Genetic analysis reveals novel roles for mei-MCMs during meiotic recombination in *Drosophila*

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Abstract

Crossover formation as a result of meiotic recombination is vital for proper segregation of homologous chromosomes at the end of meiosis I. In most organisms, crossovers are generated through two crossover pathways: Class I and Class II. Meiosis-specific protein complexes ensure accurate crossover placement and formation by promoting and inhibiting the formation of crossovers in both crossover pathways. In *Drosophila,* Class I crossovers are promoted and Class II crossovers are prevented by a complex that contains MCM (mini-chromosome maintenance) and MCM-like proteins, REC (ortholog of Mcm8), MEI-217, and MEI-218, collectively called the mei-MCM complex. However, little is known about how the mei-MCMs function within the Class I and II crossover pathways. In this study, we perform genetic analysis to understand how specific regions and motifs of REC and MEI-218 contribute to crossover formation and distribution. We see that while the N-terminus of MEI-218 is dispensable for crossover formation, REC's conserved AAA ATPase motifs exhibit differential requirements for Class I and Class II crossover formation. REC-dependent ATP hydrolysis, but not ATP binding, is required for promoting the formation of Class I, MEI-9 dependent crossovers. Conversely, the ability for REC to both bind and hydrolyze ATP is required for REC's Class II anti-crossover role, yet to varying degrees, suggesting that REC forms multiple complexes that

36 require different REC-dependent ATP binding functions. These results provide genetic insight
37 into the mechanism in which mei-MCMs promote Class I crossovers and inhibit Class II crossovers.

38 **Introduction**

39 The mini-chromosome maintenance (MCM) protein family was discovered based on its
40 essential role in DNA replication (Maine and Sinha 1984) and are structurally defined by a
41 conserved amino-terminal domain and a carboxy-terminal AAA+ ATPase domain (reviewed in
42 (Bell and Botchan 2013)). While the MCM 2-7 proteins interact to form an indispensable
43 replicative helicase that is essential in all eukaryotes, MCMs 8-9 function outside of replication,
44 are not essential for cell viability, and have been lost in many taxa (reviewed in (Forsburg 2004)).
45 Although Mcm8 and Mcm9 can complex to function in somatic homologous recombination repair
46 (Lee et al. 2015; Nishimura et al. 2012), Mcm8 also functions independently of Mcm9, particularly
47 in meiosis (Kohl, Jones, and Sekelsky 2012; Lutzmann et al. 2012; Blanton et al. 2005).

48 Meiosis is a specific type of cell division that reduces a diploid progenitor germ cell into
49 four haploid gametes through two successive rounds of division. Accurate reduction of the
50 genome at the end of meiosis I requires crossover formation between homologous chromosomes
51 during meiotic recombination. Meiotic recombination is initiated by the formation of multiple
52 double-strand breaks (DSBs); the majority of meiotic DSBs are repaired as noncrossovers, while
53 a selected subset are repaired as crossovers between homologs (reviewed in (Lake and Hawley
54 2012).

55 In meiosis, there are two distinct crossover pathways: Class I and Class II. First defined in
56 budding yeast (De los Santos et al. 2003), Class I and Class II crossovers exist in most sexually
57 reproducing organisms, and the extent to which crossover pathway is used varies among

organisms (Hollingsworth and Brill 2004). Both crossover pathways generate segregation-competent crossovers (Hatkevich et al. 2017); however, these pathways differ in key factors, such as the use of the complex Msh4/5. Only Class I crossovers are dependent upon the complex Msh4/5 (Zalevsky et al. 1999; De los Santos et al. 2003). Biochemical studies show that Msh4/5 stabilize recombination intermediates (Snowden et al. 2004), resulting in further processing and eventual crossover formation by meiosis-specific nucleases (Zakharyevich et al. 2012; De Muyt et al. 2012). Interestingly, Msh4 and Msh5 are absent from *Drosophila*. Instead, it is hypothesized that *Drosophila* utilizes a complex composed of MCM or MCM-like proteins, called the mei-MCM complex, to replace the function of Msh4/5 (Kohl, Jones, and Sekelsky 2012).

Currently, there are three recognized mei-MCM proteins: REC (the *Drosophila* ortholog of Mcm8 (Blanton et al. 2005)), MEI-217, and MEI-218. The mei-MCMs aid in DSB repair to ensure accurate meiotic crossover formation through promoting Class I crossovers. In *Drosophila*, most – if not all – crossovers are generated through the Class I pathway, with at least 90% of crossovers being dependent on both the mei-MCMs (Baker and Carpenter 1972a) and the putative catalytic unit of the Class I meiotic resolvase MEI-9 (Radford et al. 2005; Sekelsky et al. 1995; Yildiz et al. 2002, 2004; Radford et al. 2007).

While most crossovers are generated through the Class I pathway in wild-type *Drosophila*, crossovers are generated exclusively through the Class II pathway in *Blm* mutants (Hatkevich et al. 2017). Blm, or Bloom syndrome helicase, is a member of the conserved RecQ helicase family (Ellis et al. 1995), and Blm exhibits vital functions in both meiotic and somatic DSB repair (reviewed in (Hatkevich and Sekelsky 2017)). Interestingly, Blm and the mei-MCMs genetically interact; in *Blm* mutants, crossovers are reduced by 30% but in a *Blm rec* double mutant,

crossovers are significantly increased as compared to wild-type (Kohl, Jones, and Sekelsky 2012). This suggests that the mei-MCMs may also function to inhibit crossovers within the Class II pathway, in addition to their role promoting crossovers in the Class I pathway.

Among the known mei-MCMs, REC is the only bona fide MCM protein. REC harbors the conserved MCM N-terminus and the AAA+ ATPase C-terminus (Figure 1A). MEI-217, however, harbors only the conserved amino MCM domain, while MEI-218 possesses a disordered basic N-terminus (amino acids 1-500 (Brand et al. 2018)), a partially conserved central acidic region (amino acids 500-800 (Brand et al. 2018)) and a highly-conserved C-terminal ATPase-like region (amino acids 850-1116 (Kohl, Jones, and Sekelsky 2012)). The function of the disordered MEI-218 N-terminus is unknown, but gene swap studies suggest that the N-terminus, along with the central region, may contribute to differences in the recombination landscape among *Drosophila* species (Brand et al. 2018).

MEI-217 and MEI-218 together resemble one full MCM protein (Figure 1A). It appears that *mei-217* and *mei-218* have evolved from one ancestral gene, being that both genes are expressed from one bicistronic transcript (Liu et al. 2000). The mammalian ortholog of *mei-217* and *mei-218* is *Mcmdc2* (Mcm-domain containing protein 2) which expresses one protein with a conserved MCM N-terminus and AAA+ ATPase C-terminus. Mice mutant for *Mcmdc2* exhibit sterility due to an inability to repair meiotic DSBs, indicating that MCMDC2 functions in meiotic DSB repair, similar to the roles of mei-MCMs (Finsterbusch et al. 2016; McNairn, Rinaldi, and Schimenti 2017).

The C-terminal AAA+ catalytic domain of MCM proteins contain distinct signature sequences, including the Walker A and Walker B motifs that bind and hydrolyze ATP, respectively

(Iyer et al. 2004). Within the mei-MCMs, only REC contains a predicted catalytically active AAA+ ATPase domain, as catalytic residues within the Walker A and B motifs in MEI-218 and its ortholog MCMDC2 are not conserved (Kohl, Jones, and Sekelsky 2012; Finsterbusch et al. 2016; McNairn, Rinaldi, and Schimenti 2017) (Figure 1B). REC (and all Mcm8 orthologs), however, has diverged from other MCMs, as it does not contain a conserved arginine finger, a *trans* acting AAA+ ATPase motif that is present in canonical MCMs (Blanton et al. 2005; Forsburg 2004). This suggests that the mei-MCM complex may contain an additional, as-yet-unidentified, MCM protein that provides the arginine finger (Kohl, Jones, and Sekelsky 2012) or that REC may utilize a modified mechanism to hydrolyze ATP.

The mei-MCMs are a highly divergent class of proteins and appear to have evolved the function to ensure accurate recombination in *Drosophila* through promoting Class I crossovers and inhibiting Class II crossovers (Kohl, Jones, and Sekelsky 2012). While the mei-MCMs form a complex together, little is known about how individual mei-MCMs contribute to Class I and II crossover regulation. Here, we investigate specific requirements of two mei-MCMs, MEI-218 and REC, to understand how this protein class contributes to recombination in *Drosophila melanogaster*. We find, surprisingly, that the N-terminus of MEI-218 is dispensable for crossover formation and general crossover distribution. However, our study shows that REC ATP binding and hydrolysis are differentially required within the Class I and II crossover pathways. The ability for REC to hydrolyze ATP, but not bind ATP, is required for Class I, MEI-9 dependent crossovers. Lastly, we find that REC's ability to REC ATP binding and hydrolysis are required to inhibit Class II crossovers.

Materials and Methods

Drosophila stocks. Flies were maintained on standard medium at 25°C. Assays examined heteroallelic and homozygous mutant flies that have been previously described, including *mei-9^{a}* (Baker and Carpenter 1972b), *mei-218^{1}* and *mei-218^{6}* (Baker and Carpenter 1972b; McKim, Dahmus, and Hawley 1996), *Blm^{N1}* and *Blm^{D2}* (McVey et al. 2007), *rec^{1}* and *rec^{2}* (Grell 1984; Matsubayashi and Yamamoto 2003). The maternal effect lethality in *Blm$^{N1/D2}$* mutants was overcame by the *UAS::GAL4* rescue system previously described (Kohl, Jones, and Sekelsky 2012).

Generating mei-218 transgenic alleles. The transgenes for *mei-218$^{\Delta N}$* and *mei-218^{FL}* were created by cloning cDNA for *mei-218*. Cloning for full length *mei-218* included amino acids 1-1186, and the *mei-218$^{\Delta N}$* transgene included amino acid positions 527-1186. These transgenes were inserted into *p{attBUASpW}* (AddGene) by Gateway cloning. This vector was injected using PhiC31 transformation (Best Gene Inc.) into the *2A* genomic location.

Generating* rec^{KA} *and* rec^{DA} *mutants. pU6-BbsI-chiRNA plasmid (Addgene) was used to insert chiRNA oligos after *Bbs*I digestion. Oligo sequence for *rec^{KA}*: 5'F CTTCGCCGAGAAGGGATAGTAAAC 3'; oligio sequence for *rec^{DA}*: 5'F CTTCGTTGCAGTGCCTACAATCAG 3'. Resulting plasmids were co-injected with repair vector. Repair vectors were generated using pBlueScript plasmid and repair oligo G-blocks (IDT) after *Not*I and *Sac*II digestion. Oligo sequences for *rec^{KA}* and *rec^{DA}* repair vectors are available upon request. Transformant males were screened through restriction digest and were crossed to *TM3*/*TM6B* females (Bloomington Stock Center) to generate stocks.

143 ***Nondisjunction assay.*** *X*-chromosome nondisjunction (NDJ) was assayed by mating virgin
144 females with mutant background of interest to *y cv v f* / *T(1:Y)B*^{S} males. Progeny were scored for
145 viable exceptional progeny, which are *XXY* females with Bar eyes and *XO* males with Bar^{+} eyes
146 and the phenotypes from *y cv v f* chromosome. Total (adjusted) represents the total with inviable
147 exceptional progeny accounted for. NDJ rates and statistical comparisons were done as in Zeng
148 et al. 2010.

149 ***Crossover distribution assay.*** Crossover distribution on chromosome *2L* was scored by crossing
150 virgin *net dpp*$^{d\text{-}ho}$ *dp b pr cn / +* female flies with mutant background of interest to *net dpp*$^{d\text{-}ho}$ *dp*
151 *b pr cn* homozygous males. All progeny were scored for parental and recombinant phenotypes.
152 Crossover numbers in flies are shown as cM where cM = (number of crossovers / total number
153 of flies) * 100. Chi-squared tests with Bonferroni correction were performed for each interval or
154 for all intervals combined to determine statistical significance between interested backgrounds.
155 Crossover distribution is represented as cM/Mb where Mb is length of the interval without
156 transposable elements (TEs) because crossovers rarely occur within TEs (Miller et al. 2016).

157 ***Protein structure and alignment.*** Structural domains of proteins were determined by using
158 PHYRE 2. All of the MCM regions identified correspond to the protein data bank ID #c2vl6C and
159 the AAA ATPase domains identified correspond to protein data bank ID #d1g8pa. Alignment of
160 the Walker A and Walker B motifs (Kohl, Jones, and Sekelsky 2012) was done using MEGA 5 and
161 aligned with the ClustalW program. Identical and conserved residues are shaded in black.
162 Conserved residues are based on groups of amino acids with similar chemical properties
163 (nonpolar, polar, acidic, and basic).

164 ***Data availability.*** All data necessary for confirming the conclusions in this paper are included in
165 this article and in supplemental figures and tables. *Drosophila* stocks and plasmids described in
166 this study are available upon request. We have uploaded Supplemental Material to Figshare.
167 Figure S1 illustrates distribution of Msh4, Msh5, Mcm8, Mcm9, MEI-217, and MEI-218 in Diptera.
168 Figure S2 illustrates the structure of MEI-217 and MEI-218 in Diptera. Figure S3 shows sequence
169 alignment of MEI-218. Figure S4 details the cross scheme of *mei-218* over expression. Figure S5
170 illustrates the crossover distribution of *WT* and *mei-218*^{FL}. Table S1 includes analysis of genetic
171 interval differences between *WT* and *mei-218*^{FL}. Table S2 includes analysis of genetic interval
172 differences between *mei-218*^{FL} and *mei-218*$^{\Delta N}$. Table S3 includes complete data set for calculating
173 nondisjunction of *WT, rec* $^{-/+}$, and *rec*$^{DA/+}$. Table S4 includes all data sets for meiotic crossovers for
174 all genotypes discussed.

175

176 **Results and Discussion**

177 *Origins of the mei-MCM Complex*

178 The three known mei-MCM proteins are REC, MEI-217, and MEI-218. Kohl *et al.*
179 demonstrated that REC, the ortholog of Mcm8, evolved by positive selection in the lineage
180 leading to Schizophora (true flies), based on an analysis that included sequences from 12 species
181 of *Drosophila* and from the tsetse fly *Glossina morsitans*. These same species lacked orthologs
182 of Msh4, Msh5, and Mcm9. There are now genome or transcriptome sequences for more than
183 50 additional Schizophora species, all of which are like those previously described in lacking
184 Msh4, Msh5, and Mcm9 (Figure S1). There are also partial transcriptome sequences for several

185 species in the sister taxon Aschiza. We do find orthologs of Msh4, Msh5, or Mcm9 among the
186 available sequence, suggesting that the mei-MCM complex evolved as early as the split between
187 the Dipteran sub-orders of Brachycera, which includes Schizophora and Aschiza, and
188 Nematocera, which includes mosquitoes previously found have orthologs of Msh4, Msh5, and
189 Mcm9 (Kohl, Figure S1). The one other Brachycera taxon for which transcriptome sequence is
190 available is the superfamily Asiloidea. Although we do not find orthologs of Msh4 or Msh5 among
191 the available sequences, we do find clear evidence for Mcm9 orthologs. It will be interesting to
192 examine these species more thoroughly when additional sequences become available.

193 MEI-217 and MEI-218 are encoded in overlapping open reading frames (ORFs) on a
194 bicistronic transcript and are apparently derived from a single ancestral protein now called MCM
195 domain-containing protein 2 (MCMDC2) (McNairn, Rinaldi, and Schimenti 2017; Finsterbusch et
196 al. 2016). MCMDC2 appears to have arisen early in the Opisthokont (fungi and metazoan) lineage,
197 as there are apparent orthologs in several species placed in Fungi *incertae sedis*, including at least
198 the phyla Chytridiomycota, Mucoromycota, and Zoopagomycota, although we find no orthologs
199 in the Dikarya (higher fungi) subkingdom. Based on available genome and transcriptome
200 sequences, Mcmdc2 seems to have been lost in numerous lineages, from entire phyla (*e.g.*,
201 Nematoda) to subfamilies (*e.g.*, Anophelinae; we do not find orthologs in any of the 20 species
202 of *Anopheles* with sequenced genomes, but orthologs are clearly present in other Culicidae,
203 including *Aedes* and *Culex*; Figures S1 and S2). The split of Mcmdc2 into MEI-217 and MEI-218 is
204 seen in all Dipteran species that have these proteins. All Schizophora and Aschiza genomes
205 appear to encode these proteins in overlapping ORFs (Figure S2), so this configuration has been
206 maintained for more than 150 million years. In the Nematocera suborder, however, there appear

to have been numerous losses of MEI-217/218 (Figure S2). In species that have retained these proteins, they are encoded on bicistronic transcripts, but the ORFs are separated by short non-coding regions (Figure S2B). In all other taxa, including Lepidoptera, which is the most closely related order to Diptera, there is a single Mcmdc2 polypeptide that has the canonical domain structure of replicative MCM proteins.

MEI-218 N-terminus is dispensable for crossover formation

MEI-218 harbors three distinct regions: an N-terminal tail (amino acids 1-500 (Brand et al. 2018)), a central acidic region (amino acids 500-800 (Brand et al. 2018)) and C-terminal ATPase region (amino acids 850-1116 (Kohl, Jones, and Sekelsky 2012)) (Figure 1A). While the C-terminus of MEI-218 is similar to MCM AAA+ ATPase domain, the N-terminal and middle regions are predicted to be disordered (Kohl, Jones, and Sekelsky 2012) and are poorly conserved among *Drosophila* (Figure S3). Regardless the lack of conservation, results obtained during gene swap experiments suggest that the N-terminal tail and central region regulate crossover number and distribution within *Drosophila* species (Brand et al. 2018). To investigate functions of these regions, we created a transgene that expresses a truncated form of MEI-218 that eliminates the first 526 amino acids of the N-terminus (*mei-218*$^{\Delta N}$). We retained the middle region because of the presence of short sequences conserved through *Drosophila* (Figure S3). For a positive control we created a full-length MEI-218 construct (*mei-218*^{FL}) (Figure 2A). Using the *UAS*/*GAL4* system (Duffy 2002), we expressed both constructs in *mei-218* null mutants using the germline-specific *nanos* promoter and measured crossovers along five adjacent intervals that span most of *2*L and part of *2*R (Figure S4; for simplicity, we refer to this chromosomal region as *2*L.)

In wild-type females, the genetic length of *2*L is 45.8 cM (Hatkevich et al. 2017) (Figure 2B), whereas *mei-218* mutants exhibit a severe decrease in crossovers, resulting in a genetic length of 2.92 cM (Kohl, Jones, and Sekelsky 2012). Expression of *mei-218*^{FL} in *mei-218* mutants (*mei-218; mei-218*^{FL}) fully rescues the crossover defect, exhibiting a genetic length of 54.1 cM (Table S1, Figure S5). The modest increase in crossovers in *mei-218; mei-218*^{FL} may be due to overexpression of *mei-218*^{FL} as a result of the *UAS/GAL4* transgenic system. Unexpectedly, expressing MEI-218 without the N-terminus is not significantly different from full length MEI-218 (55.9 cM, Figure 2B).

When *Drosophila mauritiana* MEI-217/MEI-218 is expressed in *Drosophila melanogaster,* crossovers are increased proximally and distally, resulting in an overall change in crossover distribution (Brand et al. 2018). To determine whether the N-terminus of *Drosophila melanogaster* MEI-218 functions in regulating crossover distribution, we examined crossover distribution in *mei-218; mei-218*^{FL} and *mei-218; mei-218*$^{\triangle N}$ (Figure 2C). Overall, crossover distributions are similar, with both genotypes exhibiting a strong inhibition of crossovers near the centromere (referred to as the centromere effect (Beadle 1932)) and the majority of the crossovers placed in the medial-distal portion of *2*L.

We conclude that the N-terminal tail of MEI-218 is dispensable for both crossover formation and overall distribution. Recently, Brand et al. suggest that the variation among *Drosophila* MEI-218 N-terminal and middle-acidic regions account for the differences in recombination rate and patterning between *Drosophila melanogaster* and *Drosophila mauritiana* (Brand et al. 2018). In light of our results, it appears that the central and C-terminal regions of *D. melanogaster* MEI-218 are sufficient for the recombination landscape in *D. melanogaster*.

Regardless, the N-terminus of MEI-218 may function in other capacities; MEI-218 is expressed moderately highly in *Drosophila* male testes (Thurmond et al. 2018, FB2018_05) although males do not experience meiotic recombination. Interestingly, the predominant transcript in males does not encode MEI-217 (Thurmond et al. 2018, FB2018_05), the seemingly obligate partner for MEI-218. From these expression data, we speculate that MEI-218 may function outside of the mei-MCM complex in males, with its N-terminus being important for this unknown function.

REC ATPase activity is required for crossover formation

The mei-MCM complex is hypothesized to functionally replace the heterodimer Msh4/5 in *Drosophila* (Kohl, Jones, and Sekelsky 2012). Msh4 and 5 are part of the Walker A/B family of ATPases (Walker et al. 1982). Both proteins exhibit ATPase activity *in vitro* (Snowden et al. 2004), and mutations disrupting ATP hydrolysis in Msh4 and Msh5 confer null phenotypes *in vivo* (Pochart, Woltering, and Hollingsworth 1997). Correspondingly, it is unknown if the mei-MCM complex utilizes ATPase activity for its function *in vivo*.

Of the three known subunits, REC is the only mei-MCM that has well-conserved Walker A and B motifs, suggesting that REC has ATP binding and hydrolysis activity, respectively (Kohl, Jones, and Sekelsky 2012). To determine the importance of ATP-binding and hydrolysis motifs for the *in vivo* function of the mei-MCM complex, we introduced mutations within the Walker A and B motifs of REC using CRIPSR/Cas9 (Figure 3A). In the Walker A mutant, we substituted the conserved lysine residue to an alanine (rec^{KA}), a mutation predicted to prohibit REC from binding to ATP. In the Walker B mutant, we substituted the conserved aspartic acid for an alanine (rec^{DA}),

a mutation predicted to permit REC to bind to ATP but prohibits REC from hydrolyzing ATP. We then assayed these mutants for crossover formation along *2*L (Figure 3B).

Surprisingly rec^{KA} ATP binding mutants exhibit a genetic length of 44.9 cM, which is not statistically different from wild-type, while rec^{DA} ATP hydrolysis mutants exhibit a severe reduction of crossovers, with a genetic length of 1.6 cM. Because the genetic length of rec^{DA} is significantly lower than *rec* null mutants, we hypothesized that rec^{DA} acts as a dominant negative. To test this, we examined *X* chromosome nondisjunction (NDJ) of rec^{DA} heterozygous mutants (rec^{DA}/rec^{+}) (Figure 3C). While both wild-type and *rec* null heterozygotes exhibit near 0% *X* NDJ, rec^{DA}/rec^{+} mutants have a significantly higher NDJ rate of 1.4%, showing that rec^{DA} is weakly antimorphic. In light of these results, we propose that the mei-MCM complex binds to recombination sites independent of REC binding to ATP, and that REC-dependent ATP hydrolysis is required for the removal of the mei-MCM complex from these sites.

We conclude that REC-dependent ATPase activity is needed for crossover formation. Specifically, REC's ATP hydrolysis motif (Walker B) is required for ATPase activity, but REC's ATP binding motif (Walker A) is apparently dispensable for crossover formation. The disparate requirements for REC ATP binding and hydrolysis echo studies of Rad51 paralogs, which form multi-protein complexes and contain Walker A and B motifs (Wiese et al. 2006; Wu et al. 2004, 2005). It is proposed that the ATPase activity in human Rad51 paralogs occur in *trans* between adjacent subunits, with each subunit showing differential ATP binding and hydrolysis requirements for ATPase activity within the complex (Wiese et al. 2006; Wu et al. 2004, 2005). Additionally, in canonical MCM proteins, mutations within the different subunits' Walker A and B motifs have varying effects on ATPase activity (Gómez, Catlett, and Forsburg 2002). Because

294 neither MEI-217 nor MEI-218 possess a conserved ATPase domain (Figure 1B) (Kohl, Jones, and
295 Sekelsky 2012), we propose that ATPase activity of the mei-MCM requires REC for ATP hydrolysis
296 and an unknown mei-MCM protein for ATP binding (Figure 3D). Further studies are needed to
297 uncover this hypothesized novel mei-MCM.
298
299 *REC-dependent ATP hydrolysis is required for MEI-9-dependent crossovers*
300 Next, we examined whether the crossovers generated in rec^{KA} and rec^{DA} mutants are
301 formed by the Class I nuclease complex. In *Drosophila,* the catalytic subunit of the putative Class
302 I meiosis-specific endonuclease is MEI-9 (Radford et al. 2005; Sekelsky et al. 1995; Yildiz et al.
303 2002, 2004; Radford et al. 2007; Hatkevich et al. 2017). Accordingly, the *2*L genetic length within
304 a *mei-9* mutant is 2.75 cM (Figure 4), demonstrating that at least 90% of crossovers are
305 dependent upon MEI-9 resolution. However, *mei-9; rec* double mutants exhibit a genetic length
306 of 6.38 cM, indicating that in the absence of REC, the resulting crossovers are independent of
307 MEI-9.
308 Because rec^{KA} mutants exhibit the same distribution and number of crossovers as
309 wild-type (Figure 3), we hypothesized that rec^{KA} crossovers are dependent on MEI-9. To test this,
310 we examined genetic length across *2*L in *mei-9;* rec^{KA} double mutants (Figure 4). Mutants for
311 *mei-9;* rec^{KA} exhibit a genetic length of 2.72 cM, which is not significantly different from *mei-9*
312 single mutants, showing that crossovers in rec^{KA} are dependent upon MEI-9 nuclease. In contrast,
313 due to the dominant negative nature of rec^{DA}, we predicted that crossovers in rec^{DA} will be
314 independent of MEI-9, similar to crossovers generated in *rec* null mutants. We observe that
315 *mei-9;* rec^{DA} double mutants exhibit a genetic length of 1.1 cM, which is not significantly different

316 than *rec*^{DA} single mutants, demonstrating that crossovers in *rec*^{DA} are independent of MEI-9
317 resolution (Figure 4).

318 From these data, we conclude that MEI-9 generates the crossovers in *rec*^{KA} mutants,
319 whereas mitotic nucleases generate the crossovers formed in *rec*^{DA} mutants. These data show
320 that *rec*^{KA} appears to function as wild-type in the Class I pathway, while Class I crossovers are lost
321 in *rec* null and *rec*^{DA} mutants. We suggest that the ability of REC to hydrolyze ATP, but not bind,
322 is required for the formation of Class I crossovers.

323

324 *REC ATP binding and hydrolysis are required for REC's Class II anti-crossover role*

325 In wild-type *Drosophila*, most or all crossovers are generated through the Class I pathway
326 (Hatkevich et al. 2017), and these crossovers are dependent upon the mei-MCM complex (Kohl,
327 Jones, and Sekelsky 2012). However, in *Blm* mutants, crossovers are generated exclusively
328 through the Class II pathway (Hatkevich et al. 2017). In *Drosophila Blm* mutants, meiotic
329 crossovers are decreased by 30%, suggesting that the Class II pathway is less efficient at
330 generating crossovers than the Class I pathway. However, in *Blm rec* double mutants, crossovers
331 are increased significantly compared to *Blm* single mutants (Kohl, Jones, and Sekelsky 2012),
332 providing REC with a pro-crossover role in *Blm* mutants. From these results, we propose that REC
333 – and/or the mei-MCM complex – promotes crossovers in the Class I pathway and prohibits
334 crossovers in the Class II pathway.

335 To further understand the role of REC in the Class II pathway, we investigated whether
336 the predicted ATP binding or hydrolysis ability of REC is required for its Class II anti-crossover
337 function. To do this, we measured the crossovers across *2*L in *rec*^{KA} and *rec*^{DA} in the background

of *Blm* mutants. If REC ATP binding or hydrolysis is required for an anti-crossover role in Class II, then the genetic length of *Blm rec*^{KA} or *Blm rec*^{DA} double mutants will be similar to that of *Blm rec* double mutants. Conversely, if REC ATP binding or hydrolysis is not required, then double mutants will exhibit genetic lengths similar to that of *Blm* single mutants.

Interestingly, *Blm rec*^{KA} mutants exhibit an intermediate genetic length of 43.3 cM, which is significantly lower than *Blm rec* mutants but significantly higher than *Blm* single mutant (Figure 5A), suggesting that REC ATP binding has an anti-crossover role at a subset of Class II recombination sites. Flies mutant for *Blm rec*^{DA} have a recombination rate of 53.4 cM, which is similar to *Blm, rec* double mutants, demonstrating that REC ATP hydrolysis is required for the inhibition of crossovers at all REC-associated Class II recombination sites.

These results show that REC ATP binding is required for REC's anti-crossover role at some Class II recombination sites, while REC ATP hydrolysis is required for REC's anti-crossover role at all REC-localized Class II recombination sites. We propose that, within the Class II pathway, REC forms two types of complexes at Class II recombination sites to prohibit crossover formation (Figure 5B). Both complexes perform REC-dependent ATP hydrolysis, but only one type of complex requires REC-dependent ATP binding. We propose that these two REC-containing complexes, which may or may not require the other mei-MCMs, act together to prohibit Class II crossover formation at all REC-associated Class II recombination sites.

Conclusions

The mei-MCMs are a family of diverged proteins that help to establish the recombination landscape in *Drosophila melanogaster*. Results obtained in this study have further elucidated meiotic recombination roles for two mei-MCMs, MEI-218 and REC. While the N-terminus of

MEI-218 is dispensable for crossover formation (Figure 2), REC's conserved Walker A and B motifs exhibit differential requirements for regulating Class I and Class II crossover formation. REC-dependent ATP hydrolysis, but not ATP binding, is required for promoting the formation Class I, MEI-9 dependent crossovers (Figures 3 and 4). The weakly antimorphic phenotype of *rec*^{DA} demonstrates that an impaired REC Walker B mutant renders a poisonous complex – a complex in which we propose cannot be released from recombination sites. Conversely, both the ability for REC to bind and hydrolyze ATP are required for REC's Class II anti-crossover role, yet to varying degrees (Figure 5), suggesting that REC forms multiple complexes within the Class II pathway that exhibit differential REC-dependent ATP binding requirements. Biochemical and cytological studies are needed to support or refute these hypotheses.

Among the many remaining questions regarding the meiotic functions of the mei-MCMs, one question particularly stands out: does the mei-MCM complex in *Drosophila* truly replace Msh4/5 within the Class I crossover pathway? In mammalian meiotic recombination, MCMDC2 (a mei-MCM) is loaded early, possibly stabilizing early recombination interactions (Finsterbusch et al. 2016; McNairn, Rinaldi, and Schimenti 2017). Msh4/5 is loaded after strand invasion, stabilizing recombination intermediates (reviewed in (Gray and Cohen 2016)), placing this complex downstream of MCMDC2. Also, in budding yeast, which does not have mei-MCMs, Msh4/5 stabilizes recombination intermediates; it is unknown if there is a complex in yeast that stabilizes early recombination interactions prior to strand invasion. We propose that the *Drosophila* mei-MCMs evolved to retain the function of both MCMDC2 and Msh4/5, such that the mei-MCMs stabilize early recombination interactions *and* later recombination intermediates

to allow maturation of these sites from DSBs to crossovers. Cytological evidence from *Drosophila* is required to gain further understanding and evidence into this function of the mei-MCMs.

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Figure Legends

Figure 1. MCM protein structure and alignments. (A) Structural domains of *Drosophila melanogaster* REC, MEI-217, MEI-218 and *Mus musculus* MCMDC2. Structural domains identified using PHYRE 2 (Kohl, Jones, and Sekelsky 2012). "MCM domain" corresponds to protein data bank ID #c2vl6C and the AAA ATPase domains identified correspond to protein data bank ID #d1g8pa. The X on *Dm* MEI-218 and *Mm* MCMDC2 represents predicted inactive AAA ATPase domains. (B) Consensus sequence for Walker A motif (Walker et al. 1982), and consensus sequence for Walker B motif (Forsburg 2004). Identical or conserved amino acids are denoted with black background.

Figure 2. The role of MEI-218 N-terminus in crossover formation and distribution. (A) Schematic of transgenes for full length *mei-218* and N-terminal deleted *mei-218*, which is truncated up to amino acid 527. (B) Map units of WT (Hatkevich et al. 2017), *mei-218* (Kohl, Jones, and Sekelsky

2012), *mei-218*^{FL} and *mei-218*$^{\Delta N}$. Map units represented as centimorgans (cM). Error bars indicate 95% confidence intervals. ***$P < 0.0001$; *n.s.* = $P > 0.05$. (C) Crossover distribution (solid lines) of *mei-218*^{FL} and *mei-218*$^{\Delta N}$ represented as cM/Mb. Mb is measured distance of defined interval, excluding centromere, pericentromeric heterochromatin and transposable elements. Dotted lines represent mean crossover density across *2*L. Refer to tables S1, S2, and S4 for complete data sets.

Figure 3. REC ATPase binding and hydrolysis requirements for crossover formation. (A) Schematic representation of the mutated residues in *rec*^{KA} and *rec*^{DA}. (B) Map units of *WT* (Hatkevich et al. 2017), *rec*$^{1/2}$, *rec*^{KA}, and *rec*^{DA}. Map units represented as centimorgans (cM). Error bars show 95% confidence intervals. (C) Percent nondisjunction of *WT*, *rec*$^{1/+}$, and *rec*$^{DA/+}$. (D) Model of possible complex depicting the functional Walker B motif of REC protein interacting with a Walker A motif on a potential partner. ***$P < 0.0001$. Refer to tables S3 and S4 for complete data sets.

Figure 4. MEI-9-dependent crossovers in *rec*^{KA} and *rec*^{DA} mutants. Map units of *WT* (Hatkevich et al. 2017), *rec, mei-9, mei-9;rec, rec*^{KA}, *mei-9;rec*^{KA}, *rec*^{DA}, and *mei-9;rec*^{DA}. Map units represented as centimorgans (cM). Error bars show 95% confidence intervals. ***$P < 0.0001$; *n.s.* = not significant ($P > 0.05$). Refer to table S4 for complete data set.

Figure 5. Requirements of REC ATPase activity in Blm function. Map units of *WT* (Hatkevich et al. 2017), *Blm* (Kohl, Jones, and Sekelsky 2012) , *rec, Blm rec* (Kohl, Jones, and Sekelsky 2012), *Blm rec*^{KA}, and *Blm rec*^{DA}. Map units represented as centimorgans (cM). Error bars show 95% confidence intervals. * $P < 0.05$ and *** $P < 0.0001$. Refer to table S4 for complete data set. (B)

422 Model of REC-associated recombination sites (all circles) where filled in circles represent
423 complexes with REC-dependent ATP hydrolysis and binding, and open circles represent
424 complexes with REC-dependent ATP hydrolysis only.

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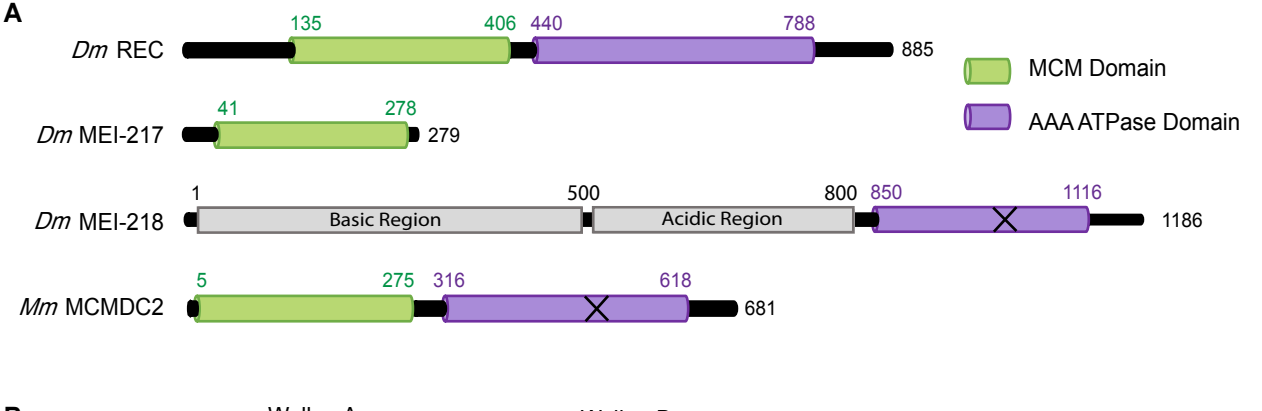
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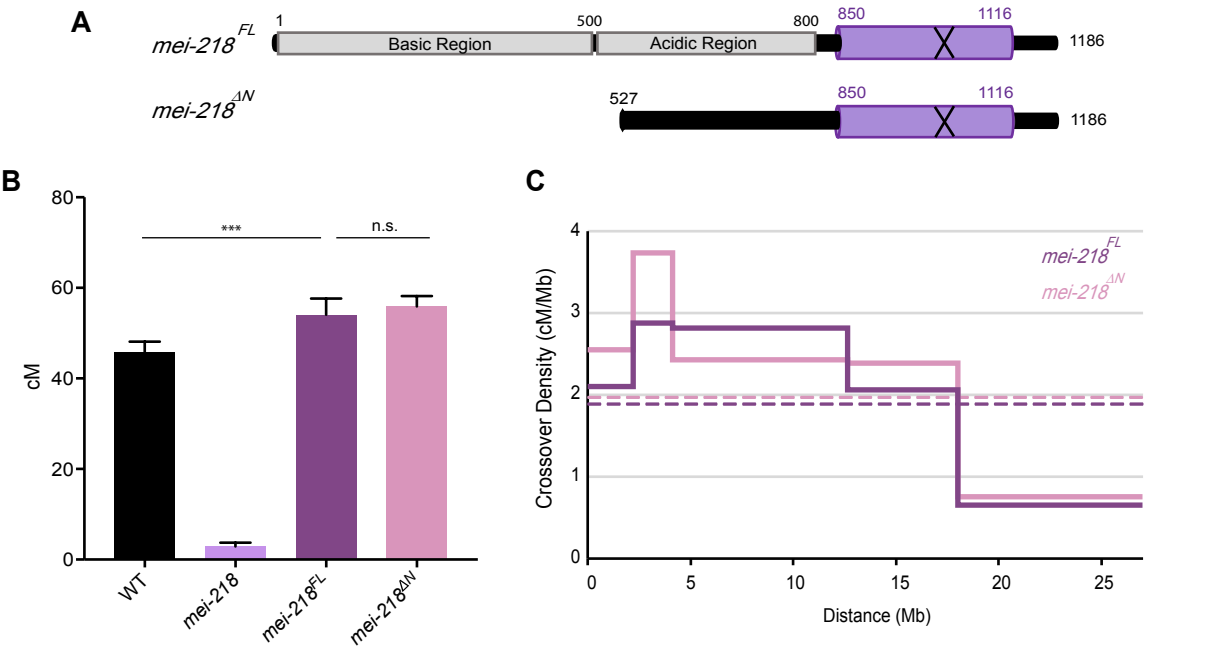
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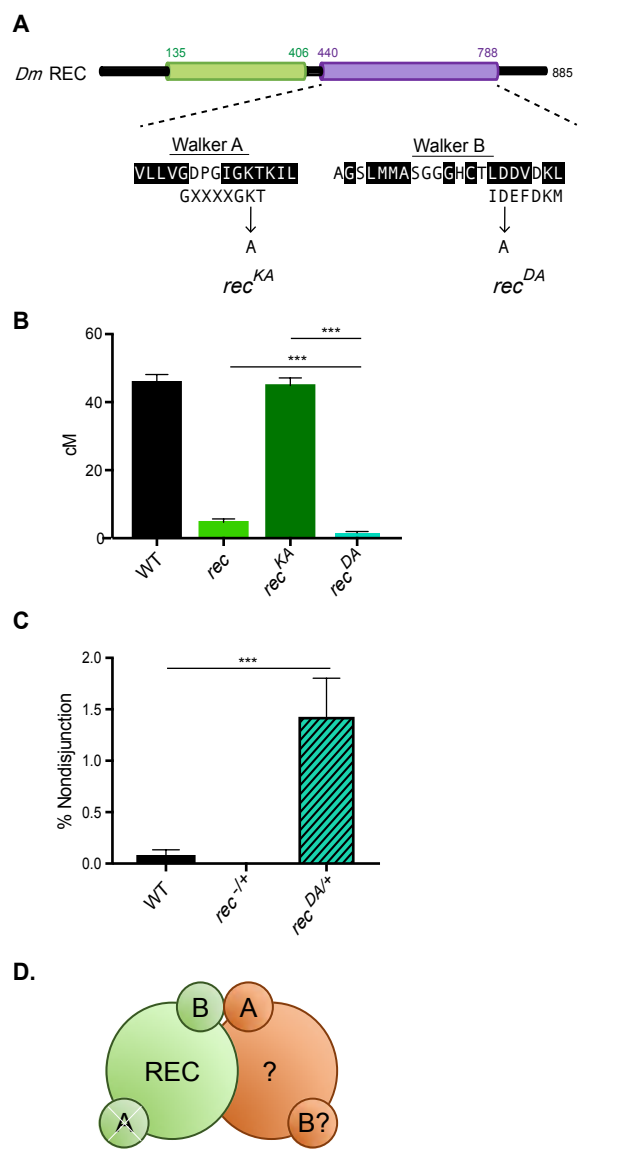
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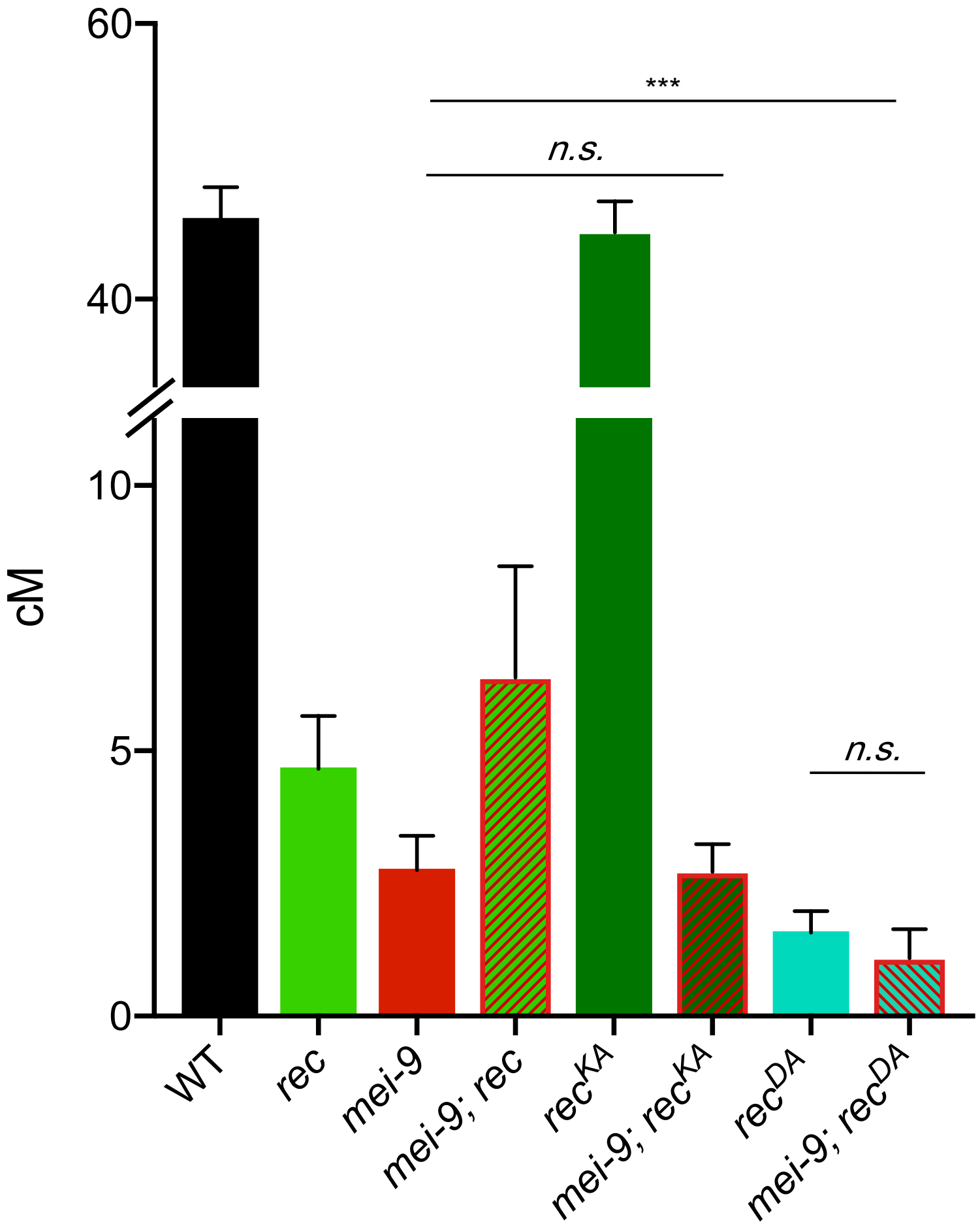
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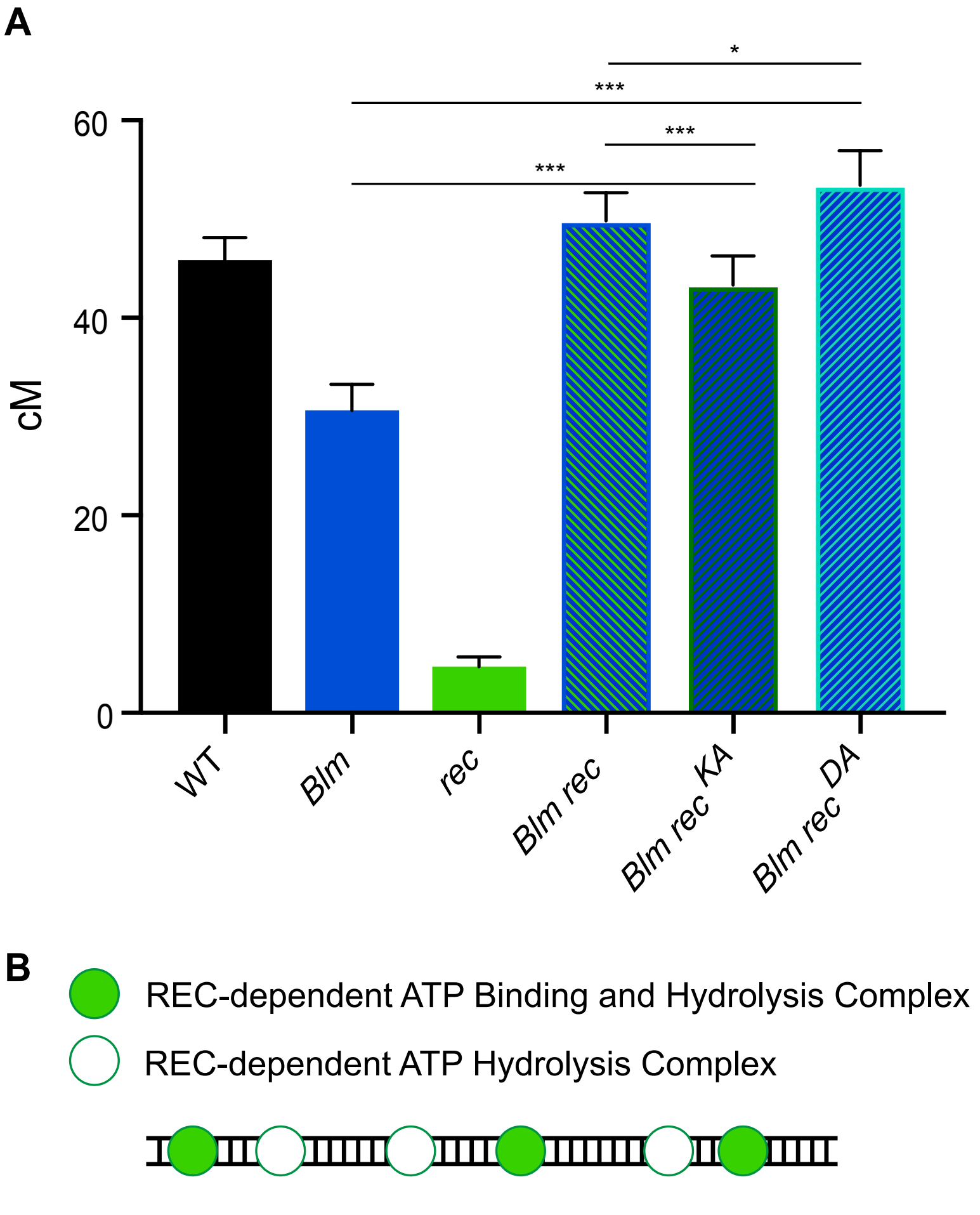
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REC-dependent ATP Hydrolysis Complex