 and MM2) which have been shown to allow for interaction with the FA complex and the Bloom syndrome helicase (BLM) complex (7). The MM2 motif, which facilitates interaction with RMI1 in the RMI1-RMI2 complex to recruit the BLM-RMI1-Topo IIIα complex (1, 19), indicates a role for FANCM in directing repair via HR.

As discussed in Chapter 1 and above, many different domains and motifs exists in the FANCM and its yeast ortholog (Mph1) that facilitate interaction with other repair proteins and promote DNA association. These motifs and domains also appear to be key in allowing FANCM to interact with additional proteins that help direct and coordinate DNA repair functions. In *D. melanogaster* Fancm many of these known motifs and domains are lacking. For instance, MM1 and MM2 are both missing in yeast Mph1 and *Drosophila* Fancm. The catalytically inactive C-terminus ERCC4-like endonuclease domain is involved in interaction with FAAP24 (11–13). While *D. melanogaster* and yeast lack this domain, both also lack FAAP24. In the C-terminus of the *S. cerevisiae* ortholog, Mph1, has two motifs that facilitate interaction with chromatin via SMC5/6 (20). While many of these motifs are not detected in *Drosophila* orthologs, there is still the potential for C-terminal interactions with other proteins involved in HR or DNA repair complexes.

This chapter focuses on the role, if any, of the C-terminus and helicase domain of Fancm in regulating repair events in *Drosophila*. To this end, I generated C-terminal truncations of Fancm *in vivo* and analyzed how these mutants respond to various DNA damaging agents and their function in CO prevention.

MATERIALS AND METHODS

Drosophila stocks

Fly stocks were maintained at 25° with standard medium. $Fancm^{0693}$ is a nonsense mutation previously described in Kuo et al. (21). Deletion of endogenous Fancm ($Fancm^{Del}$) was generated using CRISPR/Cas9 technology (22, 23). Oligonucleotides (IDT) used for guide RNA (Del1 and Del2, Table 3.1) were cloned into pU6 Bbs1 chiRNA vector, and this was injected into Cas9(X) (BestGene). $Fancm^{Del}$

deletes 3R: 21480913 to 3R:21487017. In experiments reported here, *Fancm* mutants were *st* $Fancm^{0693}$/*Sb* $Fancm^{Del}$, or *st* $Fancm^{0693}$/*w+transgene Sb* $Fancm^{Del}$. Transformants were generated from a PCR-amplified genomic fragment (F1 and F2,) and expressed under the endogenous *Fancm* promoter. The K84M mutation was introduced using QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) and Primer KMQC (Table 3.1). The truncated $Fancm^{1-645}$ construct was generated from the FL construct using endogenous *Mfe*I sites. InFusion reaction was used to add the C-terminus and 3' UTR with a PCR reaction and primer FA (Table 3.1) from the original construct. $Fancm^{1-645K84M}$ was generated in the same way, using the FLKM construct.

Mitotic crossover assay

Mitotic crossovers were measured in the male germline as previously described (24), using the genetic markers *st* and *Sb* for each genotype indicated. At least 20 individual males were assayed for each genotype indicated. Statistical analyses and graphing were done in Prism 6 (GraphPad) using Kruskal–Wallace test. P-values reported are corrected for multiple comparisons.

DNA damage sensitivity assays

Sensitivity to DNA-damaging agents was determined as previously described (25). Briefly, an aqueous solution of either MMS or HN2 at the indicated concentrations was added to the food during larval feeding. Adults in untreated vials were allowed to mate and lay eggs for 3 days before being transferred into fresh vials, allowed to lay eggs for 2 days, and treated with DNA-damaging agents. For ionizing radiation (IR) damage, larvae were exposed to gamma rays in an irradiator at 1500 rad. At least 10 individual, independent crosses were performed for each genotype indicated. Relative survival was calculated for each vial as the ratio between mutant:control flies in treated vials and normalized to the ratio of mutant:control flies in the untreated vial. Vials with less than 20 progeny were discarded. Statistical analyses were performed as described above.

RESULTS AND DISCUSSION

Helicase-dead and truncated Fancm are each able to prevent a subset of mitotic crossovers

The C-terminal region of Fancm in yeast and human orthologues contains motifs that facilitate protein-protein interactions (Figure 3.1). Human FANCM has a helix-hairpin helix region in its ERCC4-like

domain that allows for association with FAAP24, an interaction that helps stabilize the protein on chromatin (11, 12). The presence in human FANCM of motifs 1 and 2 (MM1 and MM2) allow the interaction of FANCM with the FA core complex and the Bloom Syndrome helicase (BLM) complex, respectively (1, 7). It should be noted that *Drosophila* Fancm has neither the ERCC4 domain nor MM1 or MM2. The lack of these sequences is consistent with the fact that the interacting protein partners associated with these domains, FAAP24, FANCA, and RMI1, are not present in *Drosophila* (FAAP24 appears to be missing from all insects, FANCA from holometabolous insects, and RMI1 from Schizophoran flies; unpublished observations). Nonetheless, it is likely that the C-terminal region of Fancm may contribute to the regulation and function of the protein.

To identify a potential role for the C-terminus in regulation of HR, I generated transgenic recombinant flies expressing either full length or truncated Fancm. The truncated transgenic recombinant flies are identical to the FancmΔ protein characterized *in vitro* in Chapter 2, except that it lacks the His and MBP tags used for protein purification (Figure 2.1). We refer to the transgenic truncated Fancm as tr to distinguish it from FancmΔ. To investigate the role of the helicase activity in CO prevention and DNA damage response, I generated transgenic recombinant flies that express either full-length or truncated Fancm with either a wild-type helicase domain or the helicase-dead mutation described and characterized in Chapter 2. RT-PCR was used to test for gene expression. All transgenes produce a gene product as shown in Figure 2.2 A.

Previous reports on the function(s) of *Drosophila* Fancm (21) used the nonsense mutation $Fancm^{0693}$ (L78ter) (21, 26) in *trans* to *Df(3R)ED6058* (27). This 423.1 kb genomic deletion removes more than 50 genes (28). To ensure that any mutant phenotype described was due to the loss of Fancm and not the heterozygous deletion of surrounding genes, we used CRISPR technology to generate a partial deletion of *Fancm* ($Fancm^{del}$) that should result in no protein being produced. Expression, or lack of protein expression, could not be determined as the antibody for Fancm, generated from a synthetic peptide, is highly non specific and detection of Fancm by Western blot is inconclusive. Mutants used here were heteroallelic for $Fancm^{del}$ and $Fancm^{0693}$. In all assays preformed, no significant difference was observed between the previous results using *Df(3R)ED6058* and the experiments using $Fancm^{del}$,

allowing me to conclude that *Fancm*^{del} is a null allele and the results obtained in the previous experiments with the *Df* were a result of a loss of *Fancm*. All experiments reported here used one copy of the transgene in a null background (*Fancm*^{Del}/*Fancm*^{0693}). All comparisons are made in reference to this null genotype.

As previously reported (21), *Fancm* mutants exhibit a significant increase in the number of spontaneous mitotic crossovers. Since meiotic crossovers do not occur in males, I assayed spontaneous mitotic crossovers in the male germline of the transgenic *Fancm* mutants. Crossovers were scored between the visible markers *st* and *Sb* (~20% of the genome) (Figure 3.2 B). No crossovers were detected in wild-type males or in *Fancm* null mutants with the full-length transgene (FL) (Figure 3.3, Table 3.2), indicating that the transgenes used are fully functional and that Fancm is indeed involved in preventing mitotic crossovers. Flies with the truncated Walker A mutant (trKM) transgene showed an increase in crossovers similar to that of the null mutant. The presence of an active Fancm helicase domain without the C-terminus (tr) reduced the rate of spontaneous mitotic crossovers to near wild-type levels. Interestingly, the full-length Walker A mutant (FLKM) also reduced crossover levels (Figure 3.3, Table 3.2). The fact that tr and FLKM reduced crossover levels to near wild-type yet trKM did not suggests there are at least two partially independent functions of Fancm in preventing crossovers, one that requires the helicase activity but not the C-terminus and another that is dependent on the C-terminus but does not require helicase activity.

Although both FLKM and the tr transgenes reduced the levels of spontaneous mitotic crossovers compared to the null mutant, crossovers were still detected above wild-type levels. The difference between these genotypes and WT does not cross the threshold typically considered to be statistically significant, but my lab has never detected a crossover in any wild-type male (21, 24, 29); hence, I believe the elevation is biologically significant. These data indicate that Fancm must be both full length and catalytically active to prevent all mitotic crossovers. However, the presence of either the full-length helicase-dead protein, or the absence of the C-terminus but with retention of ATPase activity, is sufficient to prevent most mitotic crossovers.

Separation of function in Fancm's roles in the response to DNA damage

Since the mitotic crossover assay measures spontaneous germline crossovers detected in progeny, I cannot determine how or when crossovers are occurring. I therefore cannot provide a mechanistic explanation for the difference between the crossovers seen in FLKM and the crossovers in tr. The difference in crossover phenotype found among the transgenes of *Fancm* led me to investigate whether there was a difference in DNA damage response using the transgenes described above.

Previous sensitivity studies using *Drosophila Fancm* (21) showed that *Fancm* mutants were sensitive to the crosslinking agent mechlorethamine (HN2), the alkylating agent methyl methanesulfonate (MMS), and strand breakage induced by ionizing radiation (IR). These types of damage engender a variety of DNA repair mechanisms. Since HN2 can induce mono-adducts, intrastrand cross-links, and ICLs, the alkylating agent MMS was tested to distinguish between the role of *Fancm* in repair of ICLs versus a broader role in damage repair. While both MMS and HN2 damage can involve replication fork impairment, the crosslinks induced by HN2 could lead to DSBs (30, 31). IR was therefore used to determine if *Fancm* is involved in repair of DSBs.

As previously reported (32), *Fancm* null mutants were sensitive to all damaging agents tested. The sensitivities seen in the null mutants are rescued when the full length (FL) transgene is present (Table 3.2). The full length mutant (FLKM) and truncated (tr) transgene both rescued sensitivity to HN2 and IR, but not MMS (Figure 3.4-3.6). The trKM transgene failed to rescue sensitivity to HN2 and IR, but did appear to rescue MMS sensitivity (Figure 3.4-3.6). However, progeny with the trKM transgene have delayed developmental timing (discussed further in Chapter 4). If MMS in unstable after addition to the culture medium, it is possible that control larvae ingested food immediately after addition of MMS, whereas trKM larvae ingested food at a later time, after a substantial fraction of MMS was already degraded. Because of this complication, we cannot be confident that the higher relative survival of trKM flies reflects functional capacity of the truncated, helicase-dead Fancm protein.

The difference in rescue among the transgenes in response to damage by HN2 and IR compared to MMS may represent different functional roles of *Fancm* in various DNA damage response pathways. The ability of both the FLKM and tr transgene to rescue the sensitivity to HN2 and IR (Figure 3.4, 3.5), is

reminiscent of the role of these transgenes in crossover prevention (Figure 3.3), and again hints at separable functions of Fancm - one that is dependent on the helicase and one that is dependent on the C-terminus. Taken together, we propose that Fancm regulates or participates in multiple DNA damage responses.

The ability to rescue sensitivity to MMS (and HN2), is representative of Fancm having more than one role in repair. The difference in response to HN2 and MMS in the FLKM and tr may be a result of whether Fancm is functioning with other proteins or independently. Kuo *et al.* investigated functions of Fancm that are independent of the FA pathway by comparing phenotypes of *Fancm* mutants to those of *FancI* mutants. Differences in sensitivity suggested a role for Fancm in DSB repair that is independent of the FA response.

I hypothesize that Fancm not only acts separately from the FA repair response, but can act both directly, through the catalytic activity of the helicase, and indirectly, in a non-helicase, non-catalytic directed manner, in repair of DSBs. A catalytic role in the formation of NCO products might be to unwind short D-loops or to initiate D-loop unwinding. A non-catalytic function might be to recruit HR repair proteins that direct repair toward NCO products, perhaps by extending unwinding of longer D-loops. For instance, if a DSB occurs, Fancm could recruit HR repair proteins, such as Blm, and direct HR toward NCO products. This recruitment and interaction with Blm would be representative of a non-catalytic activity of Fancm, as the recruitment and interaction efforts of the protein do not require the helicase activity of the protein. These dual roles are seen in the FLKM and the tr genotypes. The lack of helicase activity in FLKM prevents it from unwinding D-loop HR intermediates, resulting in some COs being made after these progress to dHJ intermediates. The COs we see in the tr genotype could result from the lack of Fancm recruiting HR repair proteins, such as Blm. Fancm's proposed interaction with BIm and involvement with HR and D-loop displacement is supported by studies in humans and yeast (21, 33–36). *Blm* mutants have more spontaneous mitotic crossovers than *Fancm* mutants (24, 32). Interestingly, *Blm Fancm* double mutants have the lower number of mitotic crossovers seen in *Fancm* single mutants, consistent with the hypothesis that Fancm recruits Blm to prevent crossovers.

FANCM and its orthologs have been shown to dissociate D-loops (35, 37–39), leading to the suggestion that they promote synthesis-dependent strand annealing (SDSA) as. As shown in chapter 2, *Drosophila* Fancm is capable of unwinding D-loop like structures. A gap repair assay demonstrated that both *Fancm* and *Blm* mutants have defects in SDSA in *Drosophila* (21). Based on the data presented in chapter 2, I propose that one role of Fancm might be to unwind short D-loops, leaving Blm to unwind D-loops that have been extended by additional synthesis or to continue unwinding those initiated by Fancm. In either case, it is possible that Fancm recruits Blm to D-loops. Use of a gap repair assay directly demonstrated roles for both Fancm and Blm in SDSA in *Drosophila* (32, 40). Unfortunately, Kuo *et al* (32) were unable to conduct this assay in *Blm Fancm* double mutants and genetic complications prevented us from using our *Fancm* transgenes in the SDSA assay (discussed further in chapter 4), so these hypotheses cannot be tested with available reagents.

Fancm's role in catalytic repair and non-catalytic repair, as well as its function in coordinating and participating in repair pathways may be reflected in the structure of the C-terminus and the various motifs found in human FANCM. For instance, the C-terminus of FANCM has motifs that allow binding with proteins that are associated with repair. Motif 1 (MM1) allows binding between FANCM and FANCA, a FA core complex protein. Motif 2 (MM2), allows FANCM to interact with the Bloom Complex through RMI1. Although *Drosophila* Fancm lacks these motifs, neither RMI1 or FANCA are found in *Drosophila.* It is possible that these motifs are diverged beyond our ability to detect them and that interaction between Fancm and other HR proteins does occur in flies.

Based on the findings presented here, I suggest that the C-terminus coordinates multiple repair efforts and is involved in the regulation of crossovers during HR, either through D-loop displacement (helicase function), or through recruitment of additional repair proteins via the C-terminus. While there are many similarities found between the orthologs of FANCM, there are also many differences in activity. These differences may indicate an evolutionary process that has diverged the function of these proteins to better maintain the fitness of the organism. Some of these differences can be reflected in the fact that conserved motifs present in some orthologs are not found in others, indicating that FANCM has co-evolved with other

proteins to fill a functional niche. Regardless, it's clear through this study, as well as work done in other organisms, that FANCM has a broad and diverse role in DNA maintenance and repair.

FIGURES

Table 3.1- Oligonucleotides used in this study

NAME	SEQUENCE
F1	GGGGACAAGTTTGTACAAAAAAGCAGCCTGGGGCTTGACATTTCCGGGC
F2	GGGGACAACTTTTGTATACAAAGTTGTTCTCATATGCGCGCCATCTGGCGGC
KMQC	GGGAATGACCTTCATCGCCGCGGTGGTTATG
FA1	AGCTTCACAGCAATTGCTAGGTAAGGTGCC
FA2	TATTAACAATCAATTGGACTTATAAAGATCTGTATCACG
Del 1	CTTCGCGGACGATTCGAACCATAG
Del1a	AAACCTATGGTTCGAATCGTCCGC
Del2	CTTCGCACATAT TTTCTCAGCCAG
Del2a	AAACCTGGCTGAGAAAATATGTGC

Table 3.2- Comparison of all transgenic Fancm genotypes and null genotype. (-) mean no rescue of the null phenotype, (+) indicate rescue.

	Null	FL	FLKM	tr	trKM
IR	-	+	+	+	-
HN2	-	+	+	+	-
MMS	-	+	-	-	+
COs	-	+	+	+	-

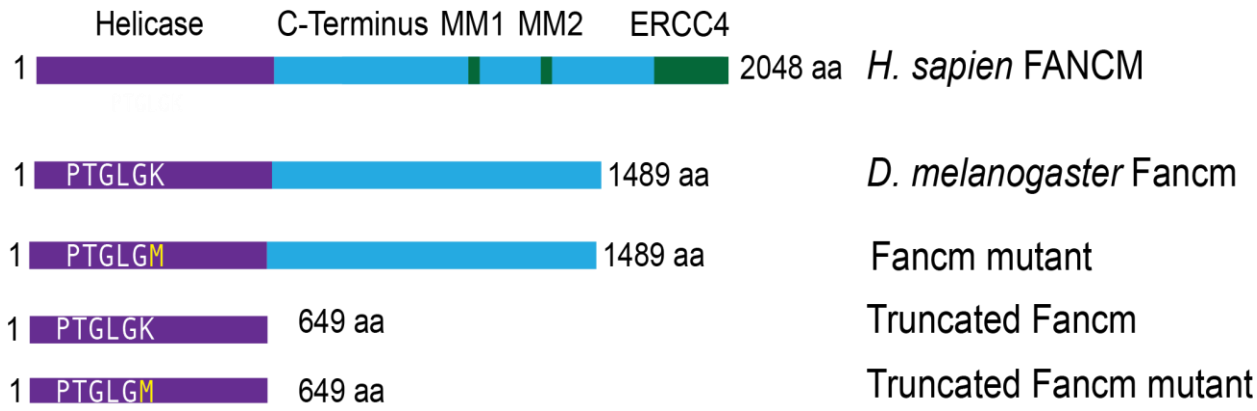
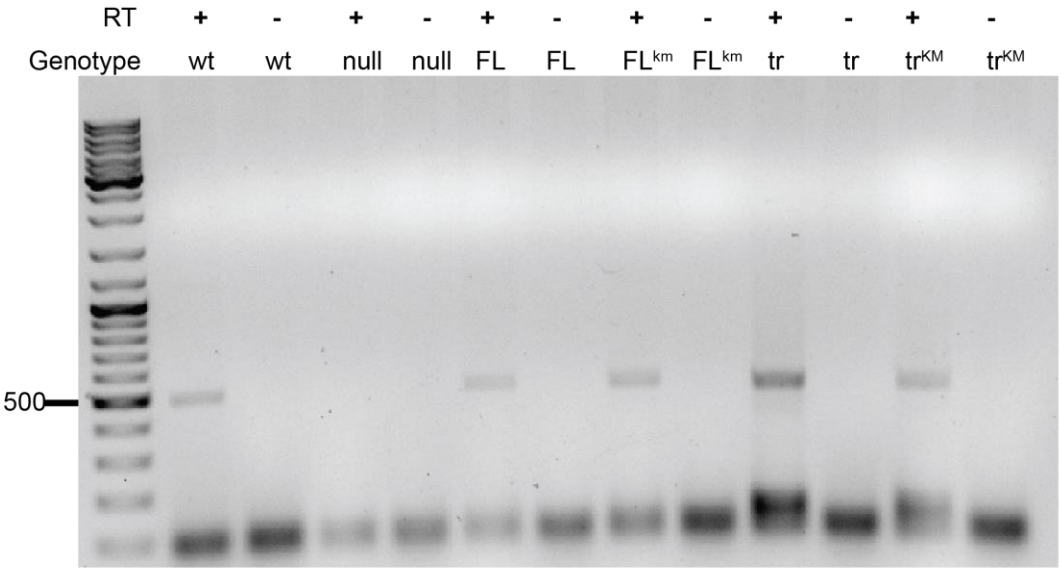


Figure 3.1 - *Schematic of Fancm* - Domains and motifs present in human FANCM are marked. Conserved domains or motifs in *Drosophila melanogaster* are noted. The Walker A motif is noted in white. Mutation of the Walker A motif is indicated in yellow.

A



B

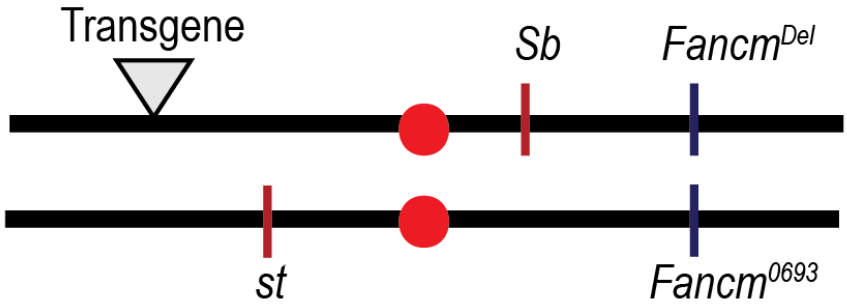


Figure 3.2 - Fancm transgenic flies - A. RT PCR was performed as described in material and methods from whole fly genomic preps. RT was either added (+) or omitted (-), to determine if the presence of a band was the result of genomic contamination. Genotypes represented here are Wildtype (wt) from yw1118; Fancm0693/Fancmdel (null); Fancm0693/Fancmdel Sb w+FancmFL (FL); Fancm0693/Fancmdel Sb w+FancmFLKM (FLKM); Fancm0693/Fancmdel Sb w+Fancmtr (tr); Fancm0693/Fancmdel Sb w+FancmtrKM (trKM); B. Map of genes used in Crossover assay. Fancm null allele, (*Fancm*^{0693}); CRISPR deletion (*Fancm*^{Del}); transgene landing site (▼), and *st* and *Sb* genes. Schematic of transgenes generated are as seen in Figure 3.1.

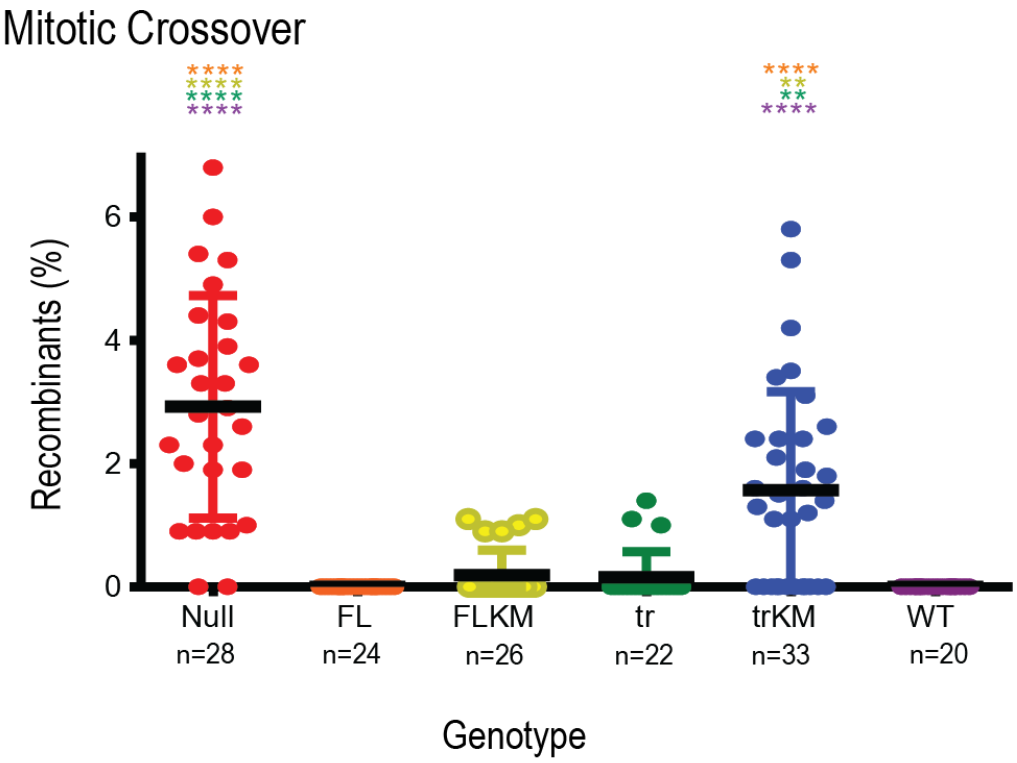


Figure 3.3 - Spontaneous mitotic crossover rates - Spontaneous mitotic crossover rates were measured between st and Sb. ● Null; ● Full length (FL); ● Full length KM (FLKM); ● truncated (tr), ● truncated KM (trKM); ● Wild-type (WT). Each dot represents one vial, n measures number of vials. Mean percentage of progeny is represented by black horizontal bar. 95% confidence intervals are represented by colored error bars. Statistical comparisons were done for Fancm compared to each other genotype. Statistically significant comparisons are indicated above error bars; ****P<0.0001 by Kruskal-Wallace test, corrected for multiple comparisons.

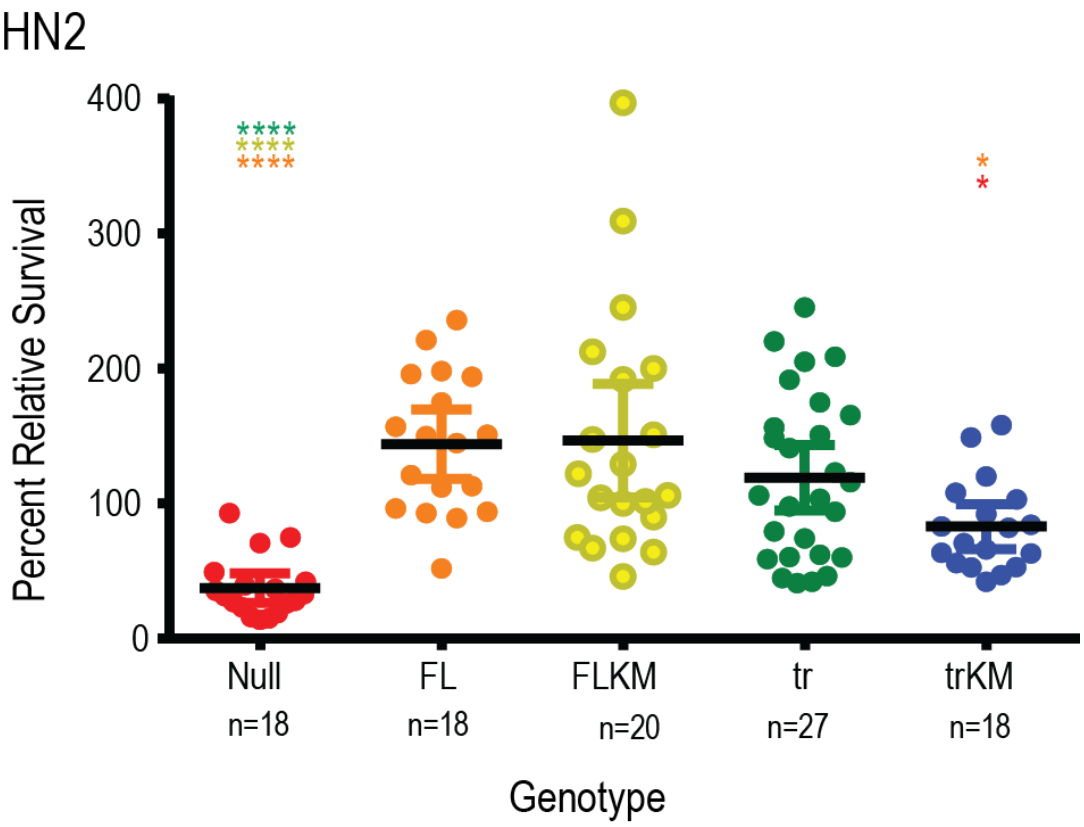


Figure 3.4 - *Sensitivity of Fancm to HN2* - Plots show the survival of the indicated phenotype relative to wildtype control flies in the same vial after exposure to 0.002% HN2 (0.1 M) ● Null; ● Full length (FL); ● Full length KM (FLKM); ● truncated (tr), ● truncated KM (trKM) Each dot represents one vial, n measures number of vials. Mean percentage of progeny is represented by black horizontal bar. 95% confidence intervals are represented by colored error bars. Statistical comparisons were done for Fancm compared to each other genotype. Statistically significant comparisons are indicated above error bars; ****P<0.0001 by Kruskal-Wallace test, corrected for multiple comparisons.

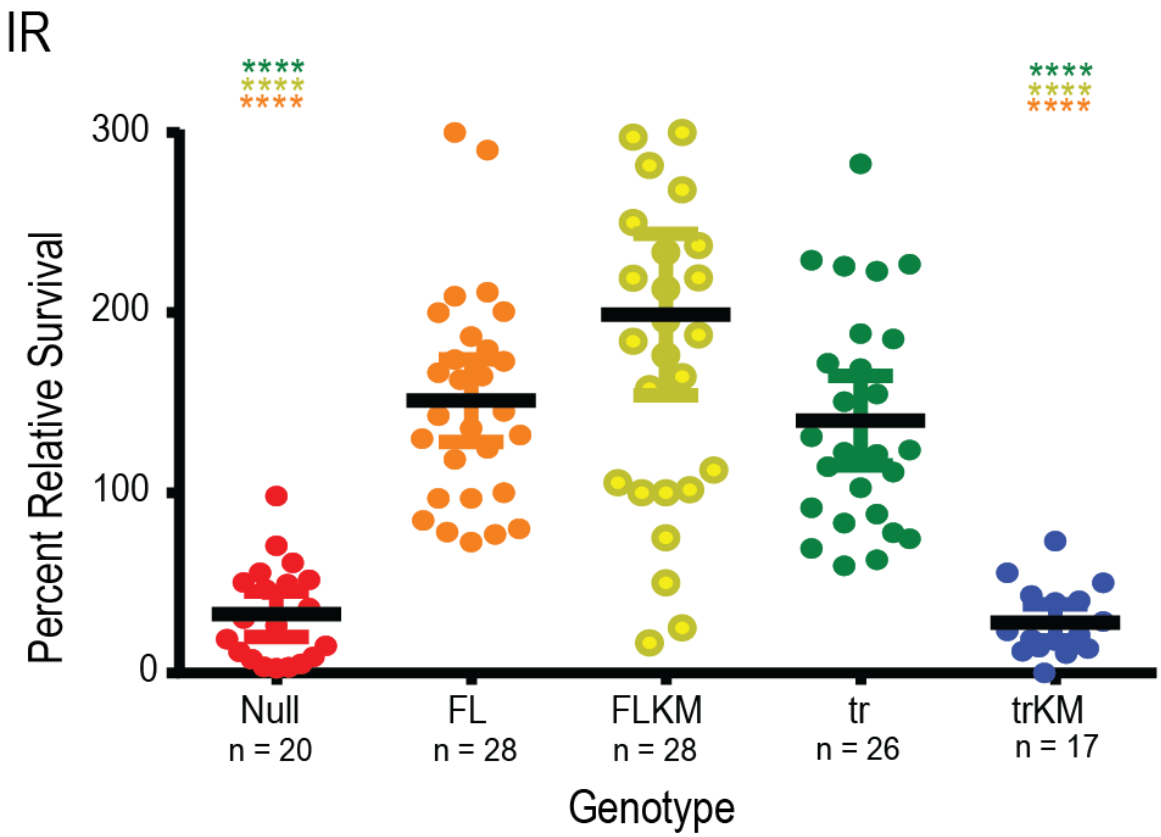


Figure 3.5- Sensitivity of Fancm to IR - Plots show the survival of the indicated phenotype relative to wildtype control flies in the same vial after exposure to IR (1500 RAD). ● Null; ● Full length (FL); ● Full length KM (FLKM); ● truncated (tr), ● truncated KM (trKM); ● Wild-type (WT). Each dot represents one vial, n measures number of vials. Mean percentage of progeny is represented by black horizontal bar. 95% confidence intervals are represented by colored error bars. Statistical comparisons were done for Fancm compared to each other genotype. Statistically significant comparisons are indicated above error bars; ****P<0.0001 by Kruskal-Wallace test, corrected for multiple comparisons.

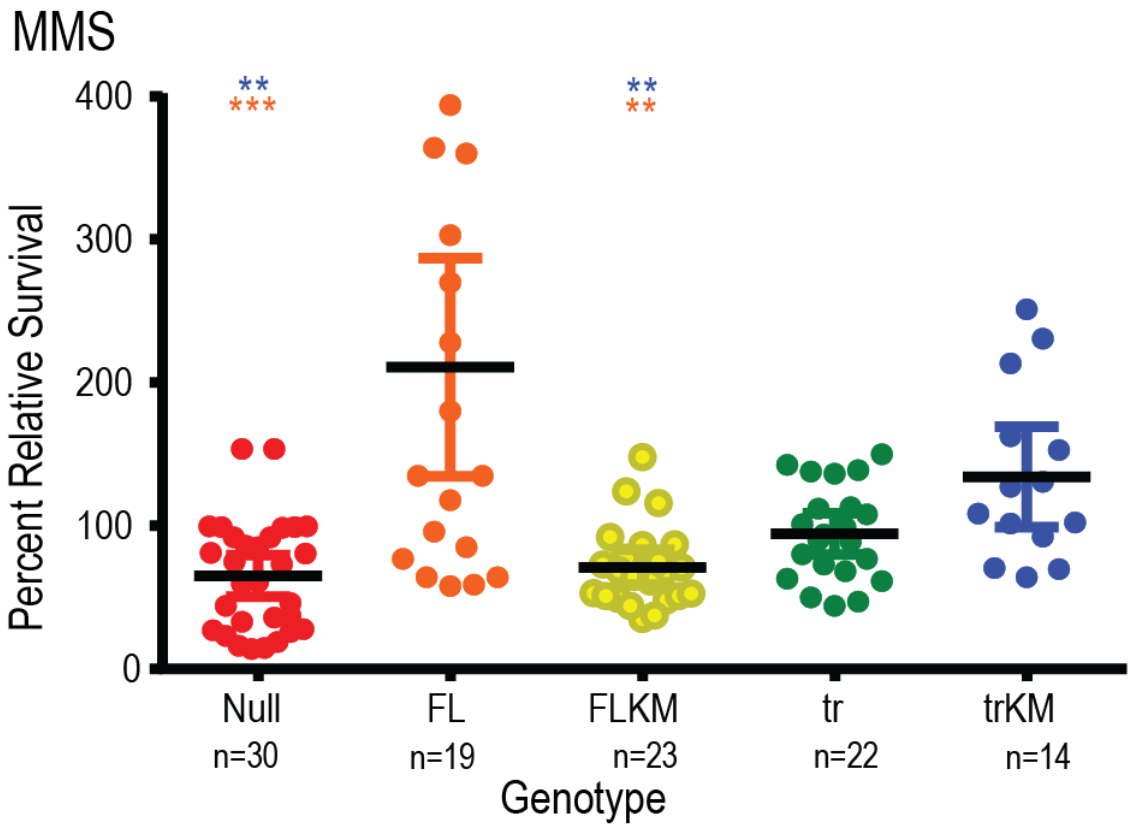


Figure 3.6 - *Sensitivity of Fancm to MMS* - Plots show the survival of the indicated phenotype relative to wildtype control flies in the same vial after exposure to 0.05% MMS (3.23 mM) ● Null; ● Full length (FL); ● Full length KM (FLKM); ● truncated (tr), ● truncated KM (trKM). Each dot represents one vial, n measures number of vials. Mean percentage of progeny is represented by black horizontal bar. 95% confidence intervals are represented by colored error bars. Statistical comparisons were done for Fancm compared to each other genotype. Statistically significant comparisons are indicated above error bars; ****P<0.0001 by Kruskal-Wallace test, corrected for multiple comparisons.

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CHAPTER 4 CONCLUDING REMARKS

Members of the FANCM family of DNA motor proteins are involved in the maintenance of genome stability. FANCM is involved in a number of processes that support the duplication and repair of DNA under a variety of conditions. While much of the work to understand the function of FANCM has been conducted in human cell lines and yeast cell cultures, the work presented here represent a novel understanding of Fancm. This work shows that Fancm is a 3' to 5' ATP-dependent DNA helicase that is limited in unwinding. This work also shows that Fancm functions during HR most likely through D-Loop displacement. Fancm also functions in crossover prevention through catalytic and noncatalytic means. Here I propose a mechanism for Fancm to function both catalytically and noncatalytically in FA dependent and independent ways.

FANCM, unlike many of the FA proteins, is highly conserved across species, although lower eukaryotes lack many other FA proteins. The simplicity of the FA pathway in organisms such as *Drosophila melanogaster*, is useful for understanding the broad and diverse roles for FANCM and provide a great model for determining how FANCM regulates repair. As discussed in chapter 1, FANCM and its orthologs are members of the SF2 helicase family and share homology in their helicase domain. The ability of FANCM and its orthologs to bind DNA is attributed to the conserved helicase domain at the N-terminus of the protein. The C-terminus, although it shows some divergence in homology between species, confers the ability of FANCM to interact with other proteins (1–6) and many motifs can be found in FANCM and its orthologs that are essential for protein-protein interaction (see chapter 3 and (7) for review).

The biochemical attributes of Fml1, Mph1, and human FANCM have been examined in detail, and these FANCM family members have been found to share several common features. All three proteins bind to ssDNA and structured DNA, with a clear preference for branched DNA structures, including the HJ, DNA fork, and D loop (8–13). As discussed in chapter 2, Fancm recognizes both single- and double-

stranded DNA with the same affinity. No clear preference was detected for branched DNA structures such as the splayed armed that was used to measure DNA binding by fluorescence anisotrophy. It is important to note that the large standard deviations make it difficult to determine clear binding preference and more DNA binding trials might help clear some of the ambiguity. This assay is also limited by the amount of protein available. The low yield of protein from purification prevents greater concentration of protein from being used in this experiment, something that is necessary to determine binding capacity and helps differentiate between binding preferences. Additional studies would be needed to determine the binding preference of Fancm.

It's important to note that DNA binding preferences for Fancm may be influenced by additional proteins. For instance, proteins that interact with FANCM and its orthologs, namely Mph1 and Fml1, also possess DNA binding capability and can influence the binding specificity of FANCM by targeting FANCM to specific DNA structures. FANCM associated with FAAP24, for instance, binds 3' flap structures and splayed arm DNA (4). As mentioned above, the N-terminal helicase domain is responsible for DNA binding in FANCM and its orthologs. The biochemical analysis conducted here represent work done using a truncated form of the *D. melanogaster* Fancm protein. The C-terminus, while not directly responsible for DNA binding, could also influence DNA binding by Fancm if the C-terminus is responsible for protein-protein interactions that affect DNA binding. Future binding studies with truncated Fancm and full length Fancm will need to be performed in order to understand Fancm's role in interacting with DNA.

As discussed in chapter 3, *D. melanogaster Fancm* appears to have catalytic and noncatalytic functions in crossover prevention. Similarly, FANCM has been shown to have diverse functions in DNA metabolism that are dependent on its translocase activity and functions that do not require this activity. FANCM's role in the repair of interstrand crosslinks in conjunction with the FA pathway seems to be to recruit other FA proteins to the site of damage, a role that is independent of its helicase/translocase activity. However, transversal of ICLs, replication fork restart, crossover prevention, and checkpoint signaling by activation of ATR require the ATPase activity of FANCM.

Kuo et al (14) examined the role of *D. melanogaster Fancm* and *Fancl* to determine if Fancm functioned independently of the *D. melanogaster* FA pathway. In testing *Fancm* and *Fancl* mutants to

DNA damaging agents, Kuo et al. found that *FancI* mutants were only sensitive to the cross-linking agent HN2, while *Fancm* mutants were sensitive to a broader range of damaging agents, suggesting that there are FA-independent roles for Fancm (14). The potential for Fancm having roles outside of the FA pathway raises several interesting questions: What prompts Fancm to initiate repair via the FA pathway versus working independently of the FA pathway? Does Fancm recruit additional repair proteins and, if so, what signals Fancm to recruit additional proteins? Are mechanisms for repair determined by Fancm and how does Fancm coordinate repair events?

The ability to function independently of the FA pathway response is not unique to FANCM as several other FA proteins function in an FA-independent manner during DNA replication (15–18). The events or signals that determine participation of FA proteins to function in the canonical FA dependent functions versus the FA independent functions are still under investigation.

FANCM's role outside of the FA pathway may be varied. FANCM has been implicated in different repair processes, replication, and recombination. As discussed in chapters 1 and 3, FANCM and FANCM orthologs function in genome maintenance through the regulation of recombination products by working to prevent mitotic crossovers, most likely through D-loop dissociation during DSB repair via HR. FANCM and the yeast orthologs Mph1 and Fml1 have also been shown to migrate Holliday junctions and dHJs (1, 9, 12, 19). This ability to work on various HR DNA intermediates suggest that FANCM functions during various steps of the HR pathway in order to ensure recombination products are resolved in a noncrossover manner.

FUNCTIONS OF FANCM IN SDSA

As seen in chapter 3, Fancm, like FANCM, Fml1 and Mph1, functions in crossover prevention during HR (1, 12, 20). This ability most likely arises from the protein's ability to dissociate D-loops (Chapter 1, (12, 19, 20)). Mazon and Symington and Kuo et al. (14, 21) proposed that Mph1 and Fancm promote noncrossover formation by acting on D-loop intermediates and directing HR down the SDSA pathway. Loss of Mph1 results in the formation of a dHJ and generates both crossover and noncrossover products through dHJ resolution (22, 23). *Drosophila Fancm* is active during SDSA, as discussed in chapter 3 (14).

Through the use of a transposable element excision-based assay, Kuo et al. (14) were able to measure Fancm's involvement in SDSA. If, as proposed, Fancm functions in a catalytic manner and can dissociate D-loops, as shown in chapter 2, then the helicase function of Fancm would be enough to rescue the effects on SDSA in the null mutant. Although genetic complications make it difficult to use the Fancm transgenes in the SDSA assay, I was able to recover a small sampling of transgenes in the SDSA assay (Table 1). The data are inconclusive as the average number of flies per vial is just above the cutoff rate to avoid bias and the number of trials is extremely low. While the data are inconclusive to determine exactly how the helicase and C-terminus influence SDSA, there is an indication that there are differences among the transgenes and between the transgenes and wild type. In order to understand the role of the helicase activity of Fancm, as well as the role of the C-terminus of Fancm in coordinating SDSA, this assay would need to be conducted using the same constructs in a different background, such as a background that doesn't contain the w^+ marker. Potentially, generating these constructs through CRISPR would eliminate the genetic interactions of the assay with the genetic background of transgenic flies and allow for examination of these mutations at the endogenous locus.

While a direct role for FANCM and its orthologs in SDSA has not been examined directly outside of *D. melanogaster,* biochemical analysis of the protein's role on SDSA intermediates, namely the D-loop, has been examined. The D-loop that forms during DSB repair via HR can either be extended through DNA synthesis or dissociated by unwinding of the invading strand after limited synthesis has been completed (24). The displacement of D-loops has been observed in FANCM, Mph1, Fml1, and *D. melanogaster* Fancm, (Chapter 2)(1, 9, 12). Dissociation of the D-loop indicates that these proteins may be using SDSA as a means of promoting noncrossover recombination products. FANCM functioning during SDSA is just one potential role for FANCM in DSB repair by HR. For instance, FANCM, Mph1 and Fml1 have been shown to regress DNA replication forks and catalyze branch migration (1, 9, 19, 25, 26), an action fueled by the hydrolysis of ATP, and loss of FANCM and its orthologs in human, mouse and chicken cells, leads to an increase in sister chromatid exchanges, indicating that FANCM functions to prevent crossovers, a function that requires the DNA ATPase activity of FANCM as well as FANCMs interaction with its partner proteins, namely FAAP24 and MHF(8, 27–29).

Drosophila Fancm is also implicated in having multiple roles during HR. Based on the crossovers that are seen in the tr and FLKM transgenic flies, there is a probability that Fancm interacts with Blm to prevent crossovers during HR, most likely during SDSA. Previous study (14) of *Blm Fancm* double mutants showed that spontaneous mitotic crossovers were slightly reduced compared to *Blm* single mutants. This indicates that a subset of repair intermediates requires Fancm's presence to recruit and direct Blm toward NCO repair outcome. When Fancm is not present then repair occurs in a Fancm-independent/Blm-independent manner, which may or may not be through HR. If this were the case, no NCO or CO product are formed, thus explaining the decrease in crossovers in the double mutant. Unfortunately, Kuo et al. (14) were unable to conduct the SDSA assay in *Blm Fancm* double mutants as they were unable to recover a *Blm Fancm* double mutant in the assays' genetic background. It would be interesting to determine how Fancm and Blm interact during SDSA. One experiment of interest would be to see if the FL transgenic Fancm fly can rescue the apparent lethality of *Blm Fancm* mutant in this background. Rescue by FL *Fancm* would indicate that Blm and Fancm both function during SDSA and that the damage or breaks that are occurring by the transposase are toxic when Blm and Fancm are missing.

PROTEIN-PROTEIN INTERACTIONS

One aspect of crossover control in which Fancm may function is with the Blm complex. The human BTR complex is composed of BLM, Topo IIIα, and the heterodimer of RMI1 and RMI2. This protein complex catalyzes branch migration of HJs, leading to the decatenation of dHJ and results in a noncrossovers product (30–32). The BTR complex is part of the the BRAFT supercomplex, a higher-order complex between the BTR complex and the FA core complex. Key in the recruitment of the BTR complex to ICLs is FANCM (6, 33). As discussed in chapter 1 and chapter 3, FANCM contains two motifs, MM1 and MM2, that are responsible for FANCM's interaction with the BTR complex (MM2) and the FA core complex (MM1). Deletion of either of these motifs increases levels of SCE (27, 34, 35).

In *Drosophila,* Fancm and Blm share many similarities and in some cases, such as crossover control, Fancm is epistatic to Blm (14). In yeast the BTR complex, referred to as the STR complex, has an anti-crossover role that is different from the role of Mph1 or Fml1 in crossover prevention, and double

mutants of *mph1* and *SGS1*, the *Blm* ortholog, are synthetically lethal (36–40). While *D. melanogaster* Fancm does not have MM1 or MM2 (Chapter 1,3), genetic interaction with *Blm* indicates potential for these two proteins to be interacting during repair processes. Further analysis of *Fancm* and *Blm* would be interesting in determining the mechanism for which the two proteins function to prevent mitotic crossovers. *In vivo* genetic analysis comparing crossover rates and sensitivity to DNA damaging agents between double mutants of *Blm Fancm* transgenic flies to a *Blm Fancm* null fly, would be useful in determining the relationship between Fancm and Blm in HR and whether the helicase function of Fancm is necessary for this interaction. Additionally, biochemical examination, discussed later in this chapter, of Fancm to determine proteins that it may interact with could provide insight into the role of Fancm in DNA metabolism.

As mentioned above, the anticrossover role of FANCM is facilitated by its interaction with its partner proteins FAAP24 and MHF (8, 27–29). Additionally, this anticrossover feature is independent of other FA proteins (7, 28, 41, 42) and it has been proposed that FANCM, through its ATP hydrolysis activity, acts in a FA-independent manner to directly regress a stalled or blocked replication fork (9, 25) allowing for the restart of the stalled fork (43), a process which is facilitated by the interaction of FANCM with MHF (7, 44–47).

MHF is a histone fold complex that partners with FANCM and Fml1 to enhance FANCM's DNA interaction and function. MHF is a dimer composed of the histone-fold proteins MHF1 and MHF2 (48–50). Mutations in MHF affect the stability of FANCM and recruitment of FA core complex members to damaged chromatin. Cells deficient in MHF are prone to chromosome rearrangements when exposed to damage. Human MHF binds to various DNA structures on its own and is believed to promote FANCM binding to various DNA structures to assist in replication fork reversal and branch migration (7, 47, 51) although the exact role of DNA binding regulation by MHF is still under investigation. MHF in *S. pombe* also displays DNA binding activity that is thought to aid Fml1 in ICL repair, replication fork repair, and crossover regulation (3, 7). *S. cerevisiae* MHF, unlike the orthologs in *S. pombe* and humans, has not been shown to have DNA binding ability nor does it affect the DNA binding capability of Mph1 (52).

Genetic interaction between *S. cerevisiae* MHF and Mph1 suggests that MHF assists Mph1 in DNA damage repair even though no DNA binding has been noted for MHF in *S. cerevisiae* (53). This interaction helps combat the regulation the Smc5/6 complex exhorts on Mph1. Smc5/6 is a conserved SMC family complex that functions in the regulation of recombination intermediates. Smc5/6 interacts with the C-terminal region of Mph1 to regulate Mph1's recombinogenic activity (10, 54). For instance, Smc5/6 inhibit Mph1's replication fork regression *in vitro* without affecting Mph1's function in D-loop dissociation (10). Such a response indicates that Mph1 is regulated to prevent fork regression which can lead to unnecessary recombination and only employs the fork reversal function of Mph1 in instances where template lesion cannot be overcome by other means, such as translesion synthesis during ICL repair.

ICLs, which block the progression of the DNA replication fork, are removed during replication via the FA pathway. FANCM works with its two obligate protein partners, FAAP24 and MHF to recognizes different DNA structures and provides a platform for targeting other FA proteins to the site of the ICL (4, 47, 55). It has been suggested the FANCM and MHF promote traversal across ICLs, a process that requires the DNA binding ability of both FANCM and MHF as well as the DNA motor activity of FANCM. This ability to traverse across ICLs suggest that DNA synthesis continues past ICLs, leaving the lesion to be removed by another repair mechanism post-replication (Figure 1.4). Although the FA pathway is believed to play a critical role in removal of ICLs, this mechanism of ICL transversal suggests that FANCM functions independently of the FA pathway and may function in multiple pathways. Although the exact mechanism for traversal of ICLs by FANCM is still unknown, this ability is preserved in several organisms (56).

FANCM, Mph1, and Fml1 often depend on partner proteins to execute certain biological functions. This is best illustrated by the role of FANCM in the FA pathway. The FANCM/FAAP24/MHF complex interacts with FANCF to recruit additional members of the FA core complex to sites of DNA lesions in the form of ICLs (6, 27, 57–60). Although Mph1, Fml1, and a number of other lower eukaryotic organisms lack members of the FA pathway aside from FANCM, multiple interactors have been identified for having a role in the function of FANCM. These interactions influence the function of FANCM family

members in a variety of ways and the differences in function and interactors between organisms likely indicate that there are specific maintenance needs.

FANCM functioning in multiple processes is supported by the many attributes that FANCM possesses such as DNA binding ability, a conserved helicase domain, and protein interaction motifs. In addition to functioning in ICL removal and traversal, FANCM also has a role in DNA replication under both normal growth conditions and under periods of DNA stress and damage, such as exposure to UV and DNA damaging agents like MMS and cell culture studies of FANCM indicate that the ATPase activity of FANCM is essential for fork restart and to prevent replication fork stalling (28, 45, 46, 61, 62).

Fml1 and Mph1, the yeast orthologs of FANCM, are also involved in DNA replication, a role that is highlighted under periods of DNA stress. These proteins have the ability to catalyze replication fork regression (1, 19) and have been proposed to regress blocked replication forks and funnel blocked replication forks into an HR repair pathway (54, 63, 64).

The protein-protein interactions described above provide insights into the function of FANCM. The interaction between FANCM and proteins that are both associated with the FA complex and not part of the FA complex suggests that FANCM functions in both FA-dependent and FA-independent ways. The C-terminus of FANCM is key in facilitating many of these interactions. As detailed in chapter 1 and chapter 3, the C-terminus of *D. melanogaster* Fancm is highly divergent from human FANCM. Many of the motifs and domains that are essential for protein-protein interaction in FANCM are missing in *D. melanogaster* Fancm. In part, this might be a result of missing interacting partners. For instance, *D. melanogaster* Fancm is missing MM1 and MM2 motifs but *Drosophila* also lack Fancf and RMI1, the proteins that interact with FANCM through these domains (Chapter 1, 3). Determining the proteins that interact with Fancm will therefore enhance our understanding of the role of Fancm in DNA metabolism.

Immunoprecipitation (IP) studies and peptide mass fingerprinting using MALDI-MS will help identify proteins that are associated with Fancm. If, as predicted, the C-terminus is essential to protein-protein interactions, full length Fancm will be needed to identify any potential protein interactors. Using *Drosophila* S2 cells, truncated Fancm and full length Fancm can be expressed with an affinity tag, either through overexpression or through CRISPR mediated recombination. A major advantage of using S2

cells is that cells can be treated with DNA damaging agents such as HN2, which induces ICLs, and IR, which induces DSBs. Treatment of S2 cells with DNA damaging agents can help in determining proteins that interact with Fancm in response to different types of DNA damage and determine which proteins function with Fancm in an FA-dependent role and FA-independent role of Fancm. Comparison of truncated Fancm to full length Fancm will allow for the identification of proteins that specifically interact with Fancm through the C-terminus. Additionally, the use of ATPase mutants in these assays can provide insight into whether the helicase/translocase function of Fancm is essential to any protein interactions.

CHECKPOINT SIGNALING

Proteins involved in HR are just one set of proteins that are predicted to interact with Fancm. The biological function, regulation and collaboration with other proteins by FANCM and its orthologs have provided insights into various DNA metabolic processes in which FANCM functions. Functions of FANCM have been linked to ATR-mediated DNA damage checkpoint (15, 65, 66). Activation of this pathway helps stabilize replication forks and inhibits replication origin firing (67, 68). Once again this function of FANCM is independent of the FA pathway but does require FANCMs interaction with FAAP24 and association with other checkpoint factors, namely CHK2.

FAAP24 assists in targeting the FA core complex to sites of ICLs and works with FANCM to engage ATR-mediated checkpoint signaling. FAAP24 and FANCM interact with HCLK2 kinase, an ATR-ATRIP associated protein required for S-phase checkpoint activation. This interaction generates long stretches of ssDNA, recruits RPA, which is a DNA stress signal that triggers ATR activation, and helps stabilize Chk1 (46, 69–71). The molecular mechanisms through which FANCM/FAAP24 triggers ATR activation under cellular stress situations is still under investigation. While FAAP24 is not found in *D. melanogaster,* Fancm may still function in ATR signaling, discussed later in this chapter.

Although FANCM works upstream of the ATR checkpoint, it is also dependent on ATR. ATR is responsible for FANCM phosphorylation in response to DNA stress. The phosphorylation of FANCM by ATR is important in the localization of FANCM to sites of ICLs and subsequent recruitment of the FA core components. The activation of the FA pathway results in the ubiquitination of FANCD2/I and leads to recruitment of repair proteins and the activation of the ATR checkpoint by CHK1 (72). The fact that

FANCM participates in ATR activation and is also acted upon by ATR suggest that there is a feedback loop between the two. Although the exact details of this connection is still unknown, it's an important connection in understanding the regulation of FANCM. Although FANCM is implicated in ATR checkpoint signaling, this may not be the case for all orthologs of FANCM. Loss of Mph1 for instance, does not reduce ATR checkpoint function like it's human counterpart (73). Examination of a potential interaction between Fancm and the ATR checkpoint pathway in *D. melanogaster* would provide insights into regulation of the DNA damage response by Fancm.

POSTTRANSLATIONAL MODIFICATIONS

Not only is FANCM phosphorylated in response to DNA stressors, but it is also phosphorylated in the absence of genotoxic stress. A large impact on the phosphorylation of FANCM is a result of cell cycle regulation. FANCM phosphorylation rises as cells proceed from S phase into the mitotic cycle and declines after mitotic exit (74). Phosphorylation of FANCM is mediated by the Polo-like kinase, PLK1, and leads to degradation by the multi-protein containing E3 ubiquitin ligase, SCF (Skp-Cullin-Fbox) (75). This programed degradation of FANCM provides a level of regulation of FANCM and allows for release of FANCM/FA core complex from chromatin during mitosis.

Cell cycle regulation of Fancm provides an interesting avenue of research. How *D. melanogaster* Fancm is regulated to coordinate repair, if it experiences any post translational modifications, and its' response to genotoxic stress and interaction with ATR, are all potential areas of study. Cell cycle profiling for Fancm to measure its activation and degradation, whether or not PTMs are responsible for its role in cell cycle function, and determining what kinases are involved in regulation the activity of Fancm throughout the cell cycle.

Based on known modified residues from human FANCM we can look for key residues that might susceptible to phosphorylation and generate phosphorylation mutants. One potential phospho-mutant to generate would be to target the ATR phosphorylation site. FANCM is phosphorylated by ATR at serine 1045 (76). Using COBALT protein alignment, serine 946 shares similarities with FANCM S1045 (Figure 4.1). By targeting this residue through CRISPR-mediated mutation we can measure the production and degradation of Fancm throughout the cell cycle, and using cell based immunoassays to measure protein

phosphorylation in the cell, we can determine when and if endogenous wild-type Fancm is phosphorylated and then compare that cell expression to potential phospho-mutants. Western blots using phospho-specific antibodies can help determine if Fancm is being phosphorylated or use of fluorometric detection or colorimetric detection systems could also be employed using the *Drosophila* S2 cell line.

It will be interesting to test whether other kinases are also involved in regulating the presence and activity of Fancm throughout the cell cycle and also determine how dephosphorylation is achieved after mitotic exit. For instance, while the known serine target for PLK1 in human FANCM (DSGXXS) (77) is not found in *D. melanogaster* Fancm, another predicted target sequence (78, 79) provides a potential target for a phospho-mutant. Using a web based tool for identifying phosphorylation sites (80), we can identify potential residues of Fancm that may be modified by kinases.

ROLE FOR FANCM DURING DNA REPLICATION

In addition to FANCM's regulation in the cell cycle by PLK1, the capability of FANCM to transverse ICLs suggests a role for FANCM during DNA replication (56, 81). One potential area of exploration for *D. melanogaster* Fancm is in replication. In examining the transgenic flies of *Fancm,* it was noted that the truncated KM (trKM) exhibited a slight delayed growth phenotype (Chapter 3). The trKM, having neither helicase function, nor a C-terminus, does not act as a wildtype copy would. Presence of this protein on DNA would cause a delay in replication as the cellular machinery would need to find alternative means to proceed in a non Fancm dependent manner. This delay is not seen in the null mutant as no Fancm protein is present and therefore replication machinery would already be functioning in a non Fancm dependent manner. If, as predicted, Fancm functions during DNA replication and the trKM causes a delay in replication, then replication tracts in the null mutant should proceed as wild type while the trKM mutants would have shorter replication tracts for the same duration of time.

The use of DNA combing analysis (82, 83) will allow for the visualization of how replication proceeds in these genetic backgrounds. Since this analysis can be conducted using whole flies, the genetic background describe here can be preserved. This analysis be performed in a 'normal' environment and in a 'toxic' environment. Treatment with DNA damaging agents, as described in Chapter 3, prior to DNA combing will enable us to determine how Fancm influences replication during DNA stress.

This is especially important as defects in the development of the eye were seen in the FL, FLKM, and tr transgenic fly when treated with HN2 or IR (Figure 4.2 A and B). This defect was not detected in the null nor the trKM fly. The defect was more prevalent in the tr and FLKM genotype than the FL genotype; 3% compared to less than 1% of total progeny.

As neither the tr or FLKM transgenic flies function as a true wild type, as seen in the level of crossover prevention in Chapter 3 (Figure 3.3), there's a possibility that there is a delay in replication caused by the inability of the protein to function in both catalytic and noncatalytic means. The slight defect seen in the FL transgenic fly of the FL transgene indicates that the FL transgene may not being expressed at wildtype levels. The inability of the tr and FLKM to perform all functions of a wildtype Fancm result in an inability of the protein to either recruit additional proteins or function as helicase. When either mutant protein encounters damage that requires both the catalytic and noncatalytic functions of Fancm, a delay in repair and a slowing of mitotic division occurs. As the eye is a highly prolific tissue, a delay or defect in mitotic division would be more noticeable than in other tissues. The restart or redirection of repair down an alternate pathway is enough of a delay that development is affected. While the eye defect we see in the FL is most likely response to a delay in repair or just general lack of abundance of Fancm, and not an indicator that there's a defect in DSB repair, the high incidence of defect found in FLKM and tr when treated with HN2 or IR, both of which can induce DSBs, is most likely because there is an inability to regulate repair via HR.

Taken together with crossovers seen FLKM and the tr transgenic flies (Chapter 3), these data indicate that Fancm functions in multiple capacities to maintain genome stability. Fancm is able to recognize damage and facilitates the recruitment of other proteins to the site of damage. Depending on the type of damage, the pathway of repair, or the protein(s) recruited, the motor ability of the protein may or may not be required. The crossovers seen in the tr and FLKM transgene could indicate that the inability of the protein to either recruit additional proteins or function as helicase causes a delay in repair and a slowing of mitotic division. In this manner, Fancm may not be catalytically involved with the repair but serves to recruit other proteins to facilitate repair. If the damage is more severe or requires Fancm catalytic activity to proceed with repair, repair stalls or slows, as seen in the FLKM mutant when the ATPase activity of the protein is defective. Taken together with the crossovers seen in these mutants, the

data indicate that a subset of damage that can result in a crossover product are dependent on both the ATPase activity of Fancm, as well as the C-terminus.

SUMMARY

The data presented throughout this work describes Fancm as a 3' to 5' ATP-dependent DNA helicase that is capable of unwinding short tracts of DNA. Genetic analysis of mutants of Fancm show that the catalytic function of Fancm as well as C-terminus of Fancm, is essential for the crossover prevention and DNA damage repair response. The need for the catalytic function of Fancm in crossover prevention indicates that Fancm functions in unwinding of HR DNA intermediates, most likely in the form of D-loops. The need for the C-terminus in crossover prevention indicates that Fancm is involved in coordinating repair through the recruitment of repair proteins. Both of these functions are necessary for full function of Fancm in the diverse roles the protein has in DNA metabolism.

The work described here provides a good framework for understanding the biochemical activities, of Fancm in DNA damage and repair via HR. Many important questions regarding this protein remain. Does Fancm function during replication? Is Fancm activated by checkpoint signals? Does Fancm influence checkpoint activation? How does Fancm coordinate repair and replication? What proteins does Fancm interact with? Ongoing studies examining the genetic, and biochemical activity of Fancm will provide insights into the role of Fancm in DNA metabolism.

FIGURES

Table 4.1 - *Percent progeny recovered from SDSA assay*

Genotype	SDSA	Precise/No	Aberrant	Avg/vial	Vials
Tr	6.1%	79.8%	10.5%	16	15
trKM	2.0%	69.7%	27.1%	12	15
FLKM	4.2%	64.5%	4.0%	12	11
FL	3.5%	90.9%	2.6%	15	14
Null	2.8%	88.2%	9.0%	N/A	N/A
WT	5.4%	84.7%	9.9%	N/A	N/A

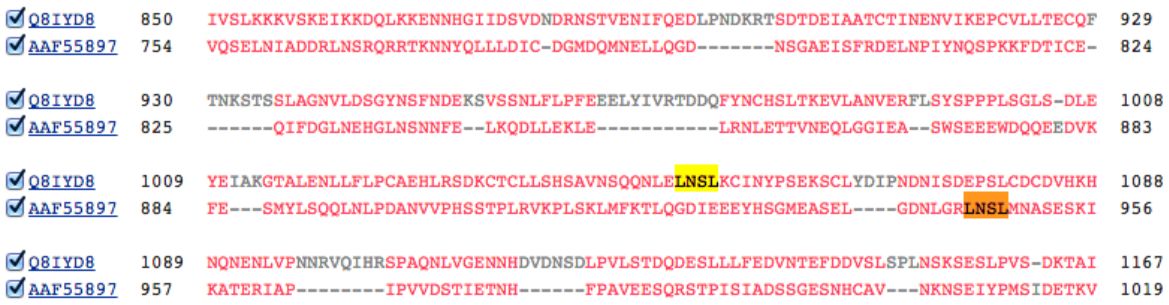

Figure 4.1 - *COBALT alignment and predicted phosphorylation site* - Protein sequence alignment of human FANCM (Q8IYD8) and *D. melanogaster* Fancm (AAF55897) by COBALT alignment program. Known phosphorylation sequence of FANCM, serine 1045, is highlighted in yellow. Predicted phosphorylation sequence of *D. melanogaster* Fancm is highlighted in orange.

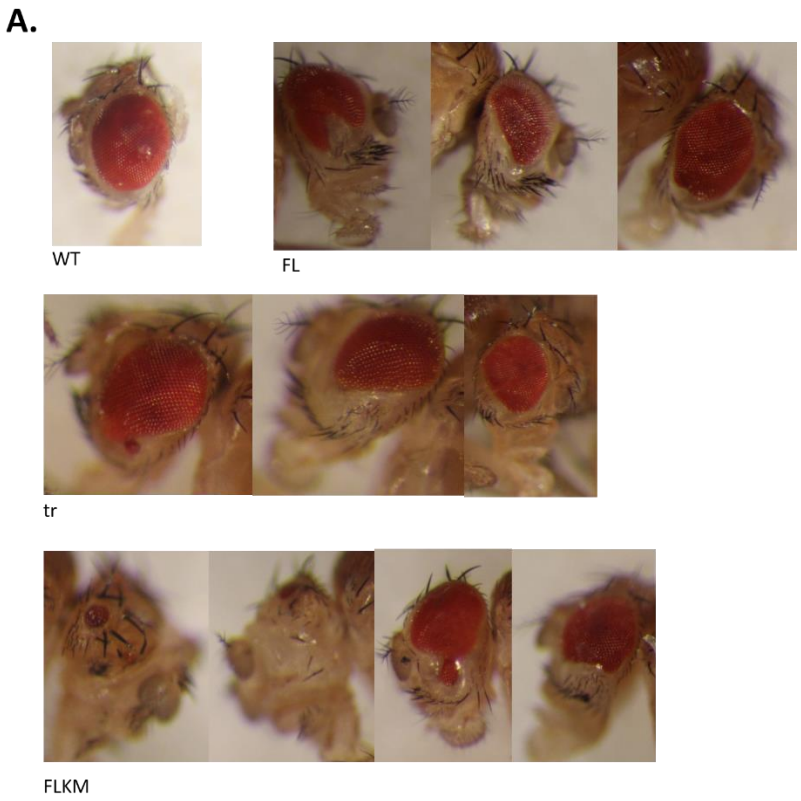

Figure 4.2 - Development defects seen in transgenic flies when exposed to DNA damage - A.) Treatment with 0.002% HN2 (0.1 M). B.) Treatment with IR (1500 RAD). Defects in eye development seen in st Fancm^{0693}/w+Fancm^{transgene} Sb Fancm^{Del}. Transgenic genotype is located under photos.

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