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New insights into the mechanism of DNA mismatch repair

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Abstract

The genome of all organisms is constantly being challenged by endogenous and exogenous sources of DNA damage. Errors like base:base mismatches or small insertions and deletions, primarily introduced by DNA polymerases during DNA replication are repaired by an evolutionary conserved DNA mismatch repair (MMR) system. The MMR system, together with the DNA replication machinery, promote repair by an excision and resynthesis mechanism during or after DNA replication, increasing replication fidelity by upto-three orders of magnitude. Consequently, inactivation of MMR genes results in elevated mutation rates that can lead to increased cancer susceptibility in humans. In this review, we summarize our current understanding of MMR with a focus on the different MMR protein complexes, their function and structure. We also discuss how recent findings have provided new insights in the spatio-temporal regulation and mechanism of MMR.

Introduction

The integrity of the genetic information largely depends on the accuracy of the DNA replication process, but also on surveillance mechanisms that increase DNA replication fidelity. On one hand, the major replicative DNA polymerases proofread their own errors by excision of misincorporated nucleotides. Moreover, cells possess a second quality control mechanism, represented by the DNA mismatch repair (MMR) pathway. This DNA repair mechanism corrects not only errors that escaped the DNA polymerase proofreading function, but also recognizes mispaired bases and branched DNAs formed during recombination, as well as chemically modified bases in DNA, introduced for example, by chemotherapeutic agents. In fact, components of the MMR machinery are required by many aspects of DNA metabolism and affect processes such as cell cycle checkpoint control, apoptosis, somatic hypermutation of immunoglobulin genes, triplet-repeat expansion, among others (Harfe and Jinks-Robertson 2000; Li 1999, 2008; Pena-Diaz and Jiricny 2012).

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Compliance with ethical standards

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Many features of MMR reactions are conserved from bacteria to humans. The core MMR machinery involves two conserved families of protein complexes, including the MutS and MutL complexes (where *Mut* stands for mutator) or their homologs. The basic reaction involves an *excision-repair* reaction in which a region of the newly synthesized strand containing the incorrect base is excised and resynthesized. Thus, MMR involves several proteins, including some components of the DNA replication machinery. Together, the core and accessory MMR proteins impart mechanistic differences specific to each organism or specific to alternative MMR sub-pathways. Although we know some of the sequential steps of the MMR reaction and have identified most of the proteins that function in MMR, some mechanistic aspects of the MMR reaction are still not well understood.

Inactivation of the MMR system leads to an increase of upto-three orders of magnitude in the overall mutation rate (reviewed in Iyer et al. 2006). Moreover, in humans, loss of MMR function results in Lynch Syndrome (previously referred to as hereditary non-polyposis colorectal cancer or HNPCC) (Boland and Goel 2010; Boland and Lynch 2013). Lynch syndrome is characterized by early-onset of cancer and the accumulation of mutations, often including frameshift mutations in repetitive sequences, a phenotype called microsatellite instability (MSI). Most Lynch syndrome patients are heterozygous carriers of germline-inactivating mutations in MMR genes, most frequently *MSH2* and *MLH1* (Peltomaki and Vasen 2004). Cells in these patients subsequently lose MMR function due to a mutagenic/epigenetic event that inactivates the remaining functional allele (Boland and Goel 2010; Huang et al. 1996; Kinzler and Vogelstein 1996; Markowitz et al. 1995). In this way, loss of MMR activity increases the rate of accumulation of mutations in genes encoding essential regulators of cell proliferation and apoptosis, thereby accelerating cancer progression. In addition, a proportion of sporadic tumors have acquired MMR defects, most notably the epigenetic silencing of *MLH1*, resulting in increased mutation rates that drive the development of cancer (Peltomaki 2014). The reader is referred to other review articles (Boland and Goel 2010; Edelman and Edelman 2004; Hsieh and Yamane 2008; Peltomaki and Vasen 2004) that have been published on MMR defects and cancer, as this topic will not be covered in detail in this article.

In the last few years, there have been a number of new developments in the MMR field. Important progress has been made toward understanding how the newly synthesized strand is identified in eukaryotes. Furthermore, new discoveries made using live-cell imaging to visualize MMR components have expanded our knowledge of the spatio-temporal kinetics of MMR. In addition, accessory proteins with important functions in MMR had been revisited and their involvement in MMR has been pinpointed to specific steps of MMR reactions, increasing our understanding of the mechanistic aspects of MMR and the function of specific MMR proteins. We also have an idea, albeit incomplete, of how epigenetic changes and posttranslational modifications might regulate the interaction of MMR with chromatin.

The aim of this review is to summarize some of the latest discoveries on MMR, focusing on protein complexes that function in MMR and highlighting the existence of MMR sub-pathways where previously identified MMR proteins have been found to have dedicated functions. The focus of this review is on MMR in *Saccharomyces cerevisiae* and humans;

because of some inconsistencies in the names of individual proteins, when the equivalent *S. cerevisiae* and human proteins have different names, the prefixes *sc* and *h*, respectively, will be used to avoid potential confusion.

The basic MMR reaction

The basic MMR reaction is best understood in *Escherichia coli*, where MMR is initiated by the MutS homodimer, which recognizes mismatches and then recruits MutL (also a homodimeric complex) to the mispair site. MutL interacts with a third MMR component called MutH, resulting in the activation of the latent endonuclease activity of MutH and nicking of the newly replicated strand. Strand discrimination, an essential feature of MMR, restricts the use of the parental strand as the template for DNA resynthesis thus eliminating misincorporation errors. In *E. coli*, strand discrimination relies on the hemi-methylated status of the newly synthesized DNA; the DNA is methylated at d(GATC) sites by the Dam methyltransferase and because this is a post-replication modification, the newly synthesized DNA strand is transiently undermethylated. MutH is a methylation-sensitive endonuclease that exclusively nicks the unmethylated, newly synthesized DNA strand at d(GATC) sites. This nick is then used as entry point for the excision reaction, targeting it exclusively to the newly synthesized strand. During the excision reaction, the DNA is unwound by DNA helicase II starting at the nick and the error-containing strand is degraded by a ssDNA specific exonuclease (ExoI, ExoVII, and ExoX act in 3' to 5' excision; RecJ and ExoVII act in the 5' to 3' excision) and resynthesized by DNA polymerase III (Table 1) (Iyer et al. 2006; Kunkel and Erie 2005). It should be noted that DNA methylation asymmetry is not used as a strand specificity signal in eukaryotes or in bacteria outside of a subset of gammaproteobacteria that includes *E. coli* (Gao et al. 2009).

Eukaryotic MMR is mechanistically similar to bacterial MMR. However, mispair recognition utilizes two *MutS* homolog (MSH) complexes, the Msh2-Msh6 (sometimes called MutS α) and Msh2-Msh3 (sometimes called MutS β) complexes that have a partial overlapping mispair recognition specificity, instead of a single MutS homodimer (Kolodner and Marsischky 1999) (Fig. 1). A third MSH complex (Msh4-Msh5) does not have an MMR-related function, but rather is important during meiotic recombination. In addition, instead of a MutL homodimer, eukaryotic MMR primarily utilizes a heterodimeric MutL homolog (MLH) complex, sometimes also referred as MutL α , which in *S. cerevisiae* is Mlh1-Pms1 and in humans is Mlh1-Pms2 (scPms1 is the homolog of hPms2). Two additional MLH heterodimers have been identified, MutL β which in *S. cerevisiae* is Mlh1-Mlh2 and in humans is Mlh1-Pms1 (scMlh2 is the homolog of hPms1) and MutL γ which is Mlh1-Mlh3; MutL β and MutL γ appear to have a minor role in MMR (Table 1 and Fig. 1). In contrast to MMR in *E. coli*, eukaryotic MMR does not utilize a MutH homolog protein, as the nicking function of MutH appears to be integral to the MutL α and MutL γ complexes (Kadyrov et al. 2006), whereas the MutL β complex is predicted to lack endonuclease activity.

Biochemical studies have demonstrated two types of MMR reactions in which human MMR proteins catalyze the repair of nicked, mispair containing substrates, similar to those catalyzed by *E. coli* MMR proteins. In one reaction, a combination of Msh2-Msh6 or Msh2-

Msh3, Replication Protein A (RPA) and Exonuclease 1 (Exo1), a 5' to 3' dsDNA specific exonuclease that also has flap endonuclease activity, promotes 5' to 3' excision of the mispaired strand from a 5' nick, resulting in a gap that is filled in by a combination of DNA polymerase Pol δ (also called Pol3), Proliferating Cell Nuclear Antigen (PCNA), Replication Factor C (RFC), and RPA (Constantin et al. 2005; Zhang et al. 2005). In a second type of reaction, a combination of Msh2-Msh6, Mlh1-Pms2, PCNA, and RFC promotes the introduction of nicks 5' to a mispair in a substrate containing a nick 3' to the mispair (Constantin et al. 2005; Kadyrov et al. 2006); this incised product is then subject to excision and gap filling essentially as seen with substrates containing a 5' nick (Constantin et al. 2005; Kadyrov et al. 2006). This mode of excision is distinct from that seen in *E. coli* where a combination of a ssDNA-specific exonuclease and a DNA helicase promote excision instead of a dsDNA specific exonuclease in eukaryotes. There are key questions raised by these studies on eukaryotic mispair excision. (1) What targets excision to the leading strand that is synthesized continuously? (2) If nicks, which are prevalent on the lagging strand due to discontinuous DNA synthesis, can target MMR to the lagging daughter strand, then why is MutL α complex absolutely required for MMR? (3) How do preexisting nicks target MutL α to incise on the already nicked strand and what role does this incision play? (4) What accounts for excision in the absence of Exo1 since this protein is not absolutely required for MMR in vivo? (Amin et al. 2001; Tishkoff et al. 1997; Wei et al. 2003).

The MutS mismatch recognition complex

Our understanding of how a DNA mispair is recognized by the MutS family of proteins has been driven by crystallo-graphic studies of MutS from *E. coli* and *Thermus aquaticus*, in combination with different types of biochemical analysis. MutS is a homodimer, in which each monomer contains six structural domains (I to VI) with distinct functions (Fig. 2a) (Lamers et al. 2000; Mendillo et al. 2007; Obmolova et al. 2000). According to the *E. coli* MutS structure (Lamers et al. 2000), domain I (residues 2–115) corresponds to the mispair recognition domain; domain II (residues 116–266) or connector domain interacts with MutL (Mendillo et al. 2009); domain III (residues 267–443 and 504–567) represents the core domain that separates DNA binding and nucleotide binding sites; domain IV (residues 444–503) forms the exterior of the DNA clamp; domain V (residues 568–765) contains the ATPase domain and dimerization sites; and domain VI (residues 766–800) or helix-turn-helix domain (HTH) is required for dimerization at moderate protein concentrations (Biswas et al. 2001; Lamers et al. 2000; Mendillo et al. 2007; Obmolova et al. 2000). This section summarizes some important aspects of the structural domains of MutS and how they function during the mispair recognition reaction.

Interaction with DNA

Biochemical and crystallography studies showed that the MutS homodimer forms a ring-like structure that encircles and sharply bends DNA when it binds a mispair (Junop et al. 2001; Lamers et al. 2000). The DNA binding complex is asymmetric; only one of the two subunits directly contacts the mispaired base via domain I, while domain I from the other subunit makes non-specific contacts with the DNA backbone. Two key amino acids in domain I that directly contact and stabilize an extra-helical base at a base:base mispair, or an extra-helical

base in a +1/-1 insertion/deletion loop (IDL), are present in a Phe-X-Glu motif (corresponding in *E. coli* MutS to residues Phe36 and Glu38).

Phe36 contacts a base at the mispair, whereas Glu38 plays a role in stabilizing the bend in the DNA. Binding to DNA provokes conformational changes in MutS that have been revealed by deuterium exchange mass spectrometry (DXMS) studies (Mendillo et al. 2010).

ATPase domain

The domain V of MutS harbors an ATPase domain that is conserved on MutS homologs and also other members of the *adenosine triphosphate (ATP)-binding cassette (ABC)* transporter family (Gorbalenya and Koonin 1990). Conformational changes resulting from the binding of ATP or ADP by MutS are transduced to the DNA binding domains. Moreover, the two ATPase sites in MutS are expected to have different functions due to the intrinsic structural asymmetry of the MutS mispair-bound complex (Junop et al. 2001; Lamers et al. 2000; Obmolova et al. 2000). A key feature of MMR is that the MutS complex must bind to mismatched DNA with higher affinity or a unique conformation than when it binds matched DNA.

Based on several biochemical, structural, and genetic studies, it has been proposed two potential models for the ATP-dependent movement of MutS along the DNA, the *translocation model* and the *molecular switch model* or *sliding clamp model* (reviewed in Iyer et al. 2006). According to the *translocation model*, ATP binding reduces the affinity of MutS for the mispair and ATP hydrolysis acts as a driving force promoting movement of the MutS along DNA (Allen et al. 1997).

On the other hand, the *sliding clamp model* proposes that the mispair is recognized by an ADP-bound MutS, resulting in a rapid ADP-ATP exchange and a MutS conformational change that releases MutS from the mismatch allowing MutS to diffuse along the DNA backbone as a sliding clamp, in an ATP-hydrolysis-independent manner (Acharya et al. 2003; Gradia et al. 1999; Hura et al. 2013; Lee et al. 2014; Mendillo et al. 2005). DXMS studies have revealed some of these ATP binding induced conformational changes (Mendillo et al. 2010). Moreover, it has been shown that the ATP binding driven conformational change is required for the mispair-dependent recruitment of MutL (Junop et al. 2001).

MutS-MutL interacting region

Upon mismatch recognition, MutS undergoes an ATP-binding dependent conformational change that allows sliding on DNA and the interaction with MutL, resulting in the recruitment of MutL by mispair-bound MutS. This higher-order complex is dynamic and transient (Galio et al. 1999; Mendillo et al. 2005), thus the identification of the interface between MutS and MutL has been difficult to determine. However, using DXMS, it was possible to identify a region in domain II of MutS (connector domain) that specifically interacts with MutL (Mendillo et al. 2009); this finding was subsequently confirmed by crosslinking and fluorescence resonance energy transfer (FRET) studies (Winkler et al. 2011). Binding of MutL requires ATP and mispair binding by MutS, which are likely

necessary for a conformational change that exposes the domain II to MutL. Consistent with this model, amino acid changes in this region of MutS (*mutS-Q211S*, *mutS-Q212S*, *mutS-N214*, and *mutS-L215S*) result in inactivation of both MutL recruitment in vitro and MMR in vivo (Mendillo et al. 2009). The MutS interface is conserved in Msh2 and analogous mutations in domain II of scMsh2 result in defects in binding of scMsh2-Msh6 to scMlh1-Pms1 in vitro and defects in MMR in vivo. Importantly, mutations in *E. coli* MutS and scMsh2 that block assembly of MutL and scMlh1-Pms1, respectively, do not alter mispair recognition or ATP-induced sliding on DNA (Mendillo et al. 2009).

Eukaryotic MSH complexes

In eukaryotes, mispaired bases are recognized by heterodimeric MSH complexes: the Msh2-Msh6 complex (MutS α), the Msh2-Msh3 complex (MutS β) (Fig. 1), and in plants also by the Msh2-Msh7 complex (Culligan and Hays 2000). These complexes share a common subunit, Msh2, and possess a unique subunit that specifies a distinct but partially overlapping substrate specificity, resulting in some genetic redundancy. Similar to the structural asymmetry of the *E. coli* MutS homodimer, each subunit of the eukaryotic MSH heterodimer has a different role, with the Msh3, Msh6, and Msh7 subunits being the mismatched base-contacting subunit. Interestingly, replacing domain I (the mispair binding domain) of Msh6 with that of Msh3 in *S. cerevisiae* resulted in a functional chimeric Msh2-Msh6 complex with Msh2-Msh3-like substrate specificity, demonstrating that domain I is the major determinant of substrate specificity (Shell et al. 2007a). There are also three other eukaryotic MSH proteins that are not involved in canonical MMR and will not be discussed further in this review: Msh1, a protein that functions in mitochondria that has no homolog in mammals (Culligan et al. 2000; Reenan and Kolodner 1992a, b), and Msh4 and Msh5 that form a heterodimeric complex that functions in the resolution of meiotic recombination intermediates in conjunction with the Mlh1-Mlh3 complex (Kolas et al. 2005; Santucci-Darmanin et al. 2002; Snowden et al. 2004).

Msh2-Msh6 (MutS α)

In humans, the Msh2-Msh6 heterodimer is about 10 times more abundant than Msh2-Msh3 and is potentially the major mispair recognition complex, although in human cell lines and in mice defects in Msh6 cause weaker mutator phenotypes than defects in Msh2, suggestive of some redundancy between these two complexes. In contrast, in *S. cerevisiae*, Msh2-Msh6 and Msh2-Msh3 appear to play more balanced roles in MMR (Marsischky et al. 1996; Sia et al. 1997). *S. cerevisiae* Msh2-Msh6 appears to functionally recognize seven of the eight possible base:base mispairs (CC mispairs are poorly recognized) and +1 and +2 insertion/deletion mispairs (Srivatsan et al. 2014). Msh2-Msh6 forms an asymmetric dimer in which the Msh6 subunit resembles the structure of the mispair-contacting subunit of *E. coli* MutS (Fig. 2a, b) (Warren et al. 2007). Similar to MutS, Msh6 contains a conserved phenylalanine (Phe337 in *S. cerevisiae* and Phe432 in humans), which interacts with mismatched DNA (Fig. 2c). Accordingly, mutations that alter this phenylalanine prevent mispair recognition (Bowers et al. 1999). Interestingly, DXMS studies have shown that *S. cerevisiae* Msh2-Msh6 forms a ring around fully basepaired DNA in which the Msh6 mispair-contacting Phe337 contacts the DNA and have identified an Msh6 DNA backbone contacting residue

Arg412, as potentially the only other residue that uniquely contacts the DNA in the mismatch-bound form of Msh2-Msh6 (Mendillo et al. 2010). Overall, the mismatch binding domains of MutS and Msh6 are structurally similar to each other, consistent with the high degree of sequence homology between MutS and Msh6 within this region.

Similar to MutS, domain V of Msh6 and Msh2 contains composite and asymmetric ATPase domains that bind ATP with different kinetics and affinities (Antony and Hingorani 2003; Mazur et al. 2006). Consistent with the view that both ATPase domains contribute to the ATPase activity of the complex, mutations altering the highly conserved lysine at the Walker A motif in either human Msh2 or Msh6 (*msh2-K675R* or *msh6-K1140R*) affect ATP hydrolysis and dissociation from the DNA (Iaccarino et al. 1998). As seen for the MutS homodimer, nucleotide binding by the Msh2-Msh6 complex modulates its interactions with mismatched and fully basepaired DNA in which ADP binding allows the binding of Msh2-Msh6 to a mismatch, while ADP-ATP exchange converts the mismatch-bound Msh2-Msh6 to the sliding clamp form that diffuses along the DNA (Gradia et al. 1999; Mazur et al. 2006). Supporting the role of this conformational change in MMR, dominant mutations have been identified in *S. cerevisiae* *MSH6* that do not affect mismatch binding but block the formation of sliding clamps and in some cases block the recruitment of Mlh1-Pms1 (Hargreaves et al. 2010; Hess et al. 2002, 2006). Furthermore, the equivalent mutations in *S. cerevisiae* *MSH2* cause essentially the same biochemical defects (Hargreaves et al. 2010).

Small-angle X-ray scattering (SAXS) studies for the *S. cerevisiae* MutS α and PCNA proteins, reveals that Msh6 contains an unstructured N-terminal region (NTR) that serves as a tether to PCNA (Shell et al. 2007b). The interaction between Msh6 and PCNA requires a conserved PCNA-interacting protein motif (PIP-box) present on the Msh6-NTR. A similar PIP-box motif is also present in Msh3-NTR, but not in Msh2; however, transferring the Msh6-NTR onto Msh2, complements deletion of the NTR on Msh6 (Shell et al. 2007b). As will be discussed later, the interaction of Msh2-Msh6 heterodimer with PCNA is necessary to recruit the mismatch recognition complexes to newly replicated DNA (Hombauer et al. 2011a; Kleczkowska et al. 2001; Shell et al. 2007b). Human Msh6 (and Msh6 from other deuterostomes) but not Msh2 or Msh3 additionally contains a PWWP domain in the N-terminal region (Laguri et al. 2008), which consequences will be discussed later. Moreover, SAXS experiments done for the human MutS α and MutS β complexes revealed that the NTR of human Msh3 and Msh6 exist rather as a globular structure, which interacts with PCNA in a well defined conformation (Iyer et al. 2008, 2010; Lang et al. 2011), and therefore differs from the unstructured NTR present on scMsh6. The potential consequences of these discrepancies between the human and the *S. cerevisiae* MutS α proteins remain unknown.

Msh2-Msh3 (MutS β)

The second MSH heterodimer that participates in mismatch recognition in eukaryotes is Msh2-Msh3. Genetic and biochemical studies in *S. cerevisiae* (Harrington and Kolodner 2007; Marsischky et al. 1996; Srivatsan et al. 2014) and in human cells (Acharya et al. 1996) have shown that the Msh2-Msh3 complex recognizes and can recruit MLH heterodimers to both small and large insertion/deletion mismatches and a limited spectrum of base:base mismatches (Acharya et al. 1996; Harrington and Kolodner 2007; Kolodner and Marsischky 1999;

Marsischky et al. 1996; Srivatsan et al. 2014). However, while domain I (the mispair binding domain) of hMsh3 has a similar fold compared to domain I of hMsh6, mispair recognition occurs quite differently. Instead of using a Phe-X-Glu motif to stabilize an extra-helical base, domain I from Msh3 bends substrate DNA and separates the DNA strands at the mispair site by insertion of key side-chain residues (Downen et al. 2010; Gupta et al. 2012). Genetic analysis done in *S. cerevisiae* is consistent with the need for Msh3 to induce additional stabilization to base:base mispairs relative to insertion/deletion mispairs that are intrinsically bent and strand separated (Downen et al. 2010). While the interactions between *S. cerevisiae* Msh2-Msh3 and ADP and ATP have not been as well studied as for scMsh2-Msh6, the ATP binding mediated conversion of mispair-bound scMsh2-Msh3 to a sliding clamp and recruitment of scMlh1-Pms1 (Srivatsan et al. 2014) appears to be similar to that of scMsh2-Msh6.

Genetics studies in *S. cerevisiae* have implicated scMsh2-Msh3, in conjunction with the Rad1-Rad10 endonuclease, in the removal of non-homologous tails in recombination intermediates (Sugawara et al. 1997). This resolution event requires the amino acid residues of the scMsh3 mispair binding domain that are critical for mispair recognition but not those required for strand separation and bending of DNA (Downen et al. 2010). In addition, studies have demonstrated that scMsh2-Msh3 binds to double-strand break-induced recombination intermediates (Sugawara et al. 1997; Surtees and Alani 2006); although whether this reflects binding to double-strand breaks or branched recombination intermediates has not yet been resolved. Furthermore, Msh2-Msh3 is known to play a role promoting trinucleotide repeat expansions, which are characteristic of several disorders including Fragile X mental retardation syndrome, Huntington's disease, myotonic dystrophy, and other types of ataxia (Lopez Castel et al. 2010; McMurray 2010; Slean et al. 2008). Initial studies in mice revealed that Msh2-Msh3 promotes genetic instability, based on the observation that wild-type as well as an *MSH6*^{-/-} mice, but not an *MSH3*^{-/-} mice, accumulate expansions at trinucleotide containing regions (Owen et al. 2005; van den Broek et al. 2002). Consistent with this, it has been shown in *S. cerevisiae* (Surtees and Alani 2006) and with human cell extracts (Panigrahi et al. 2010; Tian et al. 2009) that Msh2-Msh3 binds branched DNAs in vitro with highest affinity for branched DNAs containing single-stranded DNA tails, as well as large insertion/deletion mispairs that might play a role in trinucleotide repeat expansions. Thus, it appears that the distinctive mode of mispair recognition has allowed Msh2-Msh3 to adopt other functions that require recognition of unusual DNA structures (Owen et al. 2005).

The MutL complex

Prokaryotic MutL is a homodimeric ATPase that belongs to the GHKL superfamily consisting of Gyrase b, Hsp90, Histidine kinases and MutL homologs (Ban and Yang 1998; Dutta and Inouye 2000). In *E. coli*, MutL is a critical factor that is recruited by mismatch-bound MutS and mediates the activation of both the MutH endonuclease and the UvrD DNA helicase.

MutL as well as its eukaryotic homologs contains two distinct structural domains: a conserved N-terminal domain (NTD) and less conserved C-terminal domain (CTD), which are connected by a long proline-rich linker (Fig. 3). The NTD possesses ATPase activity

(Ban and Yang 1998), whereas the CTD is required for dimerization (Guarne et al. 2004). The crystal structures of the N- and C-terminal domains of MutL have been determined (Ban and Yang 1998; Guarne et al. 2004; Kosinski et al. 2005), whereas the linker is likely to be disordered.

The ATPase domain of *E. coli* MutL

The MutL NTD, which is about 300 amino acids in length, contains highly conserved residues clustered in four motifs, which are essential for ATP binding and/or hydrolysis (Fig. 3). ATP binding and hydrolysis by MutL induces major conformational changes that constitute a MutL ATPase cycle (Ban et al. 1999). In the nucleotide-unbound state, MutL exists as a dimer mediated by interactions between the two CTDs. ATP binding causes dimerization of the NTDs and compaction of the dimer (Sacho et al. 2008). ATP hydrolysis results in relaxation of the dimer structure and ADP release results in dissociation of the NTDs (Yang 2000).

MutL interactions with DNA

MutL binds DNA independently of MutS, a mismatch or a specific DNA sequence, with a binding preference toward ssDNA over dsDNA (Bende and Grafstrom 1991). The interaction has been proposed to occur in a groove created by the dimerization of both NTDs based on the effects of mutagenizing positively charged residues in this region (Ban et al. 1999). Mutations affecting this region disrupt MMR (Junop et al. 2003) and fail to activate the DNA helicase activity of UvrD (Guarne et al. 2004; Matson and Robertson 2006) but do not affect MutH activation (Ahrends et al. 2006). It has been suggested that binding of MutL to DNA might be important after MutH activation (and its release after incision) when MutL positions itself between the mismatch and the d(GATC) site facilitating the subsequent steps of the mispair excision reaction (Junop et al. 2003). On the other hand, the simultaneous binding to DNA and MutS might stabilize a repair intermediate during the search for the strand discrimination signal, triggering a conformational switch from an initiation mode to a processing mode (Ban and Yang 1998; Drotschmann et al. 2002).

Eukaryotic MLH complexes

In eukaryotes, there are three different heterodimeric MLH complexes called MutL α , MutL β , and MutL γ , represented in *S. cerevisiae* by Mlh1-Pms1, Mlh1-Mlh2, and Mlh1-Mlh3 complexes, respectively. In humans, the homolog MutL α , MutL β , and MutL γ heterodimers are formed by Mlh1-Pms2, Mlh1-Pms1, and Mlh1-Mlh3, respectively (Jiricny 2013; Kolodner and Marsischky 1999; Kunkel and Erie 2005). The nomenclature of these proteins in different species is described in Table 1 (*S. cerevisiae* proteins Pms1 and Mlh2 are the functional homologs of human Pms2 and Pms1, respectively). Like MutL, the eukaryotic MLH proteins have distinct NTDs and CTDs that are connected by an unstructured linker. The NTDs contain the ATPase domain, which is assembled by four conserved ATP-binding motifs characteristic of the GHKL family of ATPases (Dutta and Inouye 2000) (Fig. 3). The CTDs are not conserved in their primary sequence but are predicted to share some level of conservation with regard to their secondary structure. The CTDs contain the regions important for dimerization, and for MutL α in *S. cerevisiae*

(Gueneau et al. 2013) or humans (Schmutte et al. 2001), the interaction domain with Exo1 (Gueneau et al. 2013; Schmutte et al. 2001). Importantly, the CTD of some of the eukaryotic MutL homologs contains a conserved endonuclease motif ($DQHA(X)_2E(X)_4E$) which is not present in *E. coli* MutL or MutL in other organisms that have a MutH-dependent incision system (Table 1 and Fig. 3) (Guarne 2012; Kadyrov et al. 2006).

Mlh1-Pms1 (MutL α)

In *S. cerevisiae*, the Mlh1-Pms1 complex appears to be the major MutL homolog complex that functions in MMR, as mutations in *MLH2* and *MLH3* cause only weak MMR defects and Mlh2 and Mlh3 cannot substitute for Pms1. Importantly, MutL α possesses an endonuclease domain which resides on the CTD of the heterodimer, and which has been hypothesized to be required to make single-strand nicks in the newly synthesized DNA during MMR (Kadyrov et al. 2006). Interestingly, a similar endonuclease domain is present in Mlh3 but not in scMlh2 (or hPms1) (Fig. 3) (Kadyrov et al. 2006). The CTD of scPms1 (and hPms2) harbors in addition to the conserved $DQHA(X)_2E(X)_4E$ endonuclease motif, other conserved motifs which are part of the endonuclease domain (including several residues that participate in the coordination of two metal ions present at the catalytic active site) (Fig. 4) (Gueneau et al. 2013; Smith et al. 2013). Consequently, mutations affecting these scPms1 residues (*pms1-H703A*, *pms1-E707K*, *pms1-C817R*, *pms1-C848S*, and *pms1-H850R*) result in loss of endonuclease function in vitro and MMR defects in vivo. In addition, according to the crystal structure of the scMlh1-Pms1 CTD, the C-terminal cysteine (Cys769) of scMlh1, present in the conserved *FERC* motif, is also part of this composite endonuclease domain (Fig. 4b) (reviewed in Guarne 2012). However, the role of the Cys769 remains unclear; in one study, deletion of this cysteine caused a mutator phenotype in one mutation rate assay (Gueneau et al. 2013), whereas in another study the same mutation did not affect the endonuclease activity of scMlh1-Pms1 in vitro and did not cause a mutator phenotype in three different mutation rate assays (Smith et al. 2013). In the latter study, it was necessary to delete the last three amino acids of the *FERC* motif of Mlh1 to affect MMR function (Smith et al. 2013).

It has been shown that human MutL α is an endonuclease that can nick covalently closed circular DNA in a reaction that is stimulated by PCNA and RFC (Kadyrov et al. 2006; Pluciennik et al. 2010). However, under other reaction conditions, the MutL α endonuclease specifically nicks the already nicked strand of a nicked mismatch containing circular DNA in a reaction that requires a mispaired base, ATP, MutS α , PCNA, and RFC. It has been proposed that PCNA is loaded onto DNA at the preexisting nick by RFC and that the asymmetric nature of PCNA loaded onto the DNA directs the endonuclease to nick the already nicked strand by a mechanism that is not yet understood (Pluciennik et al. 2010). An intriguing question raised by these results is that if a pre-existing nick is required for MutL α to nick the already nicked strand, then does the endonuclease actually function in MMR by catalyzing nicks that act as the sites of initiation of the excision reaction during MMR or does the endonuclease play another role in MMR.

Mlh1-Mlh2 (MutL β)

The function of the scMlh1-Mlh2 complex (hMlh1-Pms1 in humans), also called MutL β , has remained largely unexplored, in part due to the fact that scMlh2 (or hPms1) does not contain the conserved endonuclease motifs identified on MutL α and MutL γ homologs (Fig. 3). Furthermore, deletion of *MLH2* in *S. cerevisiae* caused only a very weak mutator phenotype (Harfe et al. 2000), deletion of *PMS1* in mice did not increase cancer susceptibility (Prolla et al. 1998) and partially purified hMlh1-Pms1 showed no activity in MMR reactions in vitro (Raschle et al. 1999). However, a number of recent experiments have suggested that the scMlh1-Mlh2 heterodimer may play a direct role in MMR (Campbell et al. 2014). First, mispair-bound scMsh2-Msh6 and scMsh2-Msh3 complexes were found to recruit scMlh1-Mlh2 in vitro. Second, scMlh1-Mlh2 was found to form mispair dependent foci in vivo. And third, deletion of *scMLH2* was found to cause an increased mutator phenotype when the levels of scPms1 were reduced or in the absence of Msh6. Based on these observations, it has been proposed that scMlh1-Mlh2 acts as an accessory factor that facilitates scMlh1-Pms1-dependent MMR (Campbell et al. 2014).

Mlh1-Mlh3 (MutL γ)

The Mlh1-Mlh3 heterodimer is thought to play a minor role in MMR, as evidenced by the fact that a small fraction of insertion/deletion mispairs appear to be repaired by an Mlh1-Mlh3 dependent branch of an Msh2-Msh3 dependent MMR pathway in *S. cerevisiae* (Flores-Rozas and Kolodner 1998) and the fact that *MLH3* and *PMS2* double knockout mice have greater cancer susceptibility and a stronger mutator phenotype than *PMS2* single knockout mice (Chen et al. 2005). More strikingly, Mlh1-Mlh3 acts in the processing of meiotic recombination intermediates in conjunction with the Msh4-Msh5 complex (Zakharyevich et al. 2012). Mlh3 harbors an endonuclease motif in its CTD (Nishant et al. 2008) (Fig. 3) and recent studies have shown that the Mlh1-Mlh3 complex has endonuclease activity. In one study, Mlh1-Mlh3 was found to nick supercoiled circular DNA in a reaction that was stimulated by Msh2-Msh3 but was surprisingly not stimulated by ATP, PCNA, or RFC (Rogacheva et al. 2014) whereas in another study Mlh1-Mlh3 was shown to cleave branched DNAs (Ranjha et al. 2014), consistent with a role in resolution of meiotic recombination intermediates.

Visualization of MMR complexes

Fluorescence microscopy has recently been used to obtain mechanistic information related to the kinetics and spatial-temporal organization of MMR protein complexes in living cells. MMR complexes have been visualized in bacteria and in human cell lines (Elez et al. 2010; Hong et al. 2008; Kleczkowska et al. 2001; Roesner et al. 2013; Smith et al. 2001). However, in these studies, the use of overexpression systems has complicated the interpretation of the results. Recently, in *B. subtilis* and *S. cerevisiae*, MMR components have been fluorescently tagged at their endogenous loci and visualized by live-cell fluorescence microscopy, although the investigated MMR components remained functional after tagging them only for *S. cerevisiae* (Campbell et al. 2014; Goellner et al. 2014; Hombauer et al. 2011a; Lenhart et al. 2013a, b; Simmons et al. 2008).

Live-cell imaging of *S. cerevisiae* cells with functional fluorescent-tagged MMR proteins revealed the presence of nuclear Msh2-Msh6 foci in S phase that colocalized with components present at DNA replication factories, including the DNA polymerases, PCNA and RPA. Interestingly, the formation and colocalization of these Msh2-Msh6 foci was independent of the presence of a mispair or the ability of Msh2-Msh6 to recognize them (Hombauer et al. 2011a), however, was dependent on the interaction of Msh6 with PCNA, as mutations in *MSH6* (*msh6-F33AF34A*, *msh6 2-50*, *msh6 2-251*) (Clark et al. 2000; Flores-Rozas et al. 2000; Shell et al. 2007b) or *PCNA* (*pol30-204*) (Lau et al. 2002) which disrupt Msh6-PCNA interaction in vitro, abolished the Msh6 foci. Data obtained from studies in *B. subtilis* and human are consistent with these findings and have also identified beta clamp-dependent coupling of MutS and PCNA-dependent coupling of human Msh2-Msh6 with replicating DNA, respectively (Kleczkowska et al. 2001; Lenhart et al. 2013a, b; Liberti et al. 2011; Simmons et al. 2008).

Mutations in *S. cerevisiae* *MSH6* that disrupt the PIP-box motif only resulted in a 10–15% reduction in MMR in otherwise wild-type cells but completely abolished MMR in cells containing a deletion of *EXO1* (Hombauer et al. 2011a). These results suggest that in *S. cerevisiae*, there is a pathway that can substitute for the Msh6-PCNA interaction, although this second pathway does not appear to function in Exo1-independent MMR; the mechanism of this second pathway remains to be elucidated. In contrast, disruption of the beta clamp-MutS interaction in *B. subtilis* appears to almost completely inactivate MMR (Lenhart et al. 2013b).

In *S. cerevisiae*, after mispair recognition, Msh2-Msh6 (or Msh2-Msh3) forms a ternary complex with Mlh1-Pms1 and the mispair containing DNA (Habraken et al. 1997; Mendillo et al. 2005). Interestingly, in *S. cerevisiae*, functional fluorescent-tagged Pms1 formed nuclear foci during S-phase that were dependent on a functional mispair recognition complex, either Msh2-Msh6 or Msh2-Msh3, and the presence of mispaired bases (Hombauer et al. 2011a). Surprisingly, Mlh1-Pms1 foci rarely colocalized with DNA replication-associated Msh2-Msh6 foci, arguing that the two types of foci represent different steps in MMR. Moreover, the half-life of the Mlh1-Pms1 foci was short (1–2 min) which is consistent with in vitro experiments arguing for a rapid repair reaction (Fang and Modrich 1993; Wang and Hays 2002). The abundance of Mlh1-Pms1 foci was increased in *S. cerevisiae* strains where DNA replication fidelity was compromised or by MMR defects that act downstream of Mlh1-Pms1 recruitment (i.e., Pms1-endonuclease defective mutants, *pol30* mutants unable to stimulate the Pms1 endonuclease activity or an *exo1* mutation; Table 2). These results suggest that the Pms1 foci represent active sites of MMR repair in *S. cerevisiae*, and might be the result of loading several Mlh1-Pms1 molecules by Msh2-Msh6 at the mispair site (Hombauer et al. 2011a).

Further analysis of the alternative MutL homologs in *S. cerevisiae*, revealed that Mlh2 also formed nuclear foci that frequently colocalized with Mlh1-Pms1, recapitulating most of the behavior of Mlh1-Pms1 repair intermediates (Campbell et al. 2014). Importantly, Mlh2 foci were dependent on a functional Msh2-Msh6 complex and their abundance was increased by mutations affecting MMR downstream of mispair recognition or causing decreased DNA replication fidelity. Thus, even though Mlh2 lacks an endonuclease domain, these imaging

studies (together with genetic and biochemical data) suggest that the Mlh1-Mlh2 complex functions during MMR in conjunction with Mlh1-Pms1. Therefore, it has been proposed that Mlh1-Mlh2 plays an accessory role during the mismatch repair reaction, most likely facilitating interactions with MMR-related components (i.e., PCNA or Exo1). In contrast to Pms1, and supporting a minor role for Mlh3 in MMR, Mlh3 did not form nuclear foci in logarithmically growing *S. cerevisiae* cells or after DNA damaging treatments capable of inducing Mlh1-Pms1 and Mlh1-Mlh2 foci (Campbell et al. 2014).

In summary, visualization of mismatch repair components in living yeast cells revealed the existence of distinct recognition and repair MMR intermediates. Several mutations on MMR and DNA replication components have been reported to have drastic effects in the abundance of these repair intermediates. Moreover, a model for MMR in which MutS α loads catalytically several molecules of MutL α at the mismatch site/proximities (described in more detail later) has been proposed (Hombauer et al. 2011a). Future studies might prove this model and potentially identify similarities in the mechanism of MMR in other higher evolved eukaryotes.

It is worth mentioning that although the visualization of fluorescently tagged proteins has proven to be a valuable tool, it is restricted by the microscopy diffraction limit (range of 100–200 nm), which is below the limit of detection required to visualize single protein complexes (which are in the range of 10 nm). This limitation might be overcome by single-molecule studies in combination of high-resolution methods, which has been already applied to investigate some mechanistic aspects of the mismatch repair reaction (Lee et al. 2014).

Coupling of MMR with DNA replication

In order to prevent mutations due to DNA replication errors that escape the proofreading activity of the major leading and lagging strand DNA polymerases, Pol ϵ and Pol δ , respectively, MMR must repair the newly synthesized DNA strand prior to the next round of DNA replication. Furthermore, MMR must be able to target rare mismatches in a vast excess of basepaired DNA as replication errors are rare. Coupling of MMR to DNA replication offers a potential solution to these problems. The possibility of active coupling to replication was arguably first suggested by the observation that Msh2-Msh6 and Msh2-Msh3 interact with PCNA through a PIP-box motif located at the N-terminus of Msh6 and Msh3 potentially allowing the *molecular toolbelt* function of PCNA (Indiani et al. 2005) to have these mismatch recognition factors immediately available, in case they are needed (Clark et al. 2000; Flores-Rozas et al. 2000; Kleczkowska et al. 2001). The interaction between MutL α and PCNA also offers a possibility for replication coupling, although the molecular basis for this interaction, and hence its significance is not yet understood. In addition, the observation that nicks in mismatched DNA act as initiating signals in reconstituted MMR reactions in vitro suggests that DNA breaks present transiently on newly synthesized DNA strands, such as the lagging strand that is synthesized discontinuously, could passively target MMR to newly synthesized DNA during DNA replication. Consistent with this, it was initially suggested that the lagging DNA strand was more efficiently repaired by MMR than the leading DNA strand (Pavlov et al. 2003) but other subsequent studies have indicated that both leading and

lagging DNA strands are efficiently repaired by MMR (Hawk et al. 2005; Hombauer et al. 2011a; Lujan et al. 2012).

As discussed above, imaging of MMR and DNA replication components revealed that the Msh2-Msh6 mismatch recognition complex colocalized with DNA replication components during S-phase in human cells and in *S. cerevisiae* (Hombauer et al. 2011a; Kleczkowska et al. 2001). However, these observations did not formally prove that MMR actually occurred in a manner that was coupled to DNA replication. This hypothesis was further evaluated by restricting MMR protein expression to specific stages of the cell cycle in *S. cerevisiae* using cell cycle specific promoters to drive Msh2-Msh6 expression (Hombauer et al. 2011b). This analysis revealed a strong temporal coupling between MMR and DNA replication, and also suggested that strand discrimination in *S. cerevisiae* MMR relies on a transient DNA replication-associated signal.

In addition to the Msh6-PCNA interaction that targets the mismatch recognition complexes to the replication factories, it has been shown in human cells that epigenetic modifications promote the recruitment of Msh2-Msh6 to chromatin, which might facilitate a later interaction with PCNA at the replication fork (Li et al. 2013). This recruitment is mediated by histone H3K36me3 and the PWWP domain located at the N-terminus of human Msh6 (Laguri et al. 2008). Msh3 lacks a PWWP domain and hence the Msh2-Msh3 complex is unlikely to be recruited to chromatin by this mechanism. The histone H3K36me3 modification is cell cycle regulated and peaks during early S phase, and then declines until it is rarely detectable during G2/M (Bonenfant et al. 2007; Ryba et al. 2010); however, the role of H3K36me3 is best established for marking transcriptionally active chromatin and is less well understood in DNA replication and cell cycle control (Wagner and Carpenter 2012). The transition from H3K36me2 to H3K36me3 is catalyzed by the methyltransferase SetD2 (Wagner and Carpenter 2012). Human cells lacking SetD2 activity showed increased mononucleotide and dinucleotide repeat instability and an elevated mutation rate, although not as large as that caused by deletion of *MSH6* (Li et al. 2013). It should be noted that cells lacking *MSH6* show little if any dinucleotide repeat instability (Oki et al. 1999) because two-base insertion/deletion mismatches can still be repaired by Msh2-Msh3 (Genschel et al. 1998), which is somewhat different from the phenotype caused by loss of SetD2. In addition, cells lacking SetD2 have major alterations in transcription and chromatin structure, which could result in indirect alteration of MMR capacity. Consequently, it will be necessary to test the effect of human Msh6-PWWP domain mutations on MMR to determine if recruitment of Msh2-Msh6 to chromatin by H3K36me3 plays a direct role in MMR. The findings of Li and coworkers are not applicable to *S. cerevisiae* since scMsh6 and scMsh3 do not contain PWWP domains, however, deletion of the N-terminal tether of scMsh6 causes greater MMR defects than mutations that only affect binding of Msh6 to PCNA (Shell et al. 2007b), suggesting that in *S. cerevisiae* other features of the Msh6-NTR might regulate its recruitment/function. Additional studies are needed to address this possibility.

Mechanistic aspects of MMR

Strand discrimination during MMR in eukaryotes

In *E. coli*, after recognition of the mismatch by MutS and recruitment of MutL, the MutH endonuclease introduces a nick into the newly synthesized DNA strand. Strand discrimination is specified by the hemi-methylated status of the newly synthesized DNA, in which the daughter strand is transiently unmethylated becoming a substrate for MutH endonuclease, which exclusively nicks the unmethylated DNA at hemimethylated d(GATC) sites (Welsh et al. 1987).

In eukaryotes, methylation does not serve as a strand discrimination signal. While, the nature of the eukaryotic strand discrimination signal has remained unknown for several decades, data have accumulated that suggest that nicks or gaps present in DNA might be used to discriminate the parental from daughter DNA strands. It has been suggested that the 5' or 3' ends of Okazaki fragments could initiate the MMR reaction on the lagging strand (Fang and Modrich 1993; Holmes et al. 1990; Thomas et al. 1991), a view that has been solidified by the observation that the MutL α endonuclease is targeted by pre-existing nicks to then further nick the mismatch-containing strand. This finding, together with the physical coupling of MMR proteins to the replication machinery, provides a possible mechanism for how MMR can be targeted to the newly synthesized lagging strand. However, it has been also shown that MMR acts with a similar efficiency on the leading strand (Hawk et al. 2005; Hombauer et al. 2011a; Lujan et al. 2012), which is synthesized in a continuous manner and in principle lacks a high density of nicks. This observation then raises the question of what might be the strand specificity signal for leading-strand MMR.

Recently, it has been proposed that ribonucleotides, which can be misincorporated by DNA polymerases during DNA replication, might represent an additional source of nicks or gaps in the newly synthesized strand that might serve as a strand specificity signal. RNase H2 in mammals and *S. cerevisiae* makes single-strand breaks at the site of individual misincorporated ribonucleotides during ribonucleotide excision repair, potentially leaving behind a nick in DNA that could also be used as a strand discrimination signal (Ghodgaonkar et al. 2013; Lujan et al. 2013). However, misincorporated ribonucleotides are unlikely to provide a major strand discrimination signal during MMR for three reasons: (1) the reported weak mutator phenotype due to loss of RNase H2 was dependent upon DNA polymerase mutations that cause over a 10-fold increase in ribonucleotide misincorporation; (2) deletion of the catalytic subunit of *S. cerevisiae* RNase H2 results in little or no mutator general phenotype; and (3) the mutator phenotype seen in dinucleotide repeat reporters in RNase H2 defective mutants is dependent on nicking of the DNA by topoisomerase I and is not due to an MMR defect (Allen-Soltero et al. 2014; Ghodgaonkar et al. 2013; Kim et al. 2011; Lujan et al. 2013; Nick McElhinny et al. 2010).

PCNA is a critical replication factor that could provide a signal for DNA strand discrimination on the leading strand. PCNA is loaded onto DNA with a specific orientation relative to that of the primer termini, where it is present and acts as a processivity factor for DNA polymerases. This polarity appears to direct the strand specificity of MutL α incision (Pluciennik et al. 2010) and could in principle direct MutL α nicking of the newly

synthesized leading strand if mismatch recognition in some way promoted disassembly of the replication fork allowing MutL α to then nick the DNA. However, a recent study that reconstituted the *S. cerevisiae* DNA replisome in vitro, has found that PCNA may not be as important for the processivity of the leading strand DNA polymerase (Pol ϵ or also called Pol2) as the interaction between Pol ϵ and the CMG complex (Cdc45, Mcm2-7, and GINS) (Georgescu et al. 2014). In contrast, PCNA is essential for promoting the processivity of the lagging strand DNA polymerase (Pol δ or also called Pol3). These results suggest the possibility that either PCNA, as was initially proposed (Umar et al. 1996), or the CMG complex that is also loaded onto DNA with a distinct polarity could provide a strand discrimination signal for MMR, although this latter possibility has not been explored. Additional experimentation will be required to understand strand discrimination during MMR in eukaryotes, particularly for leading strand MMR.

Coupling mismatch recognition to downstream events

Several models have been proposed to explain how MutS recognize mispairs and how this finally leads to the excision and resynthesis of a fragment of the DNA strand that contains the mispair. These include (1) the *stationary model* where MutS remains bound to the mispair and interaction with MMR proteins bends and loops DNA to bring together mispair and strand discrimination signal (Guarne et al. 2004), (2) the *translocation model* that proposes that ATP hydrolysis is the driving force for MutS translocation along the DNA forming DNA loops that bring the mispair into the proximity of the strand discrimination signal (Allen et al. 1997), and (3) the *molecular switch model* also called *sliding clamp model*, in which ATP binding promotes a conformational change in MutS or the MSH complexes allowing them to move along the DNA followed by iterative loading of additional MutS or MSH complexes on the DNA mispair (Acharya et al. 2003). Of the three models, the *translocation model* and the *sliding clamp model* are consistent with the observation that a physical block between the mispair site and the site of an initiating nick blocks MMR (Pluciennik and Modrich 2007; Wang and Hays 2003). A general feature of these models is that they assume that one MutS or MSH complex binds one MutL or MLH complex (in a 1:1 stoichiometry) and translocates or slides away from the mispair along the DNA.

Recently, another model has been proposed in which after mispair recognition an MSH complex catalytically loads several molecules of MLH complexes at or near the site of the mispair (Hombauer et al. 2011a). This model is based on the identification in *S. cerevisiae* of DNA replication-associated Msh2-Msh6 foci and the observation of Mlh1-Pms1 foci that accumulate in an Msh2-Msh6 dependent fashion in response to mispaired bases but which rarely colocalize with Msh2-Msh6 foci or DNA replication factories. Since in order to be visualized as a focus, multiple molecules of these proteins must be in close proximity, and because the interaction between Msh2-Msh6 and Mlh1-Pms1 is unlikely to result in to simultaneous recruitment of multiple Mlh1-Pms1 complexes, it has been proposed that Msh2-Msh6 might be able to catalytically load Mlh1-Pms1 onto DNA in response to mispairs. This model is also supported by the observation that *msh6* dominant mutations (*msh6-S1036P* or *msh6-G1067D*) in *S. cerevisiae*, which prevent scMlh1-Pms1 recruitment in vitro (Hess et al. 2006), were also unable to form Pms1 foci in vivo (Hombauer et al.

2011a). Remarkably, the *msh6-G1142D*-dominant mutation that supports scMlh1-Pms1 recruitment in vitro (Hess et al. 2006) was also defective for Pms1 foci formation, suggesting that recruitment not necessarily guarantee the catalytic loading of scMlh1-Pms1 (Hombauer et al. 2011a). Further studies will be required to demonstrate that Msh2-Msh6 actually promotes catalytic loading of scMlh1-Pms1 onto DNA.

Mismatch excision

Another feature of MMR that still remains to be further explored is how mismatch excision takes place in vivo. For a long time, it has been known that Exo1 is involved during this process; nevertheless, this exonuclease is not essential for MMR. Inactivation of *EXO1* in *S. cerevisiae* or mice results in a very weak mutator phenotype (Amin et al. 2001; Edelmann and Edelmann 2004; Tishkoff et al. 1997; Wei et al. 2003), suggesting that other redundant mechanisms or exonucleases must exist.

In an attempt to identify redundant excision mechanisms, Amin et al. searched for mutations that confer a synergistic mutator phenotype in the absence of *EXO1* (*exo1* -dependent mutations or *EDM*) in *S. cerevisiae* (Amin et al. 2001). Interestingly, no additional exonucleases were identified but rather mutations in MMR genes (*MLH1*, *PMS1*, *MSH2*, and *MSH3*) and genes encoding DNA replication components (Pol30, Pol32, and Rnr1) were found. More recently, additional *EDM* mutations have been identified including mutations in the DNA polymerases Pol δ (*pol3-L612M*) (Hombauer et al. 2011a) and Pol α (*pol1-L868M*) (Liberti et al. 2013), Msh6-PCNA interaction defective mutations (*msh6 2-50* or *msh6-F33AF34A*) (Hombauer et al. 2011a) and *POL30* mutations that cause defects in activation of *S. cerevisiae* Mlh1-Pms1 endonuclease activity (Goellner et al. 2014). In addition, the fact that mutations affecting proteins that function in lagging strand DNA synthesis (Pol α , Pol δ , and Pol32) synergize with an *exo1* mutation to a greater extent than when *exo1* is combined with a low-fidelity leading strand polymerase mutation (*pol2-M644G*), suggests that Exo1 might be more important for the correction of errors generated on the lagging strand (Hombauer et al. 2011a).

The identification of *exo1* -dependent *pol30* mutations resulting in mutant PCNAs that were partially defective in activating the scMlh1-Pms1 endonuclease suggests that mispair excision becomes more Exo1-dependent when scMlh1-Pms1 endonuclease activity is reduced (Goellner et al. 2014). In addition, it was observed that an Msh6-PCNA interaction defective mutation (*msh6 2-50*) resulted in a synergistic increase in mutation rates in combination with an *exo1* mutation or with *pol30* mutations that interfere with the activation of scMlh1-Pms1 endonuclease activity but not in combination with other *pol30* mutations that affect the interaction between Pol30 and Msh6. These results have suggested a model in which Msh2-Msh6 plays a role downstream of scMlh1-Pms1 recruitment by retaining/recruiting PCNA in proximity to the mispair site, which then supports activation of the scMlh1-Pms1 endonuclease (Fig. 5). Then, in the absence of Exo1, scMlh1-Pms1 performs multiple rounds of DNA nicking (stimulated by PCNA) that either directly results in nick-directed degradation of the mutant DNA strand or possibly processing by DNA Pol δ -dependent strand displacement (Kadyrov et al. 2009) or by the 3'-5' exonuclease activity of

DNA polymerases (Tran et al. 1999). Further studies will be required to validate the existence of this type of Exo1-independent mispair excision.

Non-canonical mismatch repair functions

In addition to the *antimutator role* of MMR, which includes the correction of DNA replication errors and the suppression of homeologous recombination events, there is substantial evidence that indicate that MMR can also play a *mutagenic role* promoting genome instability. As was previously mentioned, one of these functions is related to trinucleotide repeats, in which Msh2-Msh3 complexes promote expansions at these regions, by a mechanism which is still not completely understood.

In addition, MMR plays mutagenic functions also referred as *non-canonical mismatch repair* (ncMMR) during antibody diversification, which takes place during somatic hypermutation (SHM) and class switch recombination (CSR) of immunoglobulin genes (Bak et al. 2014; Slean et al. 2008). The mutagenic role of ncMMR is driven by the correction of mispairs uncoupled from DNA replication; *repair events* that occurs in this manner are expected to be mutagenic due to the absence of a DNA strand discrimination signal (Hombauer et al. 2011b), which results in an unbiased and therefore mutagenic repair.

SHM and CSR require the action of the activation-induced deamination (AID) enzyme, as well as the base excision machinery (BER). During SHM, AID converts cytosine into uracil at regions that are actively transcribed, resulting in U:G mismatches at the immunoglobulin genes, that are then recognized by BER and/or MMR. Strand excision is followed by MMR-dependent PCNA ubiquitylation and recruitment of error-prone polymerases (Pol η), which introduces mutations at the repaired track. During CSR, incisions generated by BER and MMR lead to double-strand breaks at the Ig constant region genes, which are then repaired by recombination (non-homologous end-joining and alternative end-joining) with the upstream variable Ig region.

It has been also proposed that ncMMR might not be restricted to the process of immunoglobulin diversification occurring in B cells, but rather might occur in other cell types and potentially drive in some cases carcinogenesis of somatic and non-diving cells (MacPhee 1995). This hypothesis is supported by two recent articles that came to a similar conclusion, though having used different approaches. In a first report (Rodriguez et al. 2012), the authors used single-strand oligo-nucleotides to introduce mutations and revert a non-functional allele of the *TRP5* gene in *S. cerevisiae*. Analysis of the recovery of Trp⁺ revertants after transforming oligos targeting the transcribed or non-transcribed strand, in MMR-proficient or MMR-deficient strains, revealed that when mispair correction is uncoupled from DNA replication, it uses as template for repair either one of the two DNA strands, most likely due to the absence of a DNA strand discrimination signal and is therefore mutagenic. In agreement with mutagenic role for MMR, a second report showed that treatment of human cells with the alkylating agent MNNG results in Mlh1-dependent PCNA ubiquitylation and recruitment of Pol η , independent of DNA replication (Pena-Diaz et al. 2012). Moreover, this study also provided evidence that MMR can promote MNNG-induced mutagenesis as detected using the *HPRT* gene inactivation assay. In summary, these results indicate that MMR participates in recognition of MNNG-induced DNA damage

(during or outside S-phase) promoting mutagenesis due to the lack of a DNA strand directionality signal and the involvement of low-fidelity polymerases, resulting in mutagenic repair (Pena-Diaz et al. 2012).

Identification of new genes/mechanisms preventing mutator phenotypes

It is well known that germline inactivating mutations affecting the major MMR genes cause inherited cancer susceptibility, and that sporadic cancers showing increased mutation rates, often seen as increased microsatellite instability, have acquired MMR defects, which in most cases are consequence of epigenetic silencing of *hMLH1* (Kinzler and Vogelstein 1996; Kolodner et al. 1999; Peltomaki and Vasen 2004). However, not all microsatellite unstable tumors and tumors with increased mutation rates are associated with mutation or altered expression of the genes encoding the essential MMR components, suggesting that additional factors may contribute to mutator phenotypes linked to the development of cancer (Peltomaki 2003).

Several recent studies have demonstrated a potential role for interactions of chromatin modifications with MMR. These include the observation that the activity of H3K36 trimethyltransferase SetD2 plays a role in the recruitment of MutS α to chromatin in humans (Li et al. 2013). The observation that depletion of SetD2 causes a weak mutator phenotype and that *SETD2* may be rarely mutated in some cancers raises the possibility that defects which affect the crosstalk between MMR and chromatin can result in a mutator phenotype and cancer susceptibility, although this remains to be definitively proven. In addition, in *S. cerevisiae*, a second histone modification, the S-phase specific H3K56 acetylation, has been suggested to play a role in genome maintenance, together with MMR and DNA Pol ϵ and Pol δ proofreading activity (Kadyrova et al. 2013). Intriguingly, deletion of *MSH2* synergizes with defects in both acetylation and deacetylation, suggesting that alteration of H3K56 acetylation is distinct from a MMR defect, but causes genome instability due to the interaction of MMR defects with damage caused by deregulation of histone modification.

A second mechanism that could lead to increased mutation rates and the development of cancer is an imbalance in the expression of MMR proteins. In *S. cerevisiae*, it is known that overexpression of wild-type scMlh1, scMlh2, scMlh3, or scPms1 results in a mutator phenotype (Campbell et al. 2014; Shcherbakova et al. 2001; Shcherbakova and Kunkel 1999). This suggests that the overexpression of some MMR proteins can titrate out other MMR proteins resulting in an MMR defect; an example is the titration of scMlh1 by increased expression of either scMlh2 or scMlh3 that then interferes with the formation of sufficient scMlh1-Pms1 complex to support MMR (Campbell et al. 2014). Similarly, overexpression of Msh3 in mammalian cell lines (Drummond et al. 1997; Marra et al. 1998) and overexpression of hPms2 in mouse cells can result in elevated mutation frequencies (Gibson et al. 2006). In line with these findings, immunohisto-chemical staining of prostate cancer samples identified elevated levels of hPms2, which correlated with MSI (Norris et al. 2007) and increased recurrence after prostatectomy (Norris et al. 2009). More recently, a comparative study of genome databases available for head and neck squamous cell carcinomas (HNSCC) and human HNSCC cell lines, identified *hPMS1* as one of eight genes that were consistently found to have increased copy number (Li et al. 2014). Yet another

study that analyzed chromosomal genomic hybridization databases for human cancer identified multiple chromosomal hot spots for genome amplification that contained different oncogenes as well as MMR genes including *hMSH2*, *hPMS1*, and *hPMS2* (Myllykangas et al. 2006). These latter studies provide some support for the idea that increased copy number and increased expression of MMR genes might be linked to increased mutation rates and development of cancer although additional analysis will be required to provide definitive proof of this.

Finally, it has been known for many years that mutations affecting the active site of DNA polymerases or their exonuclease domain (reviewed in McCulloch and Kunkel 2008; Prindle and Loeb 2012; Reha-Krantz 2010) can result in increased accumulation of mutations. The high levels of misincorporation of errors likely results in saturation of MMR, such that some of the resulting mispairs escape repair (Damagnez et al. 1989; Schaaper and Radman 1989). Consistent with this, a number of studies have recently identified both inherited and acquired mutations in human leading and lagging strand polymerases (*POLE* and *POLD1*, respectively) affecting conserved residues, which are part of the proofreading domain of these DNA polymerases in colorectal and endometrial cancers (Church et al. 2013; Kane and Shcherbakova 2014; Palles et al. 2013). These defects cause a hyper-mutator phenotype characterized by the accumulation of base substitution mutations, but do not appear to result in increased microsatellite instability.

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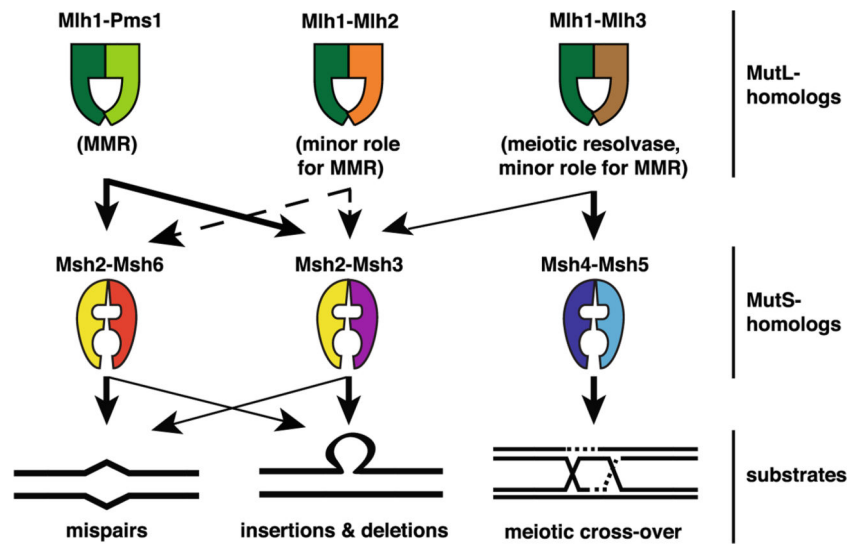


Fig. 1. *S. cerevisiae* MSH and MLH complexes. The *arrows* indicate their functional interactions and potential functions in vivo. *Arrows* denote major roles (*thick arrows*) and minor roles (*thin arrows*) in the indicated processes. *Dashed lines* represent interactions that are biologically relevant apparently only in specific genetic backgrounds

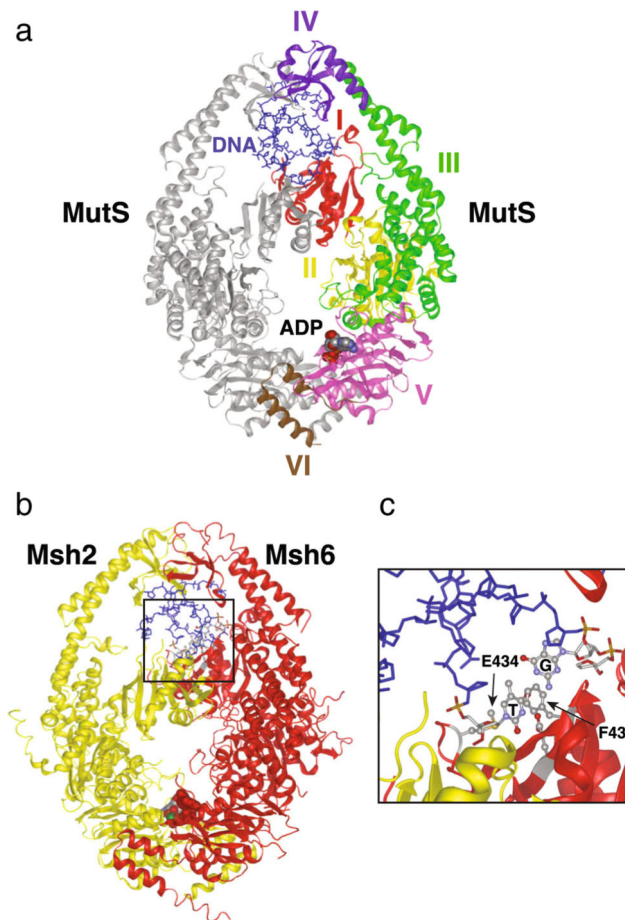


Fig. 2. Crystal structure of *E. coli* MutS and human MutS α according to previous reports (Lamers et al. 2000; Warren et al. 2007). **a** MutS homodimer, in which the mispair-contacting subunit has been colored by domain (I–VI) with red, yellow, green, purple, pink, and brown, respectively. DNA (blue sticks) and ADP are also indicated. **b** Human Msh2-Msh6 complex. Msh2 is shown in yellow and Msh6 in red. **c** Expanded view of the region in the black box in (b) that directly interacts with the mispaired base. The conserved Msh6 residues phenylalanine (F432) and glutamic acid 434 (E434), as well as the mispaired bases guanine (G) and thymine (T) are indicated

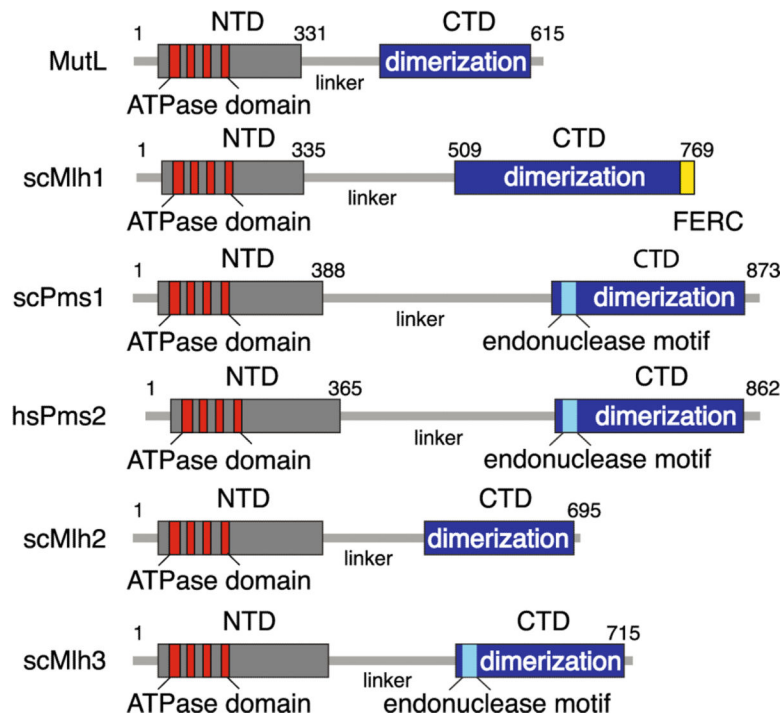


Fig. 3. Structural domains of MutL and eukaryotic MutL homologs. The N-terminal domain (NTD) is indicated by a *gray box* that contains an ATPase domain consisting of four highly conserved motifs (shown in *red*). The dimerization domain and the endonuclease motif $DQHA(X)_2E(X)_4E$ (*light blue box*) contained within the C-terminal domain (CTD) (*blue box*) are indicated. The conserved *FERC* sequence of Mlh1 is shown in *yellow*

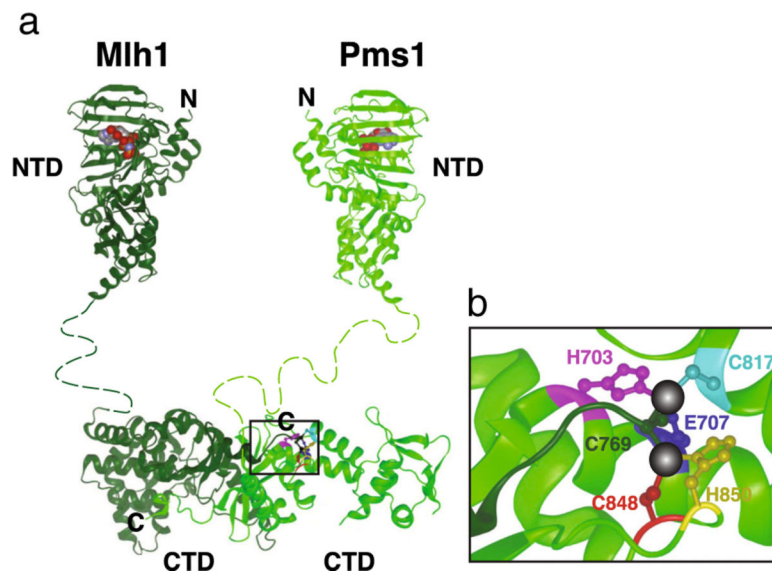


Fig. 4. Model for the Mlh1-Pms1 complex in *S. cerevisiae* adapted from Smith et al. 2013. **a** Mlh1 is shown in *dark green* and Pms1 in *brighter green*. Illustration of the N-terminal domains was generated based on structure pdb.3H4L (Arana et al. 2010), whereas the C-terminal domain structure corresponds to pdb.4e4w (Gueneau et al. 2013). N- and C-terminal domains are linked by an unstructured linker (*dashed line*). **b** Expanded view of the conserved amino acids comprising the metal binding pocket (metal ions in *black*) of the composite endonuclease site located within the *black box* in (**a**)

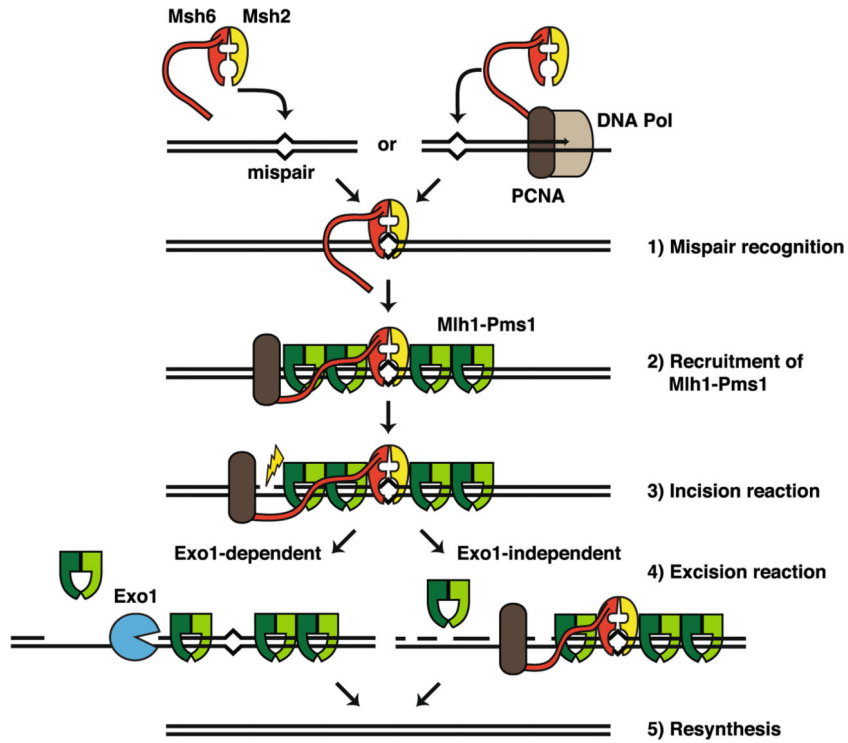


Fig. 5. Model of alternative excision pathways that act during MMR adapted from Goellner et al. 2014. The diagram describes the sequential steps of the MMR reaction. The Msh2-Msh6 heterodimer recognizes mispairs (1). A fraction of the recognition complexes is coupled to replication machinery (PCNA-DNA Pol) through PCNA. Alternatively, the Msh2-Msh6 complex scans the DNA for mispairs in a PCNA-interaction independent manner. The mispair-bound Msh2-Msh6 complex recruits Mlh1-Pms1 (2). The loading of Mlh1-Pms1 onto DNA is catalytic whereby one mispair recognition complex recruits several Mlh1-Pms1 complexes to or near the mispair site. The Mlh1-Pms1 endonuclease is then activated by PCNA to nick the DNA comprising the incision step (3). At this step, Msh6 plays a role by retaining or recruiting PCNA in proximity to the mispair site. The excision reaction (4) can be mediated by Exo1 (Exo1-dependent) or by unknown factors (Exo1-independent) potentially including multiple rounds of incision by Mlh1-Pms1, strand displacement by the DNA replication machinery or other exonucleases followed by resynthesis of the DNA (5)

Table 1

MMR proteins in *E. coli*, *S. cerevisiae* and *H. sapiens*

<i>E. coli</i>	<i>S. cerevisiae</i>	<i>H. sapiens</i>	Comments
MutS-MutS	Msh2-Msh6 (MutS α) Msh2-Msh3 (MutS β)	Msh2-Msh6 (MutS α) Msh2-Msh3 (MutS β)	Mismatch recognition complex—homodimer in <i>E. coli</i> and a heterodimer in eukaryotes. MutS α and MutS β have overlapping mismatch recognition specificity and are partially redundant
	Mlh1-Pms1 (MutL α)	Mlh1-Pms2 (MutL α)	Homodimer in <i>E. coli</i> and heterodimer in eukaryotes. MutL (<i>E. coli</i>) and MutL α (eukaryotes) play a central role during MMR. In <i>E. coli</i> , MutL promote the nicking reaction via MutH, whereas in eukaryotes MutL α possess an intrinsic endonuclease activity
MutL-MutL	Mlh1-Mlh2 (MutL β) Mlh1-Mlh3 (MutL γ)	Mlh1-Pms1 (MutL β) Mlh1-Mlh3 (MutL γ)	MutL β is an accessory factor for MMR MutL γ substitutes for MutL α in the repair of a minor fraction of mismatches, but primarily acts in the resolution of meiotic recombination intermediates
Dam methylase	Absent	Absent	Promotes N ⁶ -adenine methylation at d(GATC) sites, serves as strand discrimination signal in <i>E. coli</i>
MutH	Absent ^a	Absent ^a	Endonuclease, nicks daughter strand using d(GATC) hemi-methylated sites as strand discrimination signal
none	Exo1	Exo1	5'-3' dsDNA exonuclease, acts in the excision reaction
RecJ, ExoVII	None	None	5'-3' ssDNA exonuclease, acts in the excision reaction
ExoI, ExoVII, ExoX	None	None	3'-5' ssDNA exonuclease, acts in the excision reaction
UvrD	None or unknown	None or unknown	DNA helicase II, promotes excision reaction, activated by MutS
β -clamp	PCNA	PCNA	DNA polymerase processivity factor. In eukaryotes stimulates Mut α endonuclease activity. The gene encoding PCNA in <i>S. cerevisiae</i> is <i>POL30</i>
γ -Complex	RFC	RFC	Loading of β -clamp/PCNA
SSB	RPA1-3	RPA1-3	ssDNA binding protein, acts in the excision and DNA resynthesis reactions. The genes encoding RPA subunits in <i>S. cerevisiae</i> are <i>RFA1</i> , 2 and, 3
DNA Pol III	Pol δ	Pol delta	DNA polymerase that acts in the gap-filling step
DNA ligase	Unknown	Ligase I	Seals nicks after DNA resynthesis

^aThis endonuclease function appears to be present in MutL α and MutL γ

Table 2

Pms1-foci abundance in different MMR-related mutants in *S. cerevisiae*

Biochemical defect	Relevant genotype	Mutator phenotype (frameshift-reversion assay)	% Pms1 foci ^d	Reference
None (wild-type strain)	Wild-type	None	10	Hombauer et al. 2011a, b
1. Reduced DNA replication fidelity				
(a) exonuclease defective polymerases	<i>pol3-01; pol2-04</i>	None	20–25	Hombauer et al. 2011a
(b) polymerase active-site mutation	<i>pol3-L612M</i>	None	20	Hombauer et al. 2011a
2. Mutations that interfere with MMR				
(a) Preventing mismatch recognition	<i>msh2 ; msh3 msh6</i>	Strong	0	Hombauer et al. 2011a
(b) Msh6 dominant mutations (in an <i>msh3</i> background)	<i>S1036P; G1067D; G1142D</i>	Strong	0	Hombauer et al. 2011a
(c) Excision (5'-3' Exo1-dependent)	<i>exo1</i>	Weak	50	Hombauer et al. 2011a
(d) Nicking reaction				
PCNA (Pol30) mutations that prevent Pms1 endonuclease activation	<i>K13E; C22Y; K217E</i>	Moderate	60–95	Goellner et al. 2014
Pms1 endonuclease defective mutations	<i>G683E; E707K; C817R; C848S</i>	Strong	60–85	Smith et al. 2013
Mlh1 endonuclease defective mutation	<i>E767stop</i>	Strong	95	Smith et al. 2013

^dPercentage of yeast cells containing Pms1-foci on a logarithmically growing population for the indicated genotypes