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Biochemical activities and genetic functions of the *Drosophila melanogaster* Fancm DNA helicase in DNA repair

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ABSTRACT

Repair of DNA damage is essential to the preservation of genomic stability. During repair of double-strand breaks, several helicases function 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 important in the response to various types of DNA damage and has been suggested to prevent mitotic crossovers during double-strand break repair. The helicase activity of FANCM is believed to be important in these functions, but no helicase activity has been detected *in vitro*. We report here a genetic and biochemical study of *Drosophila melanogaster* Fancm. We 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, we found that there are helicase-independent and C-terminus-independent functions in responding to DNA damage and in preventing mitotic crossovers.

INTRODUCTION

DNA helicases are a diverse group of enzymes that separate the two strands of duplex DNA. Using the free energy derived from the hydrolysis of a 5' -nucleoside triphosphate, generally ATP, the helicase catalyzes the unwinding of duplex DNA to yield single stranded DNA (ssDNA), a process that is required in replication, transcription, recombination, and repair. Thus, helicases are involved in essentially all metabolic pathways that require the separation of duplex DNA (Brosh 2013; Khan *et al.* 2015).

Helicases exhibit a diversity of structure and mechanism that may be related to the often unique and specialized roles that these enzymes can play in the cell (Brosh 2013; Daley *et al.* 2013). Importantly, distinct helicases can interact with specific DNA substrates. For example, during repair of DNA damage different helicases often act within particular pathways and on unique DNA intermediates that are generated as repair progresses, such as Holliday junctions (HJs) or displacement loops (D-loops). This can be observed in the requirement for helicases to recognize and act on specific DNA structures during the process of double-strand break (DSB) repair via homologous recombination (HR).

DSB repair by HR is a complex process with several key events: resection of the 5' end at the strand break; invasion of the Rad51-coated 3' ssDNA tail into a homologous duplex sequence, generating a displacement loop (D-loop); DNA synthesis primed from the invading 3' end; and resolution into one of either two types of recombination product - crossovers (COs) or non-crossovers (NCOs). The formation of COs during DSB repair in mitotically dividing cells can be hazardous as they can result in loss of heterozygosity and gross chromosomal rearrangements (Lorenz and Whitby 2006; Andersen and Sekelsky 2010). Therefore, prevention

of CO pathways through the activation and promotion of NCO pathways is favored in mitotic cells undergoing HR to ensure genomic stability.

To prevent CO generation, 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 described above. After synthesis, the invading strand can be unwound from the template and annealed to the other resected end, resulting in a NCO, a process known as synthesis-dependent strand annealing (SDSA) (Adams *et al.* 2003). Alternatively, the displaced strand can anneal to the other resected end, 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 (Daley *et al.* 2013). 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 repair (Andersen and Sekelsky 2010; Heyer *et al.* 2010; Daley *et al.* 2013).

One family of conserved DNA helicases/translocases whose members are involved in HR regulation is related to archaeal Hef (Helicase-associated endonuclease for fork-structured DNA) (Komori *et al.* 2002; Prakash *et al.* 2009; Zheng *et al.* 2011; Lorenz *et al.* 2012). *Pyrococcus furiosus* Hef contains a conserved DEAD-box helicase domain and an ERCC4 C-terminal endonuclease domain. 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 (Komori *et al.* 2004). The domain structure of Hef is similar to that of the eukaryotic structure-specific endonucleases MUS81 and XPF, but they have inactive helicase domains (Nishino *et al.* 2005). Conversely, in Fanconi anemia group M (FANCM) , the nuclease domain is inactive (Meetei *et al.* 2005; Ciccia *et al.* 2007; Ciccia *et al.* 2008).

Mutations in *FANCM* cause Fanconi anemia (FA), a hereditary disorder characterized by an increased incidence of cancer, developmental abnormalities, and bone marrow failure (Meetei *et al.* 2005). 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. The primary role of FANCM appears to be to target disrupted replication forks and promote CO avoidance by processing DNA intermediates that occur during DSB repair via HR (Prakash *et al.* 2005; Prakash *et al.* 2009; Nandi and Whitby 2012).

The *S. cerevisiae* FANCM ortholog, Mph1, has also been shown to be involved in preventing crossovers (Prakash *et al.* 2009), and *mph1* mutants show sensitivity to DNA damaging agents such as ionizing radiation (IR) and methyl methanesulfonate (MMS) (Scheller *et al.* 2000). Biochemical studies using purified Mph1 show that it is a 3' to 5' DNA helicase capable of unwinding Rad51-coated D-loops (Prakash *et al.* 2005; Prakash *et al.* 2009), and that it can process DNA intermediates that form later in repair, including HJs (Prakash *et al.* 2005; Prakash *et al.* 2009; Kang *et al.* 2012). Unwinding of HJs and D-loops has also been observed using the *S. pombe* ortholog Fml1 (Sun *et al.* 2008). In contrast, no helicase unwinding activity has been detected for human FANCM (Meetei *et al.* 2005; Gari *et al.* 2008). 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 (Prakash *et al.* 2009; Lorenz *et al.* 2012; Mazón and Symington 2013; Mitchel *et al.* 2013; Kuo *et al.* 2014).

FANCM and orthologs may also have roles that are not dependent on catalytic activity. The C-terminus of human FANCM, like its Hef ancestor, has an ERCC4-like endonuclease domain. Although this domain is considered to be catalytically dead, it is involved in protein-protein interactions (Huang *et al.* 2010; Wang *et al.* 2013; Yang *et al.* 2013). Yeast and human FANCM have several motifs in the C-terminus that facilitates interaction with chromatin, additional FA proteins, and repair complexes (Deans and West 2009; Vinciguerra and D'andrea 2009). 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 (Deans and West 2009). While these two motifs are not detected in yeast 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 (Kuo *et al.* 2014). This study tested the response of Fancm in CO prevention and response to DNA damaging agents. To better understand the role of the 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*. We generated Fancm ATP hydrolysis mutants *in vivo* to examine the role of the helicase in responding to DNA damage and CO prevention. We also sought to understand the role, if any, of the C-terminus of Fancm in regulating repair events in *Drosophila*. We 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 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. *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.

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 S1) 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 S1). The protein expression plasmid was maintained in *E. coli* BL21DE3/pLysS and protein expression was induced by auto induction (Studier 2005; Studier 2014). Bacterial cultures were grown in three liters of ZYM5052 auto induction medium (Studier 2005) 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 S1) 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, isolated by centrifugation, was 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 S1) 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 $MgCl_2$. For ATPase reactions that used dsDNA, pET15b plasimid, cut with the restriction enzyme HpaI, extracted using phenol:chloroform, and precipitated with NaOAC, was used. 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 S1), 25 mM Tris-HCl (pH7.5), 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 S1), 25 mM Tris-HCl (pH7.5), 3 mM $MgCl_2$, 20 mM NaCl, 5 mM 2-mercaptoethanol (βME) and 10 µg/mL bovine serum albumin. 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.

Drosophila stocks

Fly stocks were maintained at 25°with standard medium. *Fancm^{0693}* is a nonsense mutation previously described in Kuo et al. (2014). Deletion of endogenous *Fancm* (*Fancm^{Del}*) was generated using CRISPR/Cas9 technology (Bassett *et al.* 2013; Gratz *et al.* 2013). Oligonucleotides (IDT) used for guide RNA (Del1 and Del2, Table S1) 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},* expressed under the endogenous *Fancm* promoter. Plasmid used for injections of transformants were generated from a PCR-amplified genomic fragment (F1 and F2, Table S1). The K84M mutation was introduced using QuickChange Site-Directed Mutagenesis kit (Agilent Technologies) and Primer KMQC (Table S1). 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 S1) from original construct. *Fancm$^{1-645K84M}$* was generated in the same way, using the FLKM construct. RT PCR (Qiagen) was used to determine expression (Figure S1) using primers RT (Table S1).

Mitotic crossover assay

Mitotic crossovers were measured in the male germline as previously described (Mcvey *et al.* 2007), 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 (Yıldız *et al.* 2004). 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), larvae were exposed to gamma rays in an irradiator at 1500 rad. At least 10 biological replications 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

Fancm is a ssDNA-dependent ATPase

Previous genetic studies of *Drosophila Fancm* indicated a role for the protein in SDSA and in preventing mitotic crossovers (Kuo *et al.* 2014). To further understand roles of Fancm in DNA repair, we investigated the biochemical properties of purified Fancm. SF2 helicases, including Fancm, are characterized by several conserved motifs, including a Walker A motif that binds the triphosphate tail of ATP and consequently plays a role in ATP hydrolysis (Walker *et al.* 1982; Koonin 1993). We were unable to express and purify full-length Fancm, so a truncated form of Fancm (FancmΔ) and a truncated form with a mutation in the Walker A motif (FancmΔ^{KM}) were overexpressed as His6x-MBP tagged proteins (Figure 1A) in *E. coli* and each was purified to near homogeneity (Figure S2). This truncation was generated to encompass the entire helicase domain and is based off of purified truncations of the fission yeast ortholog, Fml1 (Sun *et al.* 2008).

We confirmed the ATPase activity of purified FancmΔ and measured several biochemical parameters to characterize this activity (Figure S3). 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 1B). 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 1B). 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 (Figure 1A). ATPase activity stimulated by ssDNA as well as dsDNA has been reported for human FANCM and yeast Fml1, while Mph1 only exhibits ssDNA-dependent activity (Meetei *et al.* 2005; Prakash *et al.* 2005; Nandi and Whitby 2012). Fluorescence anisotropy was used to determine if differences in ATPase stimulation were a result of DNA binding (Figure S4). No significant differences in binding to ssDNA versus dsDN A were detected for the truncated protein.

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 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 1C, lane 3). There was no detectable unwinding of the substrate at an equal concentration of mutant protein FancmΔ^{KM} (Figure 1C, 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 1D). Fluorescence anisotropy was used to determine if there was a difference in binding of FancmΔ to these structures. No significant difference in binding affinity was detected when Fancm was incubated with partial duplex structures with either a 3' or 5' -ssDNA tail (Figure S4), indicating that unwinding of the protein is a result of a directional bias and classifies Fancm as a 3' to 5' helicase, consistent with previous work on orthologs (Prakash *et al.* 2005). These data also 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.

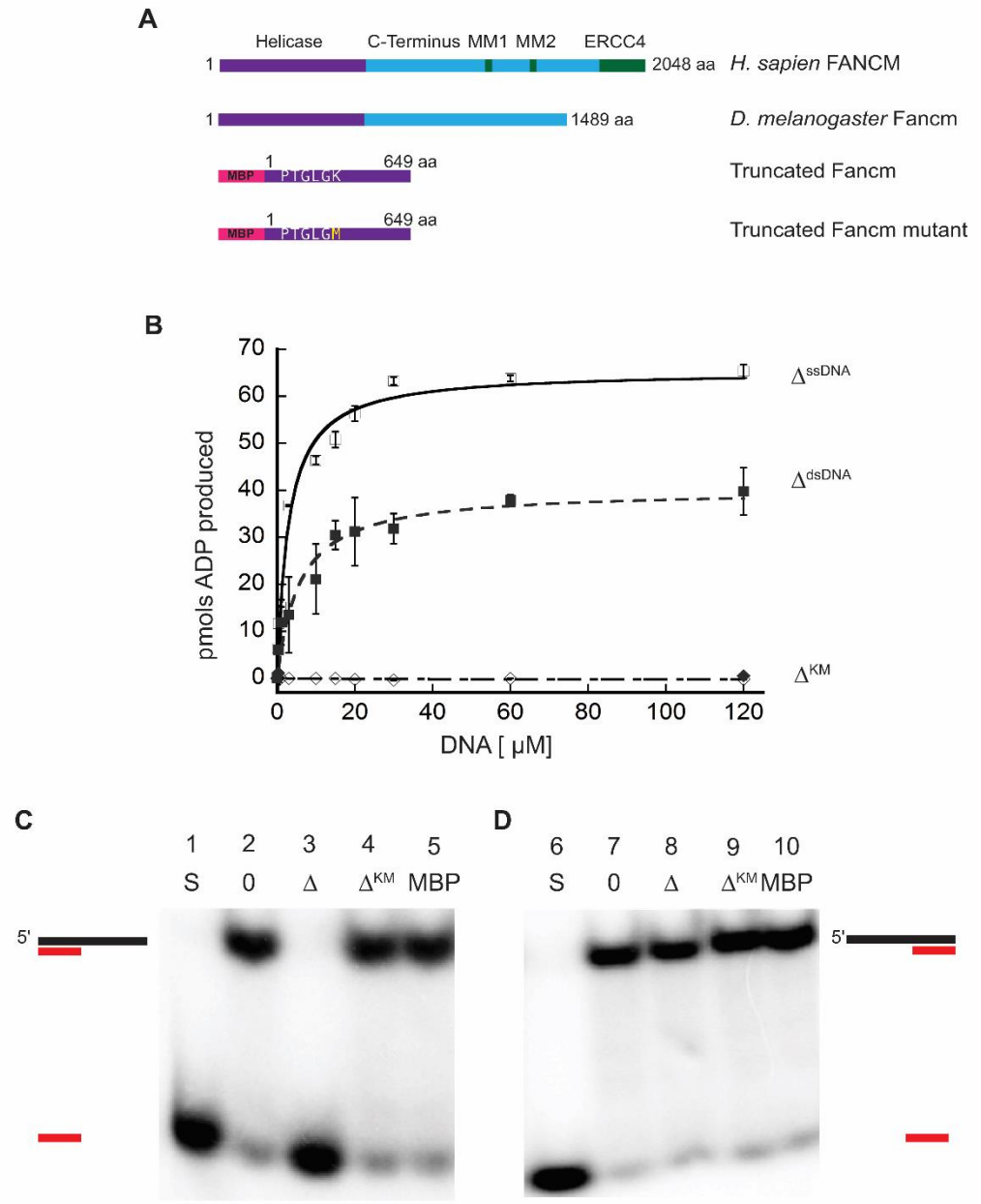


Figure 1- *Drosophila Fancm is a 3' to 5' DNA helicase dependent on ATP hydrolysis.* A.) Schematic of Fancm. Domains and motifs present in human FANCM are marked. Conserved domains or motifs in *Drosophila melanogaster* are noted. Truncated forms depicted are with an N-terminal MBP tag. B.) ATP hydrolysis 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). C.) Fancm unwinds duplex DNA. Protein (212 nM) was incubated with a 5' radiolabeled 15 bp partial duplex with a 25 nucleotide 3' overhang (15/40). D.) 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); FancmΔ^{KM} (lane 4 and 9, ΔKM); maltose binding protein (MBP) (lane 5 and 10, MBP). Colored strand represents radiolabeled strand. Substrate oligonucleotides are in Table S1.

As shown in Figure S5**,** 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 has limited unwinding capability

Further testing of the helicase activity of 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 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 2A). 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 2A). We also measured unwinding activity using two splayed-arm substrates, one with a 3' -single stranded region of 25 bp, and one with a 3' -single stranded region of 20 bp; both substrates had a 15 bp duplex region. Both substrates were completely unwound, indicating that neither the length of the 3'-tail nor the complexity of the substrate affects unwinding (Figure S6A). An additional splayed arm substrate with a 25 bp duplex region and 25 nt 5' and 3' -ssDNA arms was also tested (Figure 2A), with no detectable unwinding.

To test if the initial rate of the reaction or the duration of the reaction effected unwinding, unwinding for each substrate was determined using 10 nM and 150 nM protein at various time points for the 15/40 and 20/40 substrates used (Figure 3 A and B). At 10 nm protein concentration (Figure 3A), FancmΔ was able to fully unwind the 15/40 substrate over the course of the experiment, but could unwind only 37% of the 20/40 substrate. The same is true for reactions using 150 nm protein (Figure 3B). These data indicate that the inability to unwind the 20.40 substrate, even after extended incubation, is not due to the presence of inactive protein in the preparation or to loss of activity during the course of the reaction.

The inability of Fancm to catalyze unwinding of greater lengths of duplex DNA may result from the use of a truncated protein or missing factors such as posttranslational modifications or interacting proteins. Alternatively, the limited helicase may be an intrinsic property of this protein. Similar truncation of Fml1 did not appear to limit the length of duplex unwinding (Nandi and Whitby 2012). Conversely, there are cases where inclusion of sequences predicted to be unstructured, like the C-terminus of Fancm, impedes activity *in vitro*. An example is Blm helicase, where the full-length protein cannot unwind nucleosomal DNA but a protein that has only the conserved helicase domain can (Fujimoto *et al.* 2009). Thus, while we cannot exclude the possibility that *Drosophila* Fancm unwinds longer duplex regions *in vivo*, we hypothesize that the limited unwinding ability we observed is reflective of the protein's functions in DNA repair.

Based on *in vivo* data (Kuo *et al.* 2014), we hypothesized that Fancm may be involved in SDSA by displacing D-loops. In previous yeast studies it was shown that Mph1 can unwind the D-loop structures generated during recombination (Prakash *et al.* 2009). To test the ability of Fancm to unwind complex structures we constructed substrates resembling a recombination D-loop intermediate. We incubated Fancm with a 40 nt bubble-like structure containing an 'invading' homologous strand in which the duplex region was limited to 15 bp. To determine whether 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 2B). 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 not a result of the length of the duplex region, but rather the ability of Fancm to access the duplex region. This is possibly a result of a lack of ssDNA region to which the helicase can bind to initiate unwinding. The "middle" and "end" 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

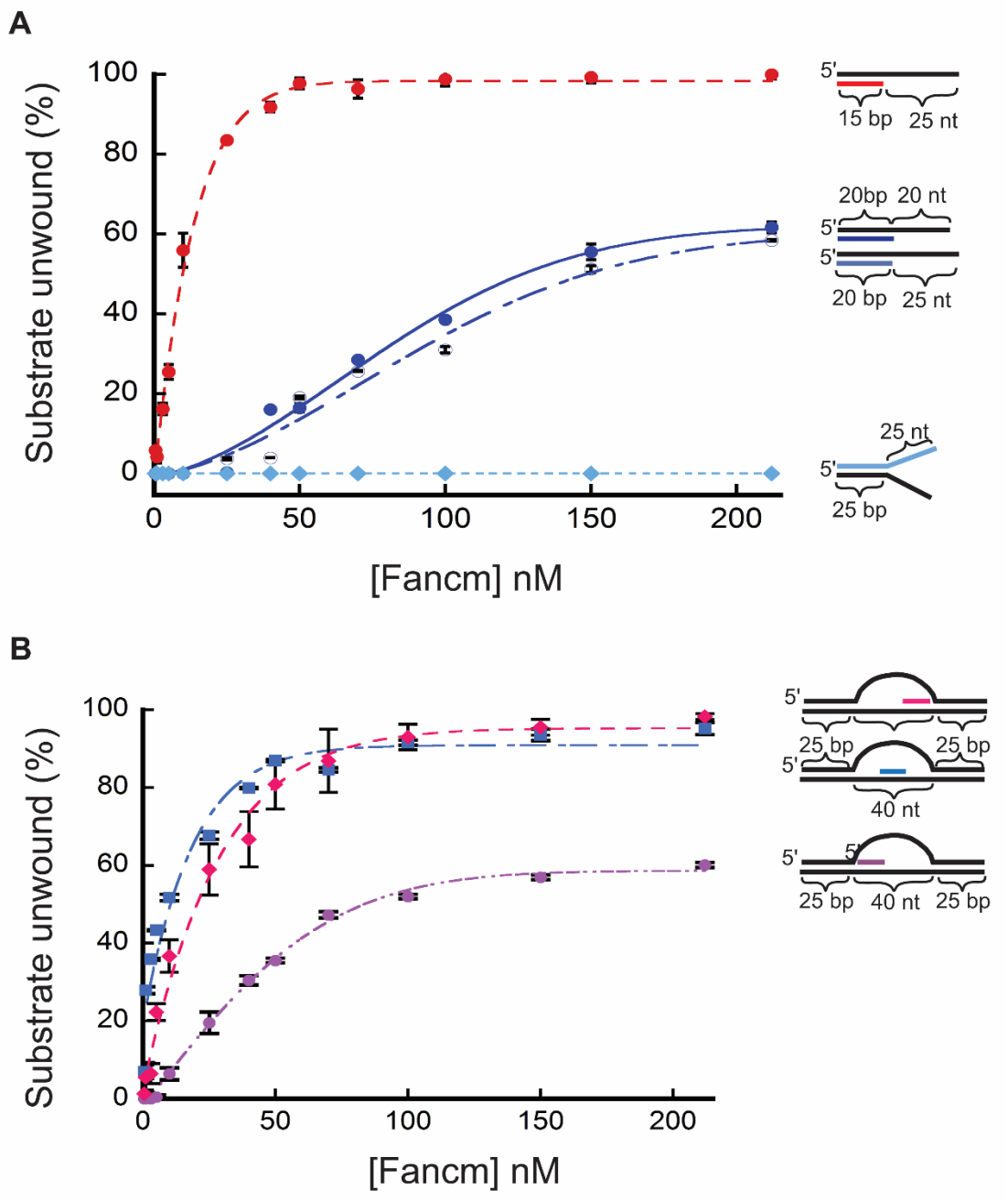


Figure 2- *Unwinding of partial duplex DNA substrates by Fancm.* Helicase reactions were performed as described under "Materials and Methods". The indicated concentrations of Fancm were incubated with 0.1 nM of the indicated substrate for 15 minutes. Colored strand on each substrate represents radiolabeled 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 S1. 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 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). Substrate oligonucleotides are in Table S1.

above, Fancm does not catalyze unwinding of a substrate with a 5' -ssDNA tail (See Figure 1D). 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 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 S6B).

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 or modification. Efforts to detect unwinding of longer duplex regions in the presence of a ssDNA binding protein (*E. coli* SSB) or under other conditions (*e.g.,* different salt concentration) were unsuccessful.

Mph1 and Fml1 have both been shown to be active helicases unwinding up to 100 bp of duplex DNA (Prakash *et al.* 2005). On the other hand, human FANCM has been shown to migrate D-loops and HJs, but no unwinding activity has been reported (Meetei *et al.* 2005; Prakash *et al.* 2005; Gari *et al.* 2008; Sun *et al.* 2008; Zheng *et al.* 2011). The data presented here suggest that *Drosophila* Fancm, while similar to both the yeast and human orthologs, is unique. Unlike human FANCM it is an active helicase, yet we could not detect unwinding of longer duplex regions like Mph1 and Fml1. 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 is stimulated by the addition of replication protein A (RPA) (Prakash *et al.* 2005); although SSB did not stimulate unwinding activity of Fancm, it is possible that *Drosophila* RPA or other proteins would do so.

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. 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 (Huang *et al.* 2010; Wang *et al.* 2013). The presence of 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 (Deans and West 2009; Hoadley *et al.* 2012). It should be noted that *Drosophila* Fancm has neither the ERCC4 domain nor recognizable MM1 or MM2 motifs. The lack of these sequences is consistent with the fact that the interacting 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, although lacking any recognizable motifs found in orthologs, may contribute to the regulation and function of the protein.

To identify the role the C-terminus has in regards to function of the protein in regulation of HR, we 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*, except that it lacks the His and MBP tags. We refer to the transgenic truncated Fancm as tr to distinguish it from FancmΔ that has these tags. To investigate the role of the helicase activity in CO prevention and DNA damage response, we generated transgenic recombinant flies that express either full-length or truncated Fancm with either a wild-type helicase domain or the helicase-dead mutation used *in vitro* (Figure 1A and 3A).

Previous reports on functions of *Drosophila* Fancm used the nonsense mutation $Fancm^{0693}$ (L78ter) in *trans* to *Df(3R)ED6058*, a 423.1 kb genomic deletion the removes more than 50 genes (Kuo *et al.* 2014). To ensure that any mutant phenotype described was an effect of 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. The mutants used here were heteroallelic for $Fancm^{del}$ and $Fancm^{0693}$. *Fancm* transcript is undetectable in the null background, indicating that no endogenous Fancm is being produced in flies of this genotype (Figure S1). In all assays performed, no significant difference was

observed between the previous results using *Df(3R)ED6058* and our experiments using *Fancm^{del}*, allowing us to conclude that *Fancm^{del}* is a null allele and the previous experiments with the deficiency can be attributed to loss of *Fancm*. All subsequent experiments reported here used one copy of the transgene in the *Fancm^{Del}*/*Fancm^{0693}* null background, and comparisons are made in reference to the null genotype.

As previously reported, *Fancm* mutants exhibit a significant increase in the number of spontaneous mitotic crossovers (Kuo *et al.* 2014). We assayed spontaneous mitotic crossovers in the male germline since meiotic crossovers do not occur in males. Crossovers were scored between the visible markers *st* and *Sb* (~20% of the genome) (Figure 4A). No crossovers were detected in wild-type males or in *Fancm* null mutants with the full-length transgene (FL) (Figure 4C), 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. 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 4C). The fact that tr and FLKM reduced crossover levels to near wild-type yet trKM did not indicates that 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 seen in the null mutant, crossovers were still detected above wild-type levels. The difference between these genotypes and WT does not cross the threshold typically used to be considered statistically significant, but we have never detected a crossover in any wild-type male (Mcvey *et al.* 2007; Kuo *et al.* 2014; Lafave *et al.* 2014); hence, we 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.

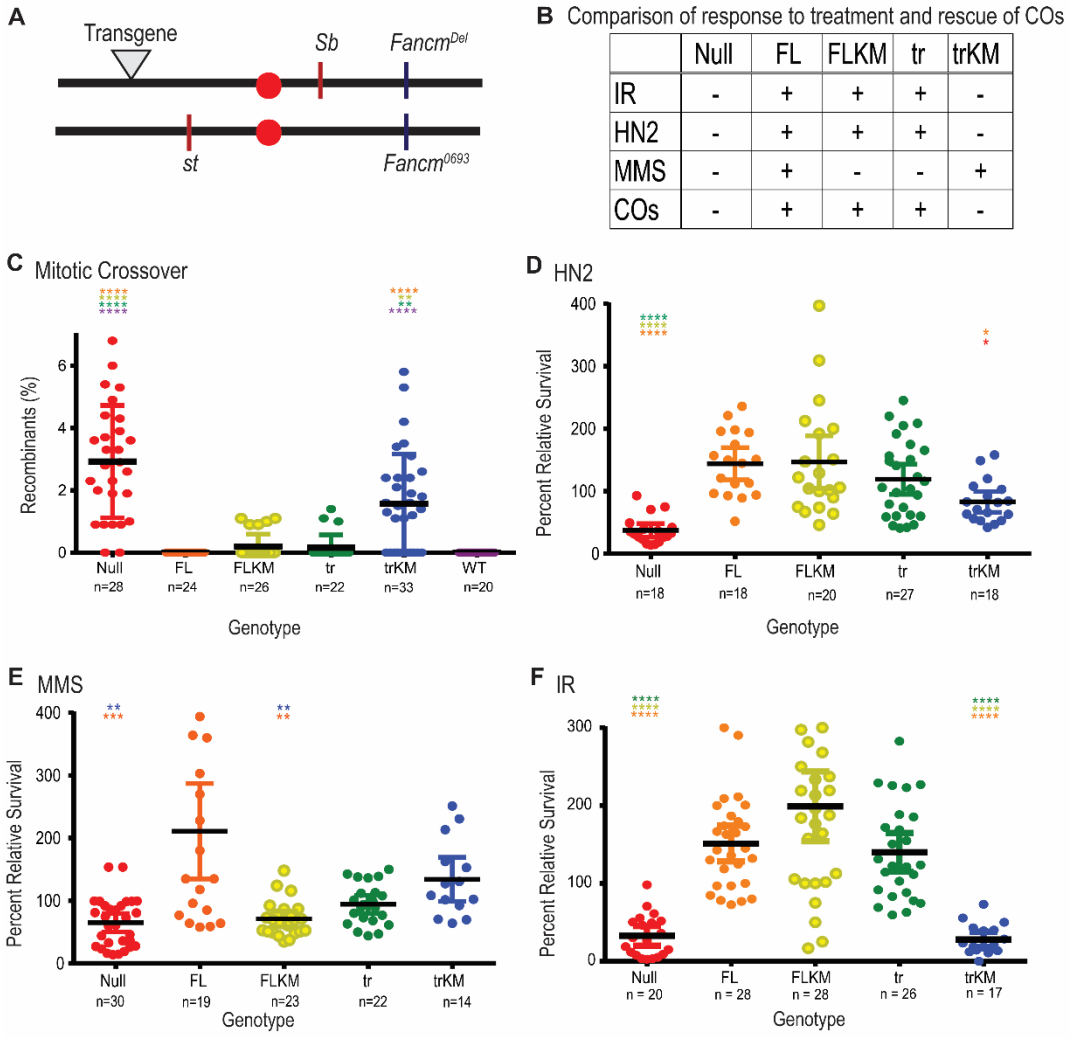
Separation of function in Fancm's roles in the response to DNA damage

Since the mitotic crossover assay measures spontaneous germline crossovers detected in progeny, we cannot determine how or when crossovers are occurring. We 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 us to investigate whether there was a difference in DNA damage response using the transgenes described above.

Previous sensitivity studies using *Drosophila Fancm* 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) (Kuo *et al.* 2014). 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 (Muniandy *et al.* 2010; Clauson *et al.* 2013). IR was therefore used to determine if *Fancm* is involved in repair of DSBs.

As previously reported, *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 (Figure 4B, D-F). The full length mutant (FLKM) and truncated (tr) transgene both rescued sensitivity to HN2 and IR, but not MMS (Figure 4B, D-F). The trKM transgene failed to rescue sensitivity to HN2 and IR, but did appear to rescue MMS sensitivity (Figure 4B, D-F). However, progeny with the trKM transgene have delayed developmental timing. 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 4D and F) is reminiscent of the role of these transgenes in crossover prevention (Figure 4C), 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.



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

Figure 4- *Fancm has genetically separable functions.* A.) Map of 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 1A, without tags. B.) Table comparison of all transgenic *Fancm* genotypes and null genotype. (-) mean no rescue of the null phenotype, (+) indicate rescue. C.) Spontaneous mitotic crossover rates were measured between *st* and *Sb.* D-F.) Comparison of Sensitivities of Fancm. Plots show the survival of the indicated phenotype relative to wildtype control flies in the same vial after exposure to (D) 0.002% HN2 (0.1 M), (E) 0.05% MMS (3.23 mM), or (F) 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.

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.

We hypothesize that Fancm not only acts separately from the FA repair response, but can act both catalytically and non-catalytically 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. These dual roles are seen in the FLKM and the tr genotypes. The lack of helicase activity in FLKM prevents it from unwinding D-loops, 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 Blm and involvement with HR and D-loop displacement is supported by studies in humans and yeast (Prakash *et al.* 2005; Nandi and Whitby 2012; Mitchel *et al.* 2013; Kuo *et al.* 2014). *Blm* mutants have more spontaneous mitotic crossovers than *Fancm* mutants (Mcvey *et al.* 2007; Kuo *et al.* 2014). 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, leading to the suggestion that they promote SDSA (Gari *et al.* 2008; Sun *et al.* 2008; Prakash *et al.* 2009). As shown above, *Drosophila* Fancm is also capable of unwinding D-loop like structures. Use of a gap repair assay directly demonstrated roles for both Fancm and Blm in SDSA in *Drosophila* (Adams *et al.* 2003; Kuo *et al.* 2014). Based on the data presented above, we 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. Unfortunately, Kuo *et al.* (2014) 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, so these hypotheses cannot be tested with available reagents.

While there are many similarities found between the orthologs of FANCM, there are also many differences that are informative. Some of these can be explained by the assay conditions, but they may also reveal functional divergence. The inability to detect conserved binding motifs is likely a consequence of co-evolution between FANCM and other proteins. 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.

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