HHS Public Access

Author manuscript

Yeast. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Yeast. 2021 January ; 38(1): 39–53. doi:10.1002/yea.3512.

Expanded roles for the MutL family of DNA mismatch repair proteins

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Abstract

The MutL family of DNA mismatch repair proteins plays a critical role in excising and repairing misincorporation errors during DNA replication. In many eukaryotes, members of this family have evolved to modulate and resolve recombination intermediates into crossovers during meiosis. In these organisms, such functions promote the accurate segregation of chromosomes during the Meiosis I division. What alterations occurred in MutL homolog (MLH) family members that enabled them to acquire these new roles? In this review we present evidence that the yeast Mlh1-Mlh3 and Mlh1-Mlh2 complexes have evolved novel enzymatic and nonenzymatic activities and protein-protein interactions that are critical for their meiotic functions. Curiously, even with these changes, these complexes retain backup and accessory roles in DNA mismatch repair during vegetative growth.

Keywords

Mismatch repair; meiotic crossing over; MLH proteins; adaptation; endonuclease; *Saccharomyces cerevisiae*

1. INTRODUCTION

During meiosis a diploid cell undergoes successive reductional and equational divisions to form haploid gametes. In most organisms, proper chromosome disjunction during the reductional division requires crossing over between homolog pairs (Figure 1). Meiotic crossing over is thought to have evolved from DNA repair mechanisms present in vegetative (somatic) growth to allow for increased genetic recombination and a shift from sister chromatid to interhomolog repair (Barton & Charlesworth, 1998; Cavalier-Smith, 2002; Hunter, 2006; Kleckner, 1996; Marcon & Moens, 2005; Villeneuve & Hillers, 2001). Meiosis-specific functions needed to accomplish these tasks are thought to have emerged from the duplication of genes involved in cellular growth and maintenance, with such events and adaptive mechanisms permitting the acquisition of meiosis-specific gene expression and function. As described below, the MutL homolog (MLH) family of DNA mismatch repair

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interests.

(MMR) proteins provides a clear example of how genes that act in vegetative growth have acquired meiotic functions (Marcon & Moens, 2005).

Yeast provides an ideal model to understand how gene duplication result in the expansion of functional roles for highly conserved proteins. The adaptation of such functions results from changes in gene expression and patterns of post-translational modifications, as well as the gain or loss of interacting protein partners and enzymatic functions. One such example in baker's yeast involves the Rad51 and Dmc1 proteins, which promote early steps in homologous recombination by catalyzing the invasion of a 3' single stranded end originating from a DNA double-strand break into a homologous template (Figure 1; Chan, Zhang, Weissman, & Bishop, 2019; Cloud, Chan, Grubb, Budke, & Bishop, 2012; Crickard, Kaniecki, Kwon, Sung, & Greene, 2018; Steinfeld et al., 2019). These proteins are thought to have evolved early in the evolution of eukaryotes from a single gene duplication of an ancestral archaea RadA recombinase. The duplication of an ancestral gene may have coincided with, or potentially enabled, meiosis (Lin, Kong, Nei, & Ma, 2006; Ramesh, Malik, & Logsdon, 2005). In support of these ideas, Rad51 is the only strand exchange protein present in vegetative growth, whereas both Rad51 and Dmc1 act in meiosis (Bishop, 2012; Bishop, Park, Xu, & Kleckner, 1992; Neale & Keeney, 2006). Rad51 and Dmc1 have biochemical and structural differences as well as unique subsets of protein interactors/regulators (Brown & Bishop, 2014; Chan et al., 2019; Lee et al., 2015; Steinfeld et al., 2019). In meiosis, Rad51 and Dmc1 are both needed to promote recombination between homologs, known as interhomolog bias (Figure 1). Interestingly, in a mutant background that lacks Dmc1 but is activated for Rad51 strand exchange activity, the bias towards interhomolog recombination is disrupted, and obligate crossovers are missing that are required for accurate chromosome segregation (Bishop 2012; Callender et al., 2016; Lao et al., 2013; Cloud et al., 2012). Additional studies showed that Rad51-mediated strand invasion activity is not required for meiotic recombination, but such an activity is critical for Dmc1 function (Cloud et al., 2012). Together, these studies identified a critical role for Dmc1 in strand exchange steps in meiosis. However, Rad51 meiotic functions are still essential in this process; Rad51 acts with the Mei5-Sae3 complex as a Dmc1 accessory factor to assist in the formation of the Dmc1 strand exchange filament (Chan et al., 2019; Cloud et al., 2012).

MutS provides another example of a single bacterial gene that duplicated and evolved early in eukaryogenesis to yield gene families with distinct vegetative growth and meiotic functions (Eisen, 1998; Lin, Nei, & Ma, 2007). In prokaryotic and eukaryotic MMR, MutS homolog (MSH) proteins recognize base-base and insertion/deletion mismatches that escape DNA polymerase proofreading, and transmit the recognition signal to downstream repair proteins such as the MLH family, which coordinate excision of the replication error and DNA resynthesis using the parental DNA strand as a repair template (Figures 2 and 3). Inactivation of MMR results in an increase in mutation rates by up to several orders of magnitude. Such an increase reduces fitness due to the accumulations of deleterious mutations yet can provide a source of beneficial mutations for adaptation to stressful environments (Kunkel & Erie, 2015; Raghavan, Aquadro & Alani, 2019). MutS is thought to have entered archaea and eukaryotes through horizontal gene transfer, followed by a series of gene duplications (Eisen, 1998; Lin et al., 2006). In baker's yeast these events gave rise to

MutS homologs (MSH) that function in the following pathways: 1. Msh2-Msh6 and Msh2-Msh3, which act to repair misincorporation errors during DNA replication and mismatches that form in heteroduplex DNA during genetic recombination; 2. Msh1, which functions in mitochondrial DNA metabolism; and 3. Msh4-Msh5, which promotes crossover formation in meiosis (Figures 1 and 3; Hollingsworth, Ponte, & Halsey, 1995; Kunkel & Erie, 2015; Reenan & Kolodner, 1992; Ross-Macdonald & Roeder, 1994; Snowden, Acharya, Butz, Berardini, & Fishel, 2004). Interestingly, Msh2-Msh6 (base-base and single nucleotide insertion/deletion mismatches) and Msh2-Msh3 (preference for insertion/deletion mismatches of up to 17 nucleotides in size) have evolved different mismatch repair specificities, and the Msh4-Msh5 complex lacks the amino-terminal mismatch recognition domain found in the other yeast MSH proteins, but recognizes Holliday junctions, a critical intermediate in meiotic recombination (Jensen, Jauert, & Kirkpatrick, 2005; Kunkel & Erie, 2015; Lahiri, Li, Hingorani & Mukerji, 2018; Pochart, Woltering, & Hollingsworth, 1997; Snowden et al., 2004).

This review focuses on how the MutL homolog (MLH) family of eukaryotic DNA MMR proteins has evolved distinct vegetative and meiotic life cycle functions; the causes of this specificity in function are not as well studied compared to those identified for the Rad51 and Dmc1 proteins. Most eukaryotes encode at least two MLH heterodimeric complexes that play critical roles in vegetative MMR and/or meiotic recombination (Kadyrov, Dzantiev, Constantin, & Modrich, 2006; Kaydrov et al., 2007; Räschle, Dufner, Marra, & Jiricny, 2002). In baker's yeast three MLH complexes have been identified: Mlh1-Pms1, Mlh1-Mlh2, and Mlh1-Mlh3 (Wang, Kleckner, & Hunter, 1999). All three function during vegetative MMR, with Mlh1-Mlh3 and Mlh1-Mlh2 displaying minor and more specialized roles. (Romanova & Crouse, 2013; Harfe, Minesinger, & Jinks-Robertson, 2000). In meiosis, Mlh1-Mlh3 acts in the biased cleavage of double Holliday junctions to yield crossovers that are critical for the formation of gametes, Mlh1-Mlh2 regulates gene conversion tract lengths, and Mlh1-Pms1, analogous to its role in MMR, repairs mismatches that form in heteroduplex DNA during genetic recombination (Figure 1; Manhart & Alani, 2016; Zakharyevich, Tang, Ma, & Hunter., 2012; Abdullah, Hoffmann, Cotton, & Borts, 2004; Campbell et al., 2014; Duroc et al., 2017; Harfe et al., 2000; Hunter & Borts, 1997). These observations suggest that MLH complexes have evolved different roles during the vegetative and meiotic life cycles of a yeast cell. Such distinct roles have been conserved in higher eukaryotes, and in humans, defects in these factors have been linked to distinct diseases such as hereditary forms of colon cancer and infertility (Gray & Cohen, 2016; Lynch, Snyder, Shaw, Heinen, & Hitchens, 2015).

In addition to being functionally conserved, the MLH family of proteins are structurally conserved (Figure 4). Each subunit in the dimeric MLH complexes contain globular amino (N-) and carboxy (C-) terminal domains connected by linker arms. Structures of some of the N- and C-terminal domains have been solved for prokaryotic and eukaryotic proteins (Figure 4; Arana et al., 2010; Ban & Yang, 1998; Guarne et al., 2004; Gueneau et al., 2013). The N-terminal domain of these proteins contains an ATP-binding site similar in structure to the GHKL family of ATPases (DNA **G**yrase, **H**sp90, histidine **k**inases, Mut**L**) (Dutta & Inouye, 2000). Upon nucleotide binding, the two ATP binding domains in the dimer physically

interact, with the linker domains becoming more ordered, and the MLH complex primed for activation (Ban & Yang, 1998; Sacho, Kadryov, Modrich, Kunkel & Erie, 2008). The main dimerization interface for the two subunits is located at the C-terminal domain, and for most MLH complexes, a metal binding domain is also found at the C-terminus that is critical for endonuclease activity (Guarne et al., 2004; Kadyrov et al., 2006).

What changes occurred in MLH family members that enabled them to acquire distinct vegetative and meiotic roles? As described below, there is strong conservation in the N- and C-terminal domains of the MLH proteins, but there are also amino acid sequences that are uniquely conserved for each MLH protein that likely confer different enzymatic functions and protein-protein interactions for the different MLH complexes. Also, the MLH proteins contain highly variable and non-conserved intrinsically disordered linker arms that range in size from about 100 amino acids (aa) in bacteria to 300 aa in yeast (Figure 5; Guarne et al., 2004; Sacho et al., 2008). In higher eukaryotes these linkers can approach 650 aa in length, as seen for the mouse and human Mlh3 linkers (Lipkin et al., 2000). The properties of these linkers have been proposed to facilitate conformational changes needed to activate latent MutL activities (Sacho et al., 2008), and recent studies in bacteria and yeast suggest that the linker arms are also important for MLH proteins to overcome barriers in the DNA landscape that would need to be traversed to locate the replication machinery and MSH proteins (Kim, Furman, Manhart, Alani, & Finkelstein, 2019; Mardenborough et al., 2019; Plys, Rogacheva, Greene, & Alani, 2012). Why linker lengths show such variability is not known, but they too could have evolved for different life cycle functions.

2. OVERVIEW OF PROKARYOTIC MUTL PROTEINS AND THEIR RELATIONSHIP TO EUKARYOTIC MLH PROTEINS

Through genetic, *in vitro* reconstitution, and single-molecule analyses, scientists have obtained a thorough understanding of MMR in the Gram-negative bacteria *E. coli*. This work has provided detailed mechanistic information with respect to mismatch recognition, strand discrimination, and repair DNA synthesis steps (Figure 2; Jiricny 2013; Lahue, Au, & Modrich, 1989; Lamers et al., 2000; Liu et al., 2016; Modrich & Lahue, 1996; Obmolova, Ban, Hsieh, & Yang, 2000). In the model for *E. coli* shown in Figure 2, MMR is initiated by the MutS homodimer binding to a mismatch. This binding causes an ATP-dependent conformational change in MutS to form a sliding clamp that recruits the MutL homodimer (Acharya et al., 2003; Liu et al., 2016). The MutS-MutL complex then activates MutH, a restriction endonuclease-like protein, which nicks the unmethylated strand of hemimethylated DNA at d(GATC) sites. Importantly, *E. coli* uses the time interval needed for DNA adenine methyltransferase (Dam) to methylate newly replicated DNA at d(GATC) sites as a strand discrimination signal for MutH incision. After activating MutH incision, MutL recruits the UvrD helicase to unwind the newly replicated strand containing the mismatch, which in turns allows for single stranded exonucleases with different polarities (RecJ, ExoI, ExoVII, ExoX) to excise mismatches 5’ or 3’ to the nicked site (Burdett et al., 2001, but see Liu et al. (2019) for an exonuclease-independent UvrD unwinding model). MMR is completed upon DNA resynthesis by DNA Polymerase III, followed by sealing of nicks by DNA ligase.

In addition to roles in MMR, MutS and MutL act to prevent recombination between divergent homologous sequences, a process that is also thought to promote speciation. This heteroduplex rejection mechanism, which involves interactions with non-MMR proteins, acts to prevent deleterious recombination events, and is also seen in eukaryotes (Chakraborty & Alani, 2016; Rayssiguier, Thaler, & Radman, 1989; Worth, Clark, Radman, & Modrich, 1994).

E. coli is an outlier among prokaryotes in having acquired DNA methylation to distinguish template from nascent strands during replication fork passage. The acquisition of Dam and MutH nuclease functions is thought to be accompanied by the loss of MutL endonuclease activity (reviewed in Putnam 2016). In fact, most prokaryotes and all eukaryotes examined lack a MutH-Dam mechanism (Lenhart, Pillon, Guarné, Biteen, & Simmons, 2016), and in these organisms, MutL family proteins display an intrinsic endonuclease activity that is thought to be directed to nick the nascent strand through orientation-specific interactions with the DNA polymerase processivity clamp (β-clamp in prokaryotes, PCNA in eukaryotes; Kadyrov et al., 2006; Kadyrov et al., 2007; Pillon et al., 2010). While it is not known how this interaction occurs, recent studies have suggested that the discontinuity of Okazaki fragments on the lagging strand, processivity clamps remaining on DNA after replication, replication restart on the leading strand, and excision of ribonucleotides could provide the correct loading of the processivity clamp for strand-specific nicking by MLH endonucleases (Heller & Marians, 2006; Kawasoe, Tsurimoto, Nakagawa, Masukata, & Takahashi, 2016; Kunkel & Erie, 2015; Pluciennik et al., 2010).

3. BAKER'S YEAST MLH PROTEINS

3.1. MLH1-PMS1 PLAYS A MAJOR ROLE IN MMR

In current models for eukaryotic MMR (Figure 3; Kunkel & Erie, 2015), once an MSH complex binds to a mismatch, it undergoes, like MutS, an ADP-ATP exchange that alters the conformation of the complex to form a sliding clamp, allowing it to recruit the major MLH MMR complex, Mlh1-Pms1 (Gradia et al., 1999; Gorman et al., 2012). During this process, MSH heterodimers can interact with the replication processivity clamp PCNA that is loaded onto DNA by clamp loader (Clark, Valle, Drotschmann, Gary, & Kunkel, 2000; Johnson et al., 1996; Kleczkowska, Marra, Lettieri, & Jiricny, 2001). The MSH-PCNA interaction has been hypothesized to connect at least a portion of the MSH proteins to the replication fork and inhibit the unloading of PCNA during MMR, thus increasing the time interval for MMR (Hombauer, Campbell, Smith, Desai, & Kolodner, 2011; Kawasoe, Tsurimoto, Nakagawa, Masukata, & Takahashi, 2016; Kleczkowska, Marra, Lettieri, & Jiricny, 2001). PCNA then activates the endonuclease activity of Mlh1-Pms1 to nick the newly replicated daughter strand (Erdeniz, Dudley, Gealy, Jinks-Robertson, & Liskay, 2005; Kadyrov et al., 2006; Kawasoe, Tsurimoto, Nakagawa, Masukata, & Takahashi, 2016; Pluciennik et al., 2010). These nicks can facilitate two excision pathways (Figure 3). One is Exo1-dependent, where a nick 5′ to the mispair serves as an entry site for ExoI, a 5' to 3' exonuclease, to enter the DNA and excise DNA containing the mismatch. The resulting single-stranded DNA gap is coated by Replication Protein A (RPA), which facilitates DNA resynthesis steps. The second is Exo1-independent; Mlh1-Pms1 makes multiple nicks in the vicinity of the mismatch,

allowing for strand displacement by Polymerase δ or ε. In both Exo1-dependent and independent mechanisms, nicks are sealed by DNA ligase, thus repairing the DNA and maintaining the original genetic information (reviewed in Goellner, Putnam, & Kolodner, 2015).

3.2. MLH1-MLH3 PLAYS A MAJOR ROLE IN MEIOTIC CROSSING OVER

Mlh1-Mlh3 plays a minor role in MMR; genetic studies have suggested that it is recruited by Msh2-Msh3 to repair primarily insertion/deletion mismatches (Flores-Rozas & Kolodner, 1998; reviewed in Manhart & Alani, 2016; Romanova & Crouse, 2013). However, Mlh1-Mlh3 plays a critical role in resolving recombination intermediates during meiotic crossing over, with *mlh1* and *mlh3* null mutants showing meiotic crossover defects (Hunter & Borts, 1997; Manhart & Alani, 2016).

Meiotic recombination is initiated by Spo11, which catalyzes 150–200 DNA double strand breaks genome-wide (Figure 1; Keeney, Giroux, & Kleckner, 1997; Pan et al., 2011; Pyatnitskaya, Borde, & De Muyt, 2019; Robine et al., 2007). The DSBs are resected in a 5' to 3' direction in steps involving Exo1 to form 3' single stranded tails at each side of the double-strand break. The RecA family proteins Dmc1 and Rad51 form a filament on the tails and invade a homologous template, creating a displacement loop (D-loop; Brown, Grubb, Zhang, Rust, & Bishop, 2015). The D-loop intermediates serve as a key substrate for interconnected recombination pathways. In one pathway, the Sgs1-Top3-Rmi1 helicase/topoisomerase complex (STR), mediates the disassembly of the D-loop intermediate to form noncrossovers in a process known as synthesis-dependent strand annealing. In a second pathway the D-loop is stabilized by a class of ZMM (yeast Zip1/Zip2/Zip3/Zip4, Msh4/Msh5, Mer3) proteins to form a single-end invasion intermediate (SEI; Hunter and Kleckner, 2001; Pyatnitskaya, Borde, & De Muyt, 2019). Second-end capture of this intermediate, followed by branch migration, DNA synthesis, and ligation, results in the formation of a double-Holliday junction (dHJ) which is resolved in a biased manner to form crossovers (class I) that display interference (Allers & Lichten, 2001; Hunter, 2006; Zakharyevich et al., 2012). In a third pathway the D-loop progresses to form dHJs that are resolved by structure selective nucleases such as Mus81-Mms4 to form noncrossovers and crossovers (class II) that lack interference and are distributed randomly (rather than the tendency for crossovers to be evenly spaced; de los Santos et al., 2003; De Muyt et al., 2012; Zakharyevich, Tang, Ma, & Hunter, 2012).

Work in yeast demonstrated that the dHJs formed in the ZMM pathway are resolved through the actions of the Msh4-Msh5, Exo1, and the Mlh1-Mlh3 endonuclease. Specifically, Msh4-Msh5 acts to stabilize single-end invasion and Holliday junctions, after which Mlh1-Mlh3 and Exo1 promote biased resolution of double-Holliday junctions to form crossovers (Borner, Kleckner, & Hunter, 2004; Manhart et al., 2017; Ranjha, Anand, & Cejka, 2014; Rogacheva et al., 2014; Snowden et al., 2004; Zakharyevich et al., 2012). This model is supported by genetic and physical studies demonstrating that Mlh1-Mlh3 acts downstream of Msh4-Msh5 in meiosis (Kolas et al., 2005; Moens et al., 2002). Relevant to these studies is the work of Marsolier-Kergoat, Khan, Schott, Zhu, & Llorente (2018), who used a high-throughput DNA sequencing approach to analyze the meiotic progeny of a hybrid yeast

strain. In their study they analyzed heteroduplex DNA tracts associated with noncrossover and crossover events. Interestingly, they observed a biased pattern of crossover resolution events consistent with the resolution of double-Holliday junctions being directed towards strands containing newly replicated DNA near the junctions. These observations suggest that cleavage of double-Holliday junctions through the actions of the Mlh1-Mlh3 endonuclease might be similar to that seen for the Mlh1-Pms1 nuclease during MMR, rather than Mlh1-Mlh3 acting as a structure-specific nuclease that binds and symmetrically cleaves Holliday junctions (see Mlh1-Mlh3 polymerization models presented in Manhart et al., 2017).

It is important to note that the Mlh1-Mlh3 complex is likely to play similar roles in higher eukaryotes. For example, *Mlh3*$^{-/-}$ mice are viable, but sterile, and display dramatic decreases in meiotic crossovers. *Mlh3*$^{-/-}$ spermatocytes undergo apoptosis after metaphase, whereas oocytes fail to complete Meiosis I (Lipkin et al., 2002). These findings suggest that like in Baker's yeast, Mlh3 plays a critical role in higher eukaryotes to direct the maturation of recombination intermediates into crossovers, promote accurate chromosome segregation, and generate viable gametes. In humans, there are few clinical studies linking *hMLH3* polymorphisms to infertility; however, a detailed analysis of population variants in *hMLH3* could provide mechanistic insights (Markandona et al., 2015).

Many questions remain for how Mlh1-Mlh3 resolves double-Holliday junctions into crossover products. It is not fully understood what kinds of chromosomal architecture or protein-protein interactions are required to stabilize Mlh1-Mlh3 or direct its function (Manhart et al., 2017). There is clear evidence for post-translational modifications regulating meiotic crossover formation (He et al., 2020; Hollingsworth & Gaglione, 2019; Sanchez et al., 2020; Sourirajan & Lichten, 2008; Wild & Matos, 2016; Wild et al., 2019). For example, the structure-specific endonucleases Mus81-Mms4 and Yen1 that act in the interference-independent crossover pathway are activated and inhibited, respectively, by phosphorylation (Figure 1; Matos, Blanco, Maslen, Skehel, & West, 2011; Wild & Matos, 2016). At present there is no evidence for phosphorylation directly regulating Mlh1-Mlh3 endonuclease activity, but it is likely that factors that interact with Mlh1-Mlh3 are regulated by phosphorylation and/or other modifications such as SUMOylation (Cheng et al., 2006; He et al., 2020; Manhart & Alani, 2016). It is also of interest to understand how the chromatin landscape is regulated to allow access of Mlh1-Mlh3 to recombination substrates (Wild et al., 2019).

Recent studies have also suggested roles for Mlh1-Mlh3 in chromosome disjunction and crossover interference (Chakraborty et al., 2017; Claeys-Bouuaert & Keeney, 2017). Crossover interference, seen in many organisms such humans, mice, *Drosophila*, and *C. elegans*, regulates the spatial distribution of crossovers; the formation of a crossover lowers the probability of a second crossover nearby (Wang et al., 2019). Widely spaced crossovers, exhibiting interference, help to ensure the fidelity of chromosome segregation. How Mlh1-Mlh3 participates in these roles is unclear.

Lastly, recent studies in yeast and higher eukaryotes have suggested a role for Mlh1-Mlh3 in mediating somatic CAG trinucleotide repeat instability (Pinto et al., 2013; Su & Freudenreich, 2017). In humans, CAG repeat expansions in exon 1 of the *HTT* gene results

in Huntington's disease, an inherited neurodegenerative disease, where age of disease onset depends on the length of the CAG repeat tract. It is unclear whether Mlh1-Mlh3 acts alone, or if this process also requires other MLH proteins (see Gomes-Pereira, Fortune, Ingram, McAbney, & Monckton, 2004). It could be that, analogous to MMR, there is some partial redundancy to Pms1 and Mlh3's role in generating expansions. Curiously, Mlh1-Mlh3 has been shown to bind specifically to, but not cleave, DNA branch structures such as Holliday junctions (Ranjha, Anand, & Cejka, 2014; Rogacheva et al., 2014), substrates that could resemble intermediates that form during trinucleotide repeat expansion.

3.3. MLH1-MLH2 MODULATES MEIOTIC RECOMBINATION TRACT LENGTH

Unlike Mlh3 and Pms1, Mlh2 lacks an endonuclease motif, and although *mlh2* mutants display sensitivity to DNA damaging agents, deletion of *MLH2* confers only a mild mutator phenotype. Harfe et al. (2000) demonstrated that although not required for vegetative MMR, Mlh2 has a minor role in the repair of frameshift mutations. Additionally, Campbell et al. (2014) showed that Mlh1-Mlh2 plays a non-essential role as an accessory factor in DNA MMR, but whose function becomes more significant when essential MMR factors become limiting. More specifically, they showed through live cell imaging that Mlh2 forms foci in the presence of mispaired DNA, and that focus formation was dependent on *MSH2*, *MSH6*, and *MLH1*. Biochemical assays revealed that Mlh1-Mlh2 is recruited to DNA mismatches by both Msh2-Msh6 and Msh2-Msh3, and *in vivo* studies showed that deletion of *MLH2* in backgrounds where essential MMR proteins were missing or reduced caused synergistic increases in mutation rate (Campbell et al., 2014). Taken together, these findings suggest that Mlh2 plays a minor role in MMR, potentially overlapping with Pms1 functions.

In contrast to minor roles in MMR, Mlh2 has been implicated through genetic analyses to interact with the Mer3 helicase to regulate meiotic gene conversion tract lengths (Figure 1; Abdullah et al., 2004; Duroc et al., 2017; Marsolier-Kergoat et al., 2018). Furthermore, Abdullah et al. (2004) suggested, in their analysis of meiotic recombination in *mlh2 msh4* and *mlh2 msh5* double mutants, that Mlh2 channels meiotic recombination intermediates into the Msh4-Msh5 interference-dependent meiotic crossover pathway. Consistent with regulating meiotic gene conversion tracts, Duroc et al. (2017) showed that the Mer3 helicase interacted with Mlh1-Mlh2 *in vitro* and *in vivo*, Mer3 and Mlh1-Mlh2 each preferentially bound to D-loop intermediates, Mlh2 was recruited to meiotic recombination hotspots through its interaction with Mer3, and Mlh2 recruitment did not require the presence of DNA mismatches or interactions with mismatch recognition factors. These studies suggested Mer3, initially in a structural role, binds to D-loop intermediates, and then recruits Mlh1-Mlh2 to prevent excessive D-loop extension and DNA synthesis. Such a mechanism was hypothesized to limit the exposure to DNA sequences that could participate in inappropriate meiotic recombination events that result in genome rearrangements, as well as to reduce the fraction of the genome converted at each meiosis (Duroc et al., 2017).

It is unclear whether Mlh2's role in repairing frameshift mutations is mechanistically related to its meiotic recombination role, especially because its meiotic role appears distinct from MMR. A phylogenetic analysis of for Mlh2 in the unikont taxa revealed that *S. cerevisiae MLH2* is the homolog to metazoan *PMS1* (*S. cerevisiae PMS1's* homolog is metazoan

PMS2), which also lacks an endonuclease motif and exhibits a weak mutator phenotype (Campbell et al., 2014). *MLH2* homologs were identified in species that diverged from *S. cerevisiae* prior to the whole genome duplication event, suggesting that the origin of *MLH2* predated the yeast whole genome duplication event. Interestingly, a subset of unikont genomes contain a yeast *MLH3* homolog but lack the yeast *MLH2* homolog (the human homolog is *PMS1*), and other unikonts lack both yeast *MLH3* and *MLH2* homologs (see Vakirlis et al., 2016). Also, in a small number of fungal species, *MLH2* homologs contained stop codons and frameshifts that could reflect inactivation of the *MLH2* gene, providing support for accessory roles for Mlh2 (Campbell et al., 2014).

4. HOW DIFFERENT ARE THE ACTIVITIES OF MLH FAMILY PROTEINS?

There are many structural and functional similarities and differences between the Pms1, Mlh2, and Mlh3 proteins (Figure 4). Pms1 and Mlh3 share highly conserved functional domains: an ATP binding site in the N-terminus that drives the conformational changes observed in MLH proteins, an intrinsically disordered linker arm, and a C-terminus containing the endonuclease motif and Mlh1 dimerization site. All three proteins compete for the same dimerization/binding site on the Mlh1 protein, and this competition could regulate the levels of each heterodimer present at each stage of the yeast life cycle (Kondo, 2001). One interesting feature of these proteins is the variability in the lengths of their intrinsically disordered linkers (Figure 5). As mentioned earlier, the linker domains are poorly conserved, and in yeast the length of the Pms1 linker is twice that of Mlh1 and three-times that of Mlh3. Curiously, the Mlh3 linker has expanded significantly in higher eukaryotes. The mouse and human Mlh3 linkers are roughly six-times larger than the yeast linker. While the functions of these linkers are thought to primarily regulate ATP-driven conformational changes, a difference in length and sequence could facilitate physical interactions with other proteins or DNA, or serve as substrates for post-translational modifications (Claeys Bouuaert & Keeney, 2017; Sacho, Kadryov, Modrich, Kunkel & Erie, 2008; Kim et al., 2019).

4.1. MLH ENDONUCLEASE DOMAINS SHOW SUBTLE DIFFERENCES

Mlh1-Pms1 and Mlh1-Mlh3 display endonuclease activities dependent on highly conserved metal binding motifs present in both Pms1 and Mlh3 (Figure 6; Gueneau et al., 2013; Kadyrov et al., 2006; Rogacheva et al., 2014). Five conserved residues predicted to form the endonuclease active site in Pms1 were found in homologous positions in Mlh3. These proteins maintain the ancestral endonuclease motif, but also contain distinct residues that are likely required for their function and pathway specificity. The outlier is Mlh2, which has very limited conservation in its C-terminal domain, has lost the conserved endonuclease motif, and has no characterized enzymatic function. However, Mlh2 has adopted a novel function in regulating the length of gene conversion tract lengths, as well as playing a role as an accessory factor in MMR, but this was only seen when Pms1 levels were reduced (Abdullah et al., 2004; Campbell et al., 2014; Duroc et al., 2017). The endonuclease motif in Mlh3's C-terminus also overlaps with the Mlh1 dimerization domain. Al Sweel et al. (2017) identified alleles in the endonuclease motif that disrupt Mlh1-Mlh3 interaction, but interestingly, these alleles are functional in meiotic crossing over and defective in MMR.

These observations suggest the presence of other protein-protein interactions during meiosis that stabilize Mlh1-Mlh3 and promote crossover resolution. The identification and characterization of such interactions would also allow us to better understand how protein-protein interactions provide pathway specificity.

4.2. MLH PROTEINS INTERACT WITH SPECIFIC SETS OF PROTEINS

The MLH proteins do not act in isolated systems. The kinds of selective forces that drove the evolutionary adaptation of these genes also likely acted on other genes in the same pathway or molecular network (Clark, Alani, & Aquadro, 2013). For example, in MMR, the endonuclease activity of Mlh1-Pms1 is activated through its interaction with the DNA processivity factor PCNA. PCNA is a ring-shaped structure that acts to enhance the processivity of DNA polymerase during replication by creating a topological link with the DNA template and enabling sliding during chain elongation (Kelman, 1997; Pillon, Miller, & Guarné, 2011). Eukaryotic Msh2-Msh6 and Mlh1-Pms1 both interact with PCNA through the PCNA-interacting motif (PIP Box), which is defined as a six amino acid residue consisting of Qxφ[L/I]xP, where φ is a hydrophobic residue and x is any amino acid (Kelman, 1997; Pillon, Miller, & Guarné, 2011). The endonuclease activity of yeast Pms1 is highly stimulated by PCNA through the PIP-motif located in close proximity to the endonuclease motif (Genschel et al., 2017; Pluciennik et al., 2010). Mlh3, however, lacks any sort of motif at this specific site (Figure 6). Historically, *in vitro* studies have shown no requirement for PCNA to stimulate the endonuclease activity of Mlh1-Mlh3 (Ranjha, Anand, & Cejka, 2014; Rogacheva et al., 2014). However, studies (Cannavo et al., 2020; Kulkarni et al., 2020), have showed that PCNA stimulates the Mlh1-Mlh3 endonuclease activity in conjunction with Msh4-Msh5 and Exo1, and one group (Cannavo et al., 2020) characterize a potential PIP-motif in Mlh1, but whether this is a bonafide PCNA interaction site is not clear (Genschel et al., 2017; Lee & Alani, 2006). One explanation for these properties is that the MLH proteins have undergone changes in their ancestral catalytic motif to bind and resolve different substrates, and/or have shifted interactions with protein partners during MMR and meiosis.

4.3. DO MLH PROTEINS SHARE FUNCTIONS?

MLH proteins are likely to share many functions, with some of their specificities driven by recruitment to different substrates or different MSH proteins. For example, Mlh1-Mlh2 meiotic functions in limiting gene conversion tracts could be explained by its interactions with Mer3, and Mlh1-Mlh3's functions actions in meiotic crossing over could be explained through its recruitment by Msh4-Msh5 to recombination intermediates (Duroc et al., 2017; Kolas et al., 2005; Moens et al., 2002). In support of this idea, work by Marsolier-Kergoat et al. (2018) showed that cleavage of double Holliday junctions, presumably through the actions of the Mlh1-Mlh3 endonuclease, was directed towards strands containing newly replicated DNA; this mechanism is analogous to Msh2-Msh6 or Msh2-Msh3 and PCNA directing the endonuclease activity of Mlh1-Pms1 to the newly replicated strand during MMR. Further support for this idea comes from studies which showed that Msh2-Msh3 recruits Mlh1-Mlh3 primarily for the repair of deletion mispairs (Flores-Rozas & Kolodner, 1998; Romanova & Crouse, 2013). Thus, the recruitment of MLH complexes to specific substrates by specialized MutS proteins could contribute to our understanding of how

protein-protein interactions influence the difference in functionality. Further studies on Mlh1-Mlh3's protein-protein interactions in meiosis would allow us to understand how DNA binding proteins and protein-protein interactions of MutL proteins evolved to adapt to meiotic processes. For example, if the residues that specify different MSH interactions were identified, and then introduced into the different MLH proteins, one could test if such interactions are sufficient for specificity, or if additional changes have occurred in the MLH proteins that specify their functions such as acquiring novel DNA binding activities.

4.4. BIOINFORMATIC AND STRUCTURAL APPROACHES

In addition to analyzing protein-protein interactions as descried above, bioinformatic strategies can be used to study the specificity of MLH complexes. Steinfeld et al. (2019) identified and analyzed amino acid residues that are well conserved within the Rad51 or Dmc1 lineage but differ between the two recombinases. This same methodology can be applied through computational approaches. For example, Multi-Harmony uses multiple sequence alignments between subfamilies of proteins, homology models, and multi-Relief and sequence-Harmony algorithms to identify amino acids that may suggest functional specificity (Brandt, Feenstra, & Heringa, 2010). By aligning Mlh3 and Pms1 amino acid sequences from 19 different budding yeast species, multi-Harmony alignments can identify residues that are well conserved within each subfamily but differ between Mlh3 and Pms1 (Figure 6). Having a three-dimensional structure of Mlh3 would allow for these residues to be mapped precisely.

4.5 REGULATION BY POST-TRANSLATIONAL MODIFICATIONS

At present there is little evidence in yeast to suggest that any of the MutL proteins are functionally regulated by phosphorylation. However, in mammalian cells, post translational modifications of MLH proteins have been proposed to regulate their functions. For example, recent studies have suggested that human Mlh1-Pms2 is phosphorylated by Casein Kinase II (CK2) at Mlh1's S477 *in vitro*. Phosphorylated Mlh1 loses MMR activity and levels of phosphorylated Mlh1 vary during the cell cycle. In tumors, CK2 is overexpressed appears to inactivate MMR. This could be analogous to mechanisms of tumorigenesis where hyper-methylation of Mlh1 drives microsatellite instability (Webbecher & Brieger, 2018). The modification of MLH proteins in yeast is not well studied but could be an interesting avenue to explore with respect to understanding unique MLH functions.

5. CONCLUSIONS AND FUTURE DIRECTIONS

This review is focused on understanding how and why eukaryotes acquired multiple MLH complexes that act in the vegetative and meiotic stages of the yeast life cycle. In addition to the MLH, MSH, and Rad51/Dmc1 examples presented, many other components of the homologous recombination machinery have been adapted to function in meiosis (Marcon & Moens, 2005; Steinfeld et al., 2019). Thus, studies performed with MLH proteins will likely provide a model to understand how large classes of proteins have adapted novel roles. Such adaptations could involve new partner interactions (see Wild et al., 2019) or yield novel biochemical functions. We believe that a combination of structural (Figure 4) and computational evolutionary (e.g. using Multi-Harmony), analyses of MLH family proteins

will encourage the creation of chimeric proteins that show how MLH proteins have evolved meiotic roles. We are optimistic that such an approach will be useful because aspects of this strategy have been successfully used by Steinfeld et al. (2019) to identify functional differences between Rad51 and Dmc1.

Molecular evolution provides another approach to study how proteins have adapted to new roles in the yeast life cycle. In work performed by Hsieh, Makrantoni, Robertson, Marston, & Murray (2020), the vegetative cohesin Scc1 was replaced with vegetative growth expression of the meiotic cohesin paralog Rec8. After passaging this strain through 1,750 vegetative growth generations, Hsieh et al. (2020) identified evolved fit populations that acquired mutations primarily in known and novel partner proteins of cohesins. Thus, this work provided a clear example of how one could “turn back the clock” to understand how proteins evolved to take on new roles. For example, a strain lacking Pms1 could be evolved to become fully functional in MMR. Such an experiment could be used to learn how the Mlh2, Mlh3, and Pms1 proteins diverged, with the goal of identifying novel protein domains and/or interacting partners.

ACKNOWLEDGMENTS

We thank Beata Mackenroth, Sara Liang, Michael Lichten, Nathan Clark, and anonymous reviewers for helpful comments on the manuscript, and Gianno Pannafino for help on the protein structure analysis of MLH proteins. C. M. F., R. E., and E.A. are supported by the National Institute of General Medical Sciences of the National Institutes of Health: R35GM134872. R. E. was also funded by the Cornell McNair Scholars Program, and the Frank L. and Lynnet Douglas Fellowship in Chemistry, Chemical Biology, and Biochemistry. The content of this review is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations:

aa	amino acid
C-terminal	carboxy terminal
D-loop	displacement loop
Dam	DNA adenine methyltransferase
HJ	double-Holliday junction
HR	homologous recombination
MLH	MutL homolog
MMR	mismatch repair
MSH	MutS homolog
N-terminal	amino-terminal
PCNA	proliferating cell nuclear antigen
RPA	replication protein A

SEI	single-end invasion
ZMM	Zip1/Zip2/Zip3/Zip4, Msh4/Msh5, Mer3

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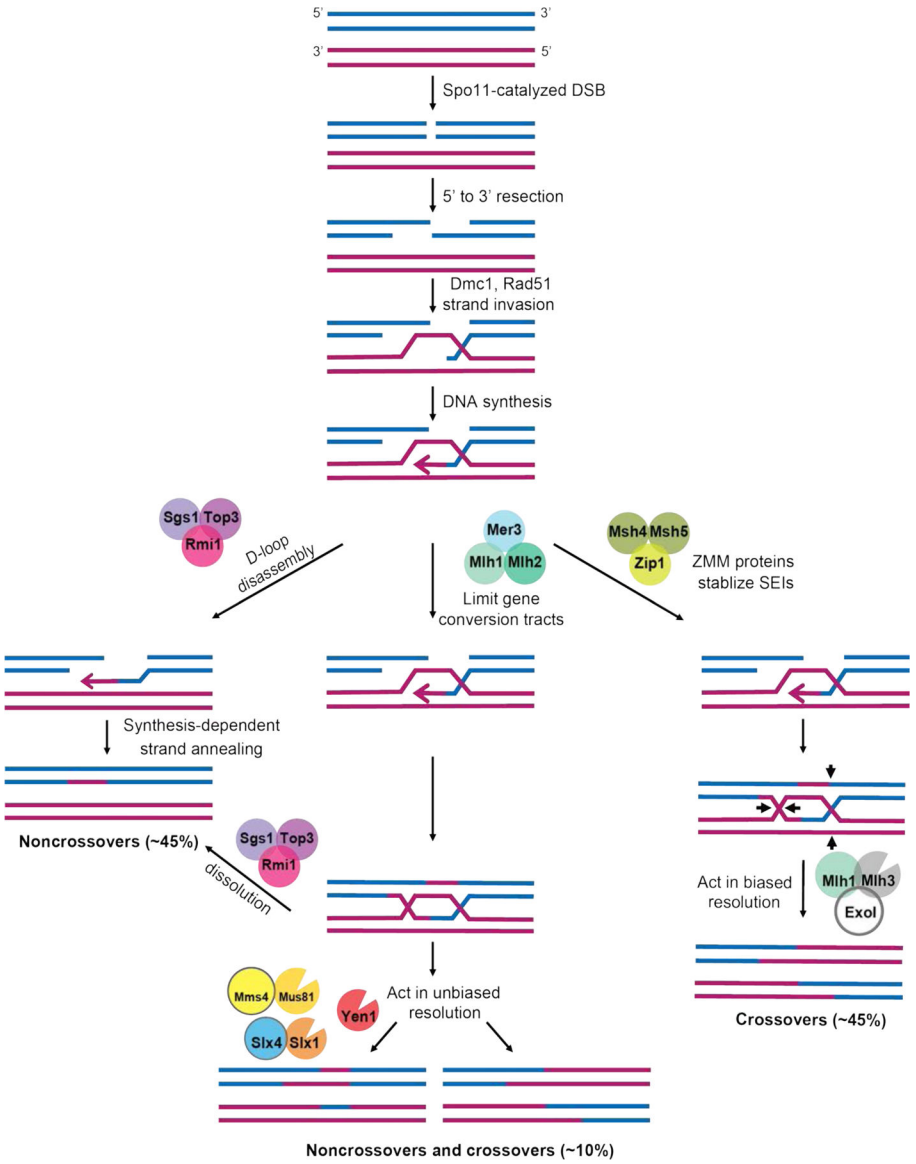

FIGURE 1. A model indicating roles for MLH proteins in meiotic recombination.

In diploid yeast induced to undergo meiosis, Spo11 catalyzes double-strand breaks (DSBs) throughout the genome, which undergo 5' to 3' resection. The resulting 3' single stranded tail can invade the homologous chromosome to form a D-loop intermediate. In the ZMM-stabilized (Class I) pathway, the single end invasion intermediate (SEI) is stabilized by the actions of Msh4-Msh5 and Zip3 to promote D-loop extension through DNA repair synthesis. A double Holliday junction (dHJ) is then formed by second end capture that is asymmetrically cleaved in an Mlh1-Mlh3-dependent step to yield primarily crossover products. Note that in this model branch migration of Holliday junctions can occur but is not shown. Noncrossovers and a minority of crossovers (class II) are thought to occur through the other pathways shown (reviewed in Manhart & Alani, 2016; Wild & Matos, 2016; Zakharyevich et al., 2012). Roles for the Mlh1-Mlh2 complex in limiting gene conversion tract length, and Mlh1-Mlh3 in biased resolution of double-Holliday junctions to form

crossovers, are shown. Mlh1-Pms1 acts to repair DNA mismatches that form in heteroduplex DNA in all pathways. The distribution of the types of meiotic recombination events in baker's yeast is approximate and was calculated based on studies showing that Spo11 catalyzed DSBs produce equal numbers of crossovers and noncrossovers (Mancera, Bourgon, Brozzi, Huber, & Steinmetz, 2008; Martini, Diaz, Hunter, & Keeney, 2006; Marsolier-Kergoat et al., 2018). Of the crossovers, 85% are estimated to be class I, and 15% class II (Cooper et al., 2018; Jessop & Lichten, 2008; Oh, Lao, Taylor, Smith, & Hunter, 2008). Of the noncrossovers, 15% are estimated to result from the resolution of dHJs in the class II pathway (DeMuyt et al., 2012), with the remainder occurring through other mechanisms such as synthesis-dependent strand annealing. See text for details and Marsolier-Kergoat et al. (2018) and Pyatnitskaya, Borde, & De Muyt (2019) for more detailed models.

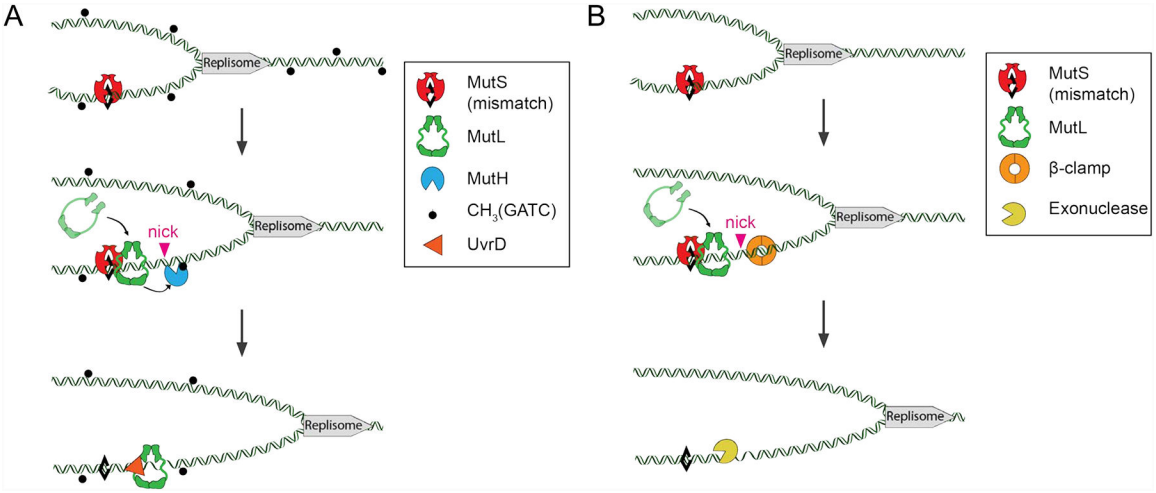

FIGURE 2. Model for prokaryotic MMR.

A model for mismatch repair in bacteria. A mismatch in DNA formed due to DNA polymerase misincorporation/DNA slippage is shown as a black diamond. MutS binds to the mismatch and undergoes an ATP-dependent conformational change to act as a sliding clamp and recruit MutL. (A) In *E. coli*, the MutS-MutL complex then acts as a sliding clamp complex that recruits MutH to nick the unmethylated (newly replicated) strand at hemi-methylated d(GATC) sites. These nicks allow for the helicase UvrD to unwind the DNA which is then excised, by a variety of nucleases, depending on the polarity of the nick relative to the mismatch. Alternatively, UvrD and MutL processively unwind the DNA between two d(GATC) sites (depicted). The resulting gap is then filled in by DNA polymerase. (B) In most other bacteria, MutL contains an intrinsic endonuclease activity that is stimulated by the DNA replication processivity factor β-clamp. These nicks act as entry sites for exonucleases to excise the mismatch, followed by re-synthesis of the gapped DNA by DNA polymerase. It is not clear if a UvrD helicase acts during MMR in bacteria that do not use MutH and Dam methylase. In such bacteria a recent model suggests that analogous to eukaryotic MMR, an exonuclease acts on double-stranded DNA to excise the mismatch, thus not requiring a UvrD helicase type activity (as shown, see Lenhart, Pillon, Guarne, Biteen, & Simmons, 2016).

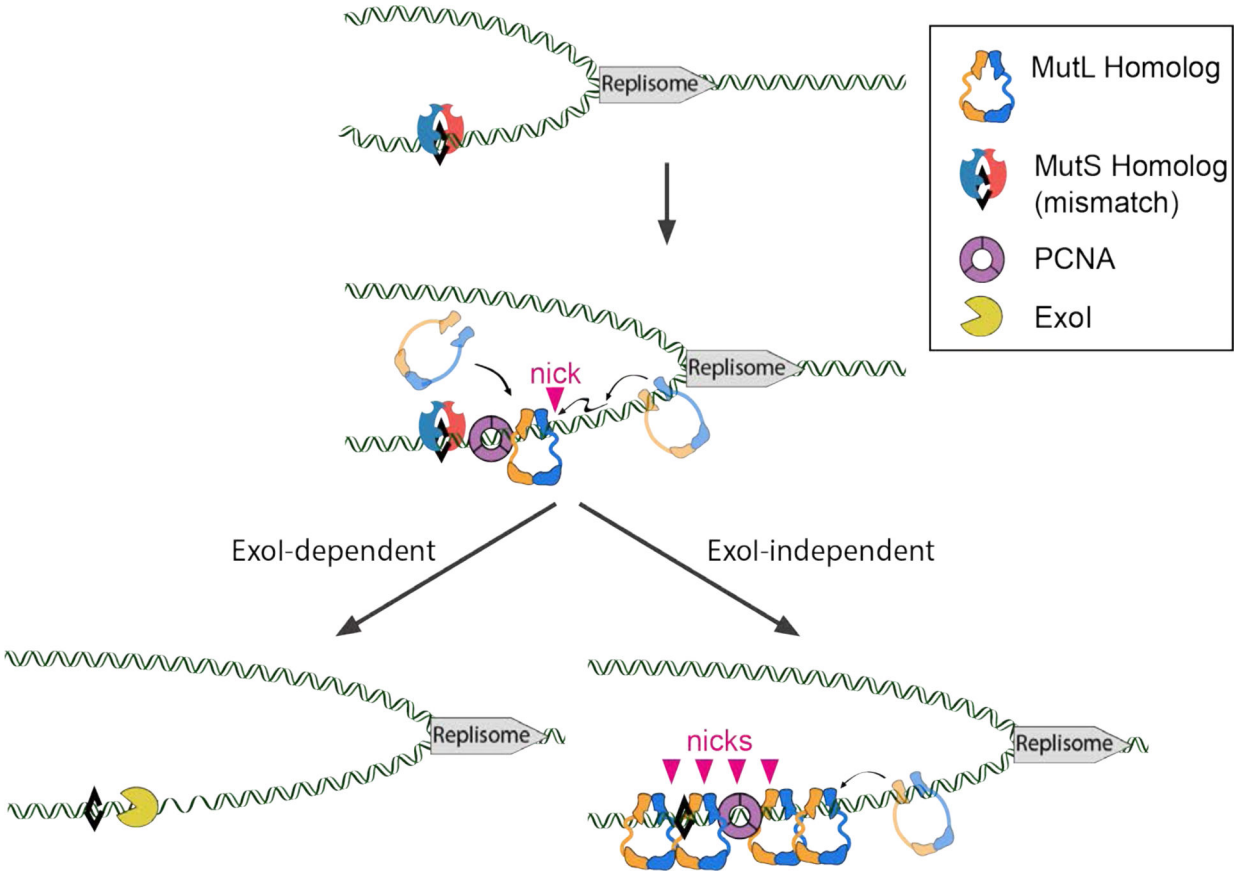

FIGURE 3. Model for MMR in baker's yeast.

A mismatch in DNA formed due to DNA polymerase misincorporation is shown as black diamond. MSH complexes (Msh2-Msh3 or Msh2-Msh6) bind to the mismatch and undergo an ATP-dependent conformational change that allows them to act as a sliding clamp and recruit MLH complexes. Mlh1-Pms1 or Mlh1-Mlh3 undergo ATP-dependent conformational changes and orientation-specific interactions with PCNA (see text for a review of hypotheses for how these interactions with PCNA could be initiated), resulting in incision of the newly replicated DNA strand. This is followed by two potential pathways, ExoI-independent and -dependent, that can recruit downstream repair factors to excise and resynthesize the DNA and ligate the repaired strand. In the ExoI-dependent pathway, ExoI uses the nicks made by MLH complexes as an entry site to excise the mismatch containing DNA in a 5'−3' direction. In the ExoI-independent pathway, multiple nicks made by MLH complexes in the vicinity of the mismatch promote DNA strand displacement and synthesis by DNA polymerase to repair the mismatch. Adapted from Kim et al., (2019).

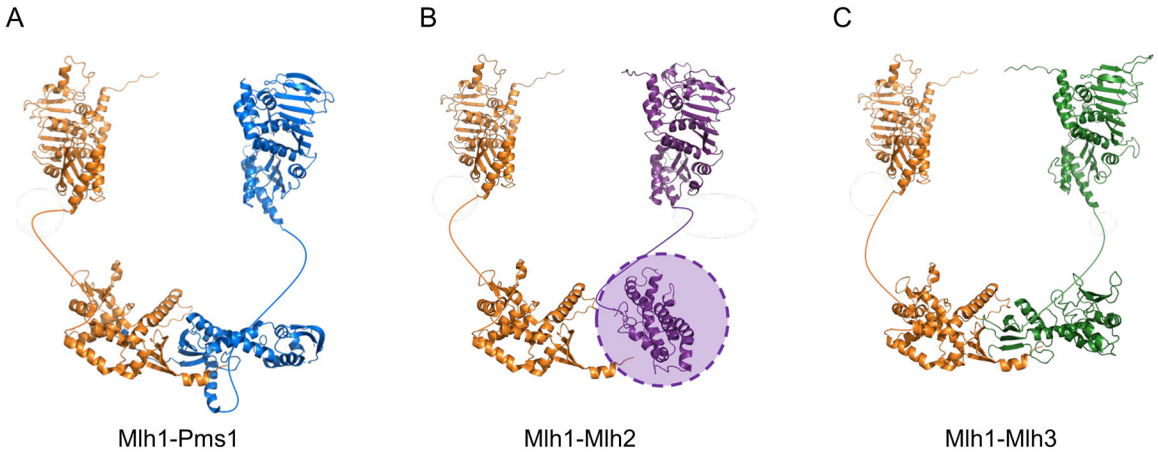

FIGURE 4. Structural homology of yeast Mlh1-Pms1, Mlh1-Mlh2, and Mlh1-Mlh3.

(A) For yeast Mlh1-Pms1, the N-terminal domain of Pms1 was obtained from PDB 3h4l, and the N-terminal domain of Mlh1 was modeled by a Phyre2 (amino acids 1–367 of Mlh1; http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index). The structure of the Mlh1-Pms1 C-terminus (PDB 4e4w) was solved by Gueneau et al. (2013). (B) For yeast Mlh1-Mlh2, the N-terminal domains of Mlh1 and Mlh2 were created using a Phyre2 homology model (amino acids 1–371 of Mlh2). The C-terminal domain of Mlh1 was obtained from PDB 4e4w. The C-terminal domain of Mlh2 (amino acids 503–695), created using a Phyre2 homology model, did not align well with the C-terminal domains of the other MLH proteins; for this reason, it is indicated by a shaded oval. (C) For yeast Mlh1-Mlh3, the N-terminal domains of Mlh1 and Mlh3 were created by a homology model presented in Al-Sweel et al. (2017). The C-terminal domain of Mlh1 was obtained from PDB 4e4w, and the C-terminal domain of Mlh3 (amino acids 491–715 of Mlh3) was created by a homology model presented in Al-Sweel et al. (2017). In all panels the unstructured linker domains are shown as curved lines.

A

Linkers in MLH proteins; length in amino acids (location)

Species	Mlh1 (MutL for *E. coli* and *B. subtilis*)	Pms1 (Pms2)	Mlh3	Mlh2 (Pms1)
E. coli	97 (334-431)	-	-	-
B. subtilis	108 (327-435)	-	-	-
S. cerevisiae	164 (335-499)	295 (364-659)	113 (375-488)	245 (372-617)
S. pombe	92 (359-451)	234 (326-560)	-	-
M. musculus	171 (331-502)	284 (383-667)	612 (419-1031)	290 (416-706)
H. sapien	170 (331-501)	283 (385-668)	642 (396-1038)	303 (419-722)
D. melanogaster	87 (354-441)	307 (399-695)	-	-
C. elegans	145 (347-496)	249 (367-616)	-	-
A. thaliana	100 (357-457)	364 (344-698)	511 (357-868)	-
D. rerio	160 (322-482)	288 (374-662)	303 (358-661)	334 (342-676)

B

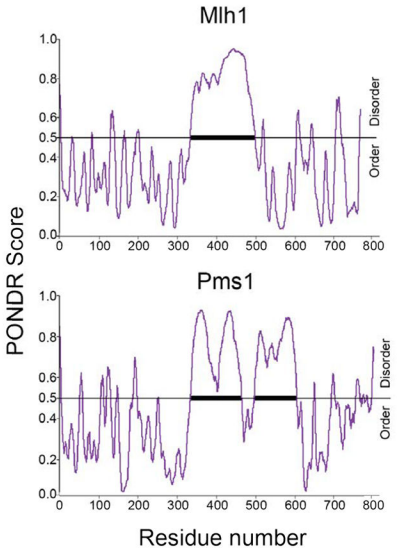

FIGURE 5. Composition of linker variation among MutL and MLH proteins.

(A) Proposed linker lengths of various MutL and MLH proteins from bacteria to higher eukaryotes. Amino acid sequences were obtained from UniProt (https://www.uniprot.org), and linker lengths were determined using the Predictor of Natural Disorder (PONDR) VSL2 disorder prediction algorithm. (B) Shown is a PONDR analysis of *S. cerevisiae* Mlh1 and Pms1 (Kim et al., 2019; Obradovic et al., 2003).

FIGURE 6. Alignment of the endonuclease domain of *S. cerevisiae* Pms1 (Gueneau et al., 2013) with Pms1 and Mlh3 homologs from 18 budding yeast (family *Saccharomycetaceae*) species (Clark, Alani, & Aquadro, 2012).

Saccharomyces cerevisiae (Scer) Pms1 and Mlh3 amino acid sequences are shown, followed by sequences from homologs from six of the 18 species. The metal binding site of Pms1 (green font), which forms the endonuclease active site, contains five residues (H703, E707, C817, C848, H850) that are highly conserved (100% identity in 18 Pms1 and 18 Mlh3 sequences; Clark, Alani, & Aquadro, 2012). The QXLXXP motif (blue font), important for interactions with PCNA (PIP), is highly conserved in the Pms1 sequences (>94% identity; Genschel et al., 2017), but is absent in Mlh3 sequences. Also shown are residues in red that are uniquely conserved (61 to 100% identity) within each Pms1 and Mlh3 homolog family. Homologs were obtained from *Saccharomyces cerevisiae (Scer), Ashbya gossypii (Agos), Candida albicans (Calb), Candida dubliniensis (Cdub), Candida glabrata (Cgla), Candida guilliermondii (Cgui), Candida tropicalis (Ctro), Saccharomyces paradoxus, Saccharomyces mikatae, Saccharomyces bayanus, Saccharomyces kluyveri, Kluyveromyces thermotolerans, Kluyveromyces waltii, Kluyveromyces lactis, Kluyveromyces polysporus, Candida lusitaniae, Lodderomyces elongisporus, Debaryomyces hansenii*, and *Pichia stipitis*. Because of incomplete sequence information, the Pms1 homolog for *Debaryomyces hansenii*, and the Mlh3 homolog of *Candida lusitaniae* were not included.