The multifaceted mismatch-repair system

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Abstract | By removing biosynthetic errors from newly synthesized DNA, mismatch repair (MMR) improves the fidelity of DNA replication by several orders of magnitude. Loss of MMR brings about a mutator phenotype, which causes a predisposition to cancer. But MMR status also affects meiotic and mitotic recombination, DNA-damage signalling, apoptosis and cell-type-specific processes such as class-switch recombination, somatic hypermutation and triplet-repeat expansion. This article reviews our current understanding of this multifaceted DNA-repair system in human cells.

Microsatellite instability
Alterations in the length of short repetitive sequences (microsatellites), which can be detected by the appearance of new bands after PCR amplification of microsatellite DNA from MMR-deficient cells, or tumours, that were not present in PCR products of the corresponding DNA from normal cells.

Heteroduplex
A DNA duplex formed by association between two homologous but non-identical strands, or a duplex that contains one or several mismatches that arose during replication.

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Over the past decade, a significant proportion of cancers of the colon, endometrium and other organs were found to exhibit a phenotype known as microsatellite instability (MSI; see REFS 1,2 for reviews). Microsatellites are repeated-sequence motifs, such as $[A]_n$ or $[CA]_n$, which are present in our genome in large numbers. During DNA synthesis, the primer and template strands in a microsatellite can occasionally dissociate and re-anneal incorrectly[3]. This gives rise to heteroduplex DNA molecules, in which the number of microsatellite-repeat units in the template and in the newly synthesized strand differ. These heterogeneities, in which the partnerless nucleotide is partially extrahelical, are known as insertion/deletion loops (IDLs). Together with base–base mismatches, which are caused by errors of DNA polymerases that escape their proofreading function, IDLs are addressed by the mismatch repair (MMR) system, which degrades the error-containing section of the newly synthesized strand and therefore provides the DNA polymerase with another chance to generate an error-free copy of the template sequence. In the absence of MMR, IDLs and base–base mismatches remain uncorrected, which results in a mutator phenotype that is accompanied by MSI and, eventually, in cancer.

Thanks to the evolutionary conservation of this complex pathway, much of our understanding of mammalian MMR has come from the study of *Escherichia coli* and *Saccharomyces cerevisiae* (for a recent review, see REF. 4). However, the importance of MMR malfunction for human disease can only be appreciated in mammalian systems. These studies revealed that MMR proteins are also implicated in other DNA-metabolic pathways, ranging from DNA-damage signalling to recombinogenic and mutagenic processes that are limited to multicellular organisms. This review will focus on the latest advances in our understanding of human MMR (for other reviews, see REFS 2,4–9).

Criteria for successful MMR

The MMR machinery has to satisfy two criteria: first, it must efficiently recognize base–base mismatches and IDLs; second, it must direct the repair machinery to the newly synthesized DNA strand, which carries the erroneous genetic information. How these tasks are fulfilled was first elucidated in *E. coli*, where studies of the mutator strains *mutS*, *mutL*, *mutH* and *uvrD* culminated in the reconstitution of this prototypic MMR system from individual purified components[10].

In *E. coli*, the recognition of biosynthetic errors is mediated by a MutS homodimer, which then recruits a homodimer of MutL (FIG. 1a). The ATP-dependent formation of this ternary complex activates the endonuclease activity of MutH, which is bound to a hemi-methylated GATC site. These sites are normally methylated on adenines, but because the modifying enzyme, deoxyadenine methylase, lags behind the replication fork by ~2 minutes, the newly synthesized strand is transiently unmethylated. MutS–MutL-activated MutH uses this time window to incise the unmethylated strand. The UvrD helicase unwinds the ends of the nicked error-containing strand from the template. This enables one of several exonucleases to digest the unwound DNA, either in the 5′→3′ direction when the nearest hemi-methylated GATC site lies 5′ from the mismatch, or in the 3′→5′ direction if it lies 3′ from the mismatch. The exonucleolytic degradation stops once the mismatch has been removed. The resulting gap is then filled by DNA polymerase III and the repair is completed when DNA ligase seals the remaining nick.

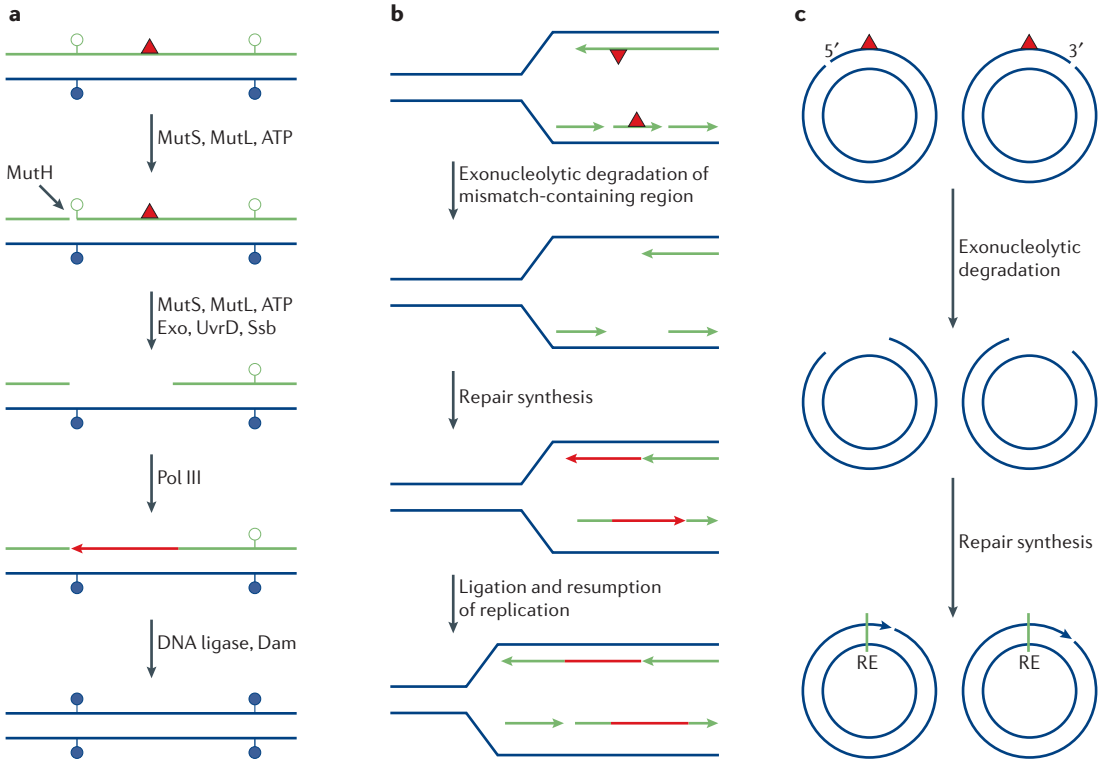


Figure 1 | **Postreplicative mismatch repair. a** | In *Escherichia coli*, newly synthesized DNA (green) is transiently unmethylated at GATC sites (empty circles). The mismatch (red triangle)-activated MutS–MutL–ATP complex licenses the MutH endonuclease to incise the nearest unmethylated GATC sequence (either 5′ or 3′ from the mismatch). UvrD helicase, together with one of several exonucleases (Exo), generate a gap that extends from the nick to ~100 nucleotides past the mispair, and which is stabilized by the single-stranded DNA-binding protein Ssb. This gap is filled (shown in red) by DNA polymerase III (pol III) and the remaining nick is sealed by DNA ligase. The GATC sites are then methylated (solid blue circles) by deoxyadenine methylase (Dam). **b** | In mammalian cells, the degradation of the mismatch-containing leading strand (green) might begin at the 3′ terminus of the primer strand. Once the mispair is removed, the polymerase resynthesizes the degraded region. Mismatch repair (MMR) in the lagging strand might remove an entire Okazaki fragment, with degradation commencing at either end. Extension of the fragment closest to the replication fork would replace the degraded one. Removal of the RNA termini of the Okazaki fragments, followed by ligation, would give rise to a continuous, error-free, lagging strand. **c** | Substrates used in the *in vitro* MMR assays[14,15]. These circular heteroduplexes contain a single-strand break that is either 5′ or 3′ from a mismatch, which lies within a recognition sequence of a restriction enzyme (RE), and therefore makes the molecule refractory to cleavage by this enzyme. Mismatch-provoked degradation of the discontinuous strand, followed by repair synthesis, restores the RE-cleavage site.

Although the MutS and MutL proteins are highly conserved, MutH is found only in Gram-negative bacteria and no functional homologue has been identified in other organisms. This led to the suggestion that the processing of mismatches that arise during replication could be directed by strand breaks[11] such as the 5′ or 3′ termini of Okazaki fragments in the lagging strand, or the 3′ terminus of the leading strand (FIG. 1b). Similarly, the repair of mismatches that arise during recombination could be directed to the invading strand by its 3′ terminus. This hypothesis was supported experimentally; *E. coli* strains that lack MutH were shown to process mismatches *in vivo*[12,13] and *in vitro*[10], provided that the substrate contained strand breaks in the vicinity of the mispair. The same is true for human *in vitro* MMR assays[14–16] (FIG. 1c).

Mismatch recognition in mammalian cells

The situation in eukaryotes is more complex than in *E. coli*. Of the five MutS homologues (MSH) that have been identified in human cells, MSH2, MSH3 and MSH6 participate in MMR in the form of heterodimers (see REF. 4 for a recent review). The most abundant mismatch-binding factor is composed of MSH2 and MSH6. This factor, which is often referred to as MutSα, initiates the repair of base–base mismatches and IDLs of one or two extrahelical nucleotides[17–19], whereas the repair of larger IDLs is initiated by MutSβ, which is a heterodimer of MSH2 and MSH3 (TABLE 1).

The partial redundancy between MutSα and MutSβ helps to explain the different tumour phenotypes of *Msh2*-, *Msh3*- and *Msh6*-null mice. As *Msh2*$^{-/-}$ animals lack all mismatch recognition functions, they have the most severe phenotype[20]. The phenotype of *Msh6*$^{-/-}$ mice is less severe compared with *Msh2*-null animals[21] because MutSβ can deal with most IDLs. *Msh3*$^{-/-}$ animals are not tumour prone[22], presumably because MutSα can initiate the repair of most replication errors.

Okazaki fragments
Fragments of ~200–1000 nucleotides in length that are generated on the lagging strand during replication. They contain short RNA sequences at their 5′ termini, which are made by the primase complex. During the later stages of replication, the RNA sequences are removed and the Okazaki fragments are joined together to form a continuous strand.

Table 1 | **Human MutS and MutL homologue complexes that are involved in mismatch repair**

Complex	Components	Function	References
MutSα	MSH2, MSH6	Recognition of base–base mismatches and small IDLs	17–19,30
MutSβ	MSH2, MSH3	Recognition of IDLs	19,25
MutLα	MLH1, PMS2	Forms a ternary complex with mismatch DNA and MutSα; increases discrimination between heteroduplexes and homoduplexes; also functions in meiotic recombination	43,65,71,73
MutLβ	MLH1, PMS1	Unknown	44
MutLγ	MLH1, MLH3	Primary function in meiotic recombination; backup for MutLα in the repair of base–base mismatches and small IDLs	45,46,110

IDL, insertion/deletion loop; MLH, MutL homologue; MSH, MutS homologue; PMS, post-meiotic segregation protein.

The MutSα sliding clamp. What is the molecular mechanism of mismatch recognition? The MSH proteins are ATPases that possess the Walker ATP-binding motif[23], which is highly conserved among polypeptides that are involved in DNA repair[24]. However, nucleotides are dispensable for the initial binding of MutSα and MutSβ to their respective substrates[17–19,25], and mismatch binding is largely unaffected by amino-acid substitutions that alter the ATP-hydrolysis or ATP-binding properties of one or both subunits[26,27].

The lack of effect of ATP-binding-site mutations on initial mismatch recognition was explained when the MutS homologue protein of *Thermus aquaticus* was shown to contact mismatched DNA through a Phe residue within a highly conserved Phe-X-Glu motif[28] — this motif is in the N terminus and therefore far removed from the C-terminal ATP-binding sites of MutS homologues. Interestingly, in eukaryotes, the Phe-X-Glu motif is conserved only in MSH6, which indicates that only MSH6 might be involved in the initial mismatch recognition. Indeed, when the conserved Phe residue in human MSH6 was mutated to Ala, MutSα lost its DNA-binding activity[29] (how MutSβ recognizes IDLs is currently unknown).

Although nucleotide binding might not be required for the initial recognition of a mismatched substrate, ATP controls the functions of MutSα that occur immediately after its initial contact with DNA. So, on the addition of ATP, MutSα rapidly dissociates from complexes with both homoduplex and heteroduplex oligonucleotides. Interestingly, when the ends of the oligonucleotides are blocked with streptavidin[26,30,31], or with the lac repressor[32], the heterodimer remains selectively trapped on the mismatch-containing substrates while it dissociates from homoduplexes. These findings indicated that mismatch binding in the presence of ATP and magnesium brings about a rapid conformational change in MutSα, which allows it to release the mismatch and move along the DNA contour in the form of a sliding clamp.

Whether the movement of MutSα on the DNA is driven by ATP hydrolysis[31] or whether it is hydrolysis independent[33] has been the subject of discussion, mainly because it is difficult to design experiments that can separate the different functions of the heterodimer. So, although the ATPase activity of MutSα is clearly essential for its function in MMR, it is not easy to study its many functions — mismatch binding, clamp formation, translation of the DNA and dissociation from the DNA (either directly or by sliding off an end) — in isolation, and determine which require ATP binding and which require hydrolysis of the nucleotide. Moreover, because MutSα has two highly homologous ATP-binding sites that are functionally interdependent, it might exist in nine different nucleotide-occupancy states[34]. Not even the determination of the crystal structures of the MutS proteins from *E. coli*[35] and *T. aquaticus*[36] could elucidate the role of nucleotides in the function of MutSα.

However, the MutS structures clearly substantiated the findings of the earlier biochemical studies. So, in the absence of DNA, the ATP-binding domains of the two MutS subunits were interlocked and clearly structured, whereas the remainder of the protein was disordered (FIG. 2a). In a co-crystal with a mismatched oligonucleotide, the MutS homodimer became highly ordered and was clamped around the DNA like a pair of praying hands[37]. The protein 'anchored' itself on the substrate by inserting the Phe residue of the Phe-X-Glu motif of one of the subunits (a thumb of one of the hands) into the minor groove of the helix at the mismatch site. This resulted in the bending of the DNA by ~60° (FIG. 2b).

Human MutSα that is purified from cell extracts carries ADP[38], but its binding to DNA was shown to bring about an ADP→ATP exchange[30]. This exchange was accompanied by the factor's conversion into a sliding clamp, which seemed to require ATP binding but not hydrolysis for its translocation. In the structure of bacterial MutS, the ATP-binding site of the subunit that contacts the mismatch was occupied by an ADP molecule, and the ATP-binding site of the other subunit was empty. Together with the fact that the Phe residue was inserted into the helix at the mismatch site, this indicates that the structure that was trapped in the crystal represented an early stage of binding. By diffusing different nucleotides into the MutS–DNA–ADP co-crystals[39,40], the composite MutS ATP-binding sites became more compact. This compaction was postulated[40] to release the MutS–ATP complex from the mismatch by forcing the Phe-containing 'thumb' out of the DNA through steric interactions, while keeping the 'fingers' closed around it (FIG. 2c).

In this 'thumb-out, fingers-closed' structure, ATP was bound in both subunits, but was hydrolysed in neither. This differs from human MutSα, which has one

Walker ATP-binding motif
A protein fold consisting of ~200 amino-acid residues that is found in many DNA-repair proteins. The nucleotide-binding domain consists of three conserved motifs; the type A and B motifs are the most highly conserved.

Sliding clamp
A protein complex that encircles DNA and allows it to pass freely through the hole in its centre. The term was first coined to describe the β-subunit of *E. coli* DNA polymerase III and proliferating cell nuclear antigen (PCNA), which tether DNA polymerases to DNA, thereby increasing their processivity (that is, the number of nucleotides that are incorporated into DNA per polymerase–template binding event).

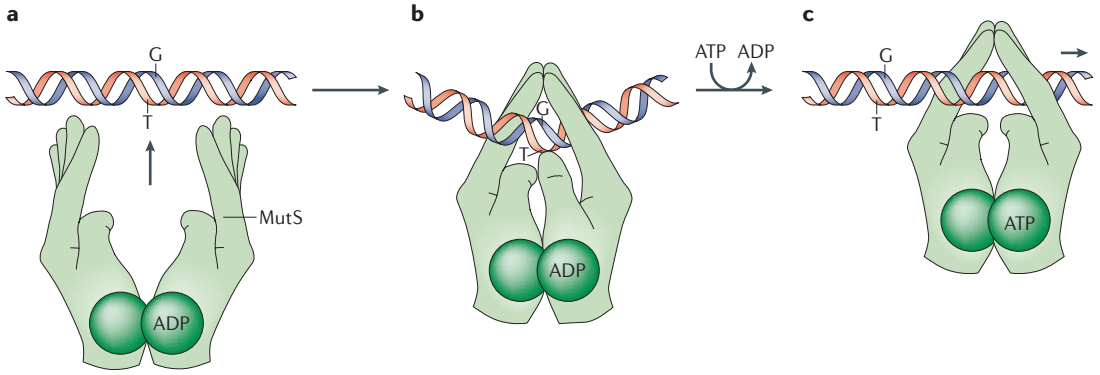


Figure 2 | **The MutS sliding clamp and its activation. a** | The ADP-bound MutS homodimer binds to a G–T mismatch in duplex DNA. In the absence of DNA, the finger domains are unstructured and open, and the ATP-binding sites are dimerized. **b** | I the presence of the mismatched DNA, the ADP-bound form of MutS is wrapped around the DNA like a pair of praying hands, and is anchored at the mismatch site by a Phe-X-Glu wedge (the thumb of one of the hands) that is inserted into the minor groove of the duplex. **c** | ADP/ATP exchange brings about a conformational change that releases the Phe-X-Glu thumb from the mismatch site, but leaves the fingers closed around the duplex. The clamp is now free to translocate along the DNA in either direction (only one direction shown here). Although no eukaryotic MutSα structure is available, biochemical experiments indicate that these heterodimers function analogously to the bacterial proteins. The idea of the 'praying hands' was taken from REF. 37.

high-affinity ADP-binding site and one high-affinity ATP-binding site; the protein apparently cannot bind two ATP molecules concurrently[41]. It remains to be shown whether the ADP–MutSα–ATP species is actually involved in mismatch recognition, and which structural changes and/or changes in the nucleotide-binding site occupancy take place that help differentiate between heteroduplex and homoduplex substrates, such that MutSα forms a sliding clamp on the heteroduplex substrates and dissociates directly from the homoduplex substrates.

MutL homologues: molecular matchmakers?

The MutL proteins are ATPases of the GHKL (gyrase/Hsp90/histidine-kinase/MutL) family[42], with the ATPase situated in the N-terminal domain and the dimerization domain at the C terminus. Human cells express four MutL homologues: MLH1, MLH3, PMS1 (post-meiotic segregation protein-1) and PMS2, which function as three distinct heterodimers (TABLE 1). The complex that is composed of MLH1 and PMS2 — MutLα — has the most important role in MMR, as cells that lack either protein exhibit mutator phenotypes and MSI that is comparable to cells that are mutated in *MSH2* (reviewed in REF. 2). However, as *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ mice do not have identical phenotypes (*Pms2*$^{-/-}$ mice, although cancer-prone, do not get gastrointestinal tumours[43]), it was postulated that there must be a 'backup' for PMS2 in DNA metabolism. MutLβ, which is composed of MLH1 and PMS1, could conceivably fulfil this function. Although this heterodimer could not be shown to participate in MMR *in vitro*[44], *Pms1*-knockout mice exhibit MSI in mononucleotide runs[43], so MutLβ might be involved in MMR to some extent. Similarly, MutLγ, which is composed of MLH1 and MLH3, and which has hitherto been believed to be predominantly involved in meiotic recombination, might also have a backup role in mammalian MMR. *Mlh3*-knockout mice exhibit weak instability in mononucleotide microsatellites and are tumour prone[45], and recombinant MutLγ participates, albeit inefficiently, in the repair of base–base mismatches and single-nucleotide IDLs[46]. The role of this factor, therefore, seems to differ from that of the yeast MutLγ, which has been implicated in the repair of a subset of IDLs[47].

How do MutL homologues function in MMR? In *E. coli*, the ATP-activated mismatch-bound MutS sliding clamp was shown to first interact with a homodimer of MutL. This interaction is believed to modulate the ATP-hydrolysis-dependent turnover of the complex and/or its interaction with the MMR factors bound at the excision-initiation site, such as MutH and UvrD (reviewed in REF. 4). How this interaction might be mediated is subject to discussion. The first of three favoured models indicates that the MutS–MutL complex forms at the mismatch and then translocates in either direction and helps to initiate the excision process on encountering MutH and UvrD[48]; the second model indicates that MutS interacts with MutL at the site of repair initiation; and the third model indicates that the MutS–MutL complex remains bound at the mismatch and interacts with the MutH and UvrD proteins by looping out the intervening DNA[49]. Evidence in support of these models is largely indirect; the MutS–MutL complex, however, was shown to migrate during *E. coli* MMR *in vivo*[50], which argues against the third scenario.

Similar to the bacterial MutS and MutL proteins, human MutSα and MutLα can form relatively stable ATP-dependent ternary complexes on oligonucleotide substrates that have free ends[51–53]. This evidence, coupled with the *in vitro* finding that mismatch-provoked degradation of the nicked strand can be initiated on substrates that contain physical barriers between the mismatch and the excision-initiation site[54,55], has led to the proposal that the MutSα–MutLα complex remains

bound at the mismatch and initiates the repair reaction by looping out the intervening DNA. However, surface-plasmon-resonance studies that have been carried out with the human[51] and yeast[32] MutSα–MutLα complexes provided evidence that the MutSα–MutLα complex can travel along the DNA contour similarly to the MutSα sliding clamp.

Unfortunately, none of the experimental systems deployed so far show where the ternary complex assembles. The propensity of the proteins to associate also with DNA ends[53,56] complicates the interpretation of the results of these studies. In some experiments, the formation of the ternary complexes was mismatch independent and was very susceptible to changes in experimental conditions. The fact that the ternary complex possesses four ATP-binding sites on the MutL and MutS homologues further complicates data interpretation. Most importantly, it is unclear whether these studies reflect the function of these polypeptides during MMR, given that the repair process involves several important accessory proteins (see below), which were not included in the above assays.

PCNA and MMR

In addition to the MutS and MutL homologues, MMR requires several other factors, most of which are involved in DNA replication. One such factor is the homotrimeric proliferating cell nuclear antigen (PCNA). During DNA replication, the PCNA sliding clamp is loaded onto the 3′ terminus of an Okazaki fragment or onto the 3′ end of the leading strand by replication factor C (RFC). Once loaded, it associates with the replicating DNA polymerase and functions as its processivity factor. Because of this function, the involvement of PCNA in MMR synthesis was anticipated.

Surprisingly, experiments in the Kunkel laboratory implicated PCNA in a step of the MMR process that preceded repair synthesis[57]. This finding was substantiated when MSH6 and MSH3 were shown to interact with PCNA both *in vitro* and *in vivo* through highly conserved PCNA-interacting motifs near their N termini[58]. This indicated that MutSα and/or MutSβ might be present in the DNA replication complex, where they might control the progress of DNA synthesis by, for example, interacting with the PCNA–DNA polymerase complex on mismatch binding.

In more recent studies, the binding of MutSα to mismatched substrates was shown to lead to the factor's dissociation from PCNA[59], which indicated that the processivity factor might bind MutSα during DNA replication, but hand it over to the MMR machinery when a mismatch is detected.

Exonucleases in MMR

Given that the mismatch-dependent degradation of heteroduplex substrates can initiate at a strand break or gap that is either 5′ or 3′ from the mispair (FIG. 1b), it was anticipated that the MMR process would involve exonucleases of both 3′→5′ and 5′→3′ polarity. In agreement with this expectation, yeast genetic studies implicated the 3′→5′ proofreading activities of DNA polymerase δ and/or DNA polymerase ε in MMR[60,61], and recent findings indicated that the 3′→5′ exonuclease activity of MRE11 (REF. 62) might also be required *in vivo*. As far as 5′→3′ exonucleases are concerned, the only enzyme with this directionality that has been implicated in MMR *in vivo* and *in vitro* is exonuclease-1 (EXO1, reviewed in REF. 63). Unlike mice that lack MSH2 or MLH1, *Exo1*$^{-/-}$ mice are prone to lymphomas but not to gastrointestinal tumours. They are sterile, like the *Mlh1*$^{-/-}$ animals, but the meiotic defect seems to take place later. The extracts of embryonic stem (ES) cells are deficient in the repair of base–base mismatches and one-nucleotide IDLs, but not of larger IDLs. Correspondingly, the mutator phenotype of the cells is weaker than that seen in MSH2- and MLH1-deficient cells and MSI is limited to mononucleotide repeats. Taken together, the above evidence indicates that EXO1 is involved in some, but not all, MMR-dependent events *in vivo*[64]. It was therefore surprising to learn that a reconstituted MMR system (see below) required only EXO1 for both 5′→3′ and 3′→5′ mismatch correction.

Reconstitution of the human MMR system

The reconstitution of the bidirectional MMR system from its individual purified components represents one of the main challenges of the DNA-repair field. Initial studies attempted to fractionate HeLa-cell extracts and identify the MMR constituents in the active fractions. This led not only to the identification of MutSα[18] and MutLα[65], but also pointed to DNA polymerase δ[66], the single-stranded binding-factor replication protein A (RPA)[67,68], EXO1 (REF. 69) and the non-histone chromatin component high-mobility group box 1 (HMGB1)[70] as MMR factors. Together with PCNA, this spectrum of proteins seemed comprehensive enough to attempt MMR reconstitution. The initial studies focused on the mismatch-provoked exonucleolytic degradation of the discontinuous strand in heteroduplex substrates (FIG. 1c).

5′→3′ strand degradation. On a heteroduplex substrate that contained a single G–T mismatch and a strand break that was situated 125 nucleotides away in a 5′ direction, EXO1 catalysed the 5′→3′ degradation of the discontinuous strand, such that the mismatch was removed in a single step[69]. However, the loading of EXO1 at the break was very inefficient. In the presence of MutSα–ATP, EXO1 was loaded at the break much more efficiently, and its processivity was increased. This differed from the situation in cell extracts, in which the degradation tracts terminated ~150 nucleotides past the mismatch site.

In subsequent studies[71,72], the system was examined in greater detail. In the presence of HMGB1, the efficiency of MutSα-dependent EXO1-mediated 5′→3′ degradation was optimal[72]. RPA also stimulated EXO1 activity in the presence of MutSα, but only so long as the mismatch was present. Once the mispair was removed, RPA inhibited EXO1, presumably by displacing it from the homoduplex[73]. The 5′→3′ degradation reaction did not require MutLα, even though the presence of this factor improved the selectivity of the reaction for heteroduplex

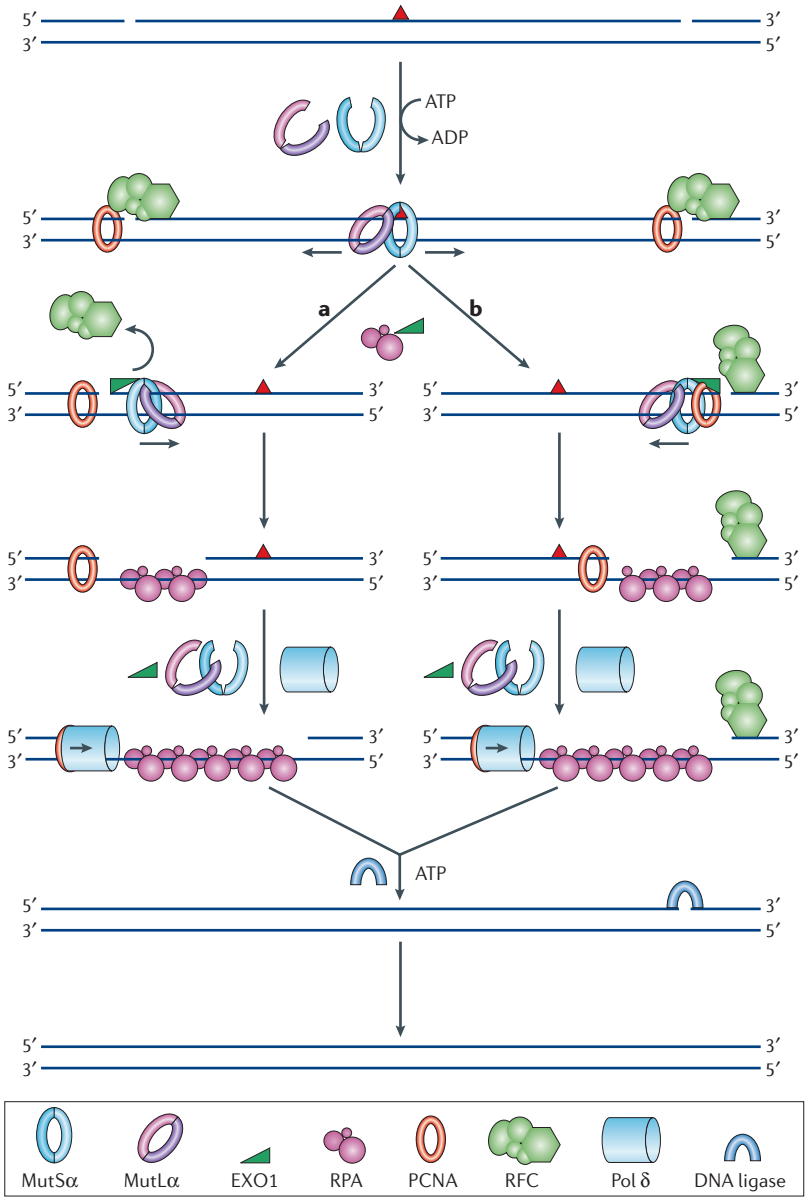


Figure 3 | **The reconstituted human mismatch-repair system.** The mismatch repair (MMR) process was recently reconstituted[72,74] from either MutSα or MutSβ, MutLα, replication protein A (RPA), exonuclease-1 (EXO1), proliferating cell nuclear antigen (PCNA), replication factor C (RFC), DNA polymerase δ (Pol δ) and DNA ligase I. The following is proposed to take place. The mismatch (red triangle)-bound MutSα (or MutSβ) recruits MutLα. The ternary complex undergoes an ATP-driven conformational switch, which releases the sliding clamp from the mismatch site. **a** | Clamps that diffuse upstream encounter RFC that is bound at the 5′ terminus of the strand break, and will displace it and load EXO1. The activated exonuclease commences the degradation of the strand in a 5′→3′ direction. The single-stranded gap is stabilized by RPA. When the mismatch is removed, EXO1 activity is no longer stimulated by MutSα, and is actively inhibited by MutLα. Pol δ loads at the 3′ terminus of the original discontinuity, which carries a bound PCNA molecule. This complex fills the gap and DNA ligase I seals the remaining nick to complete the repair process. **b** | Clamps that migrate downstream encounter a PCNA molecule that is bound at the 3′ terminus of the strand break. The recruitment and the activation of EXO1 results in the degradation of the region between the original discontinuity and the mismatch, possibly through several iterative EXO1-loading events. RFC that is bound at the 5′ terminus of the discontinuity prevents degradation in the 5′→3′ direction (away from the mismatch). Once the mismatch is removed and the EXO1 activity is inhibited by bound RPA and MutLα, the gap is filled by Pol δ. DNA ligase I seals the remaining nick to complete the repair process.

substrates. Moreover, the inhibition of EXO1 activity after the mismatch had been removed was stronger when MutSα, MutLα and RPA were present[72,73].

3′→5′ strand degradation. When the four-component (MutSα–MutLα–RPA–EXO1) system encountered a strand break that was 3′ from the mismatch, it initiated a 5′→3′ strand-degradation reaction, which travelled away from the mismatch[71]. As extracts of human cells catalyse MMR reactions in both the 5′→3′ and 3′→5′ direction[16], Dzantiev and colleagues fractionated HeLa-cell extracts and searched for activities that enabled the reconstituted system to catalyse also mismatch-activated 3′→5′ strand degradation. The fractions that possessed such an activity were shown to contain RFC[71].

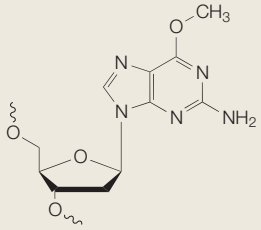
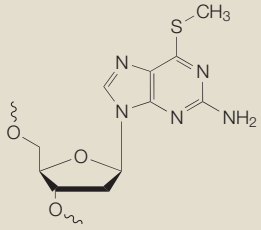
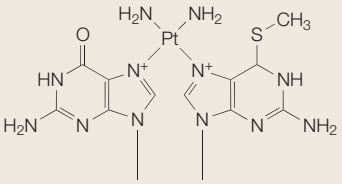
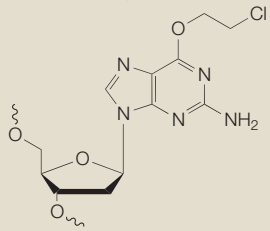
How can RFC change the directionality of the *in vitro* degradation reaction? When PCNA is loaded onto circular DNA that contains a strand break, it associates preferentially with the 3′ terminus, whereas RFC will remain bound at the 5′ phosphoryl terminus. It was suggested that the formation of a MutSα–MutLα–EXO1–PCNA–RFC complex might trigger a cryptic 3′→5′ exonuclease activity of EXO1, which could catalyse the degradation of the error-containing strand towards the mismatch. It is presently unclear how this might come about. However, the process requires catalytically active EXO1, which would seem to implicate this enzymatic activity in the 3′→5′ strand-degradation reaction.

***Bidirectional MMR* in vitro.** In the above *in vitro* experiments, only the mismatch-activated strand-degradation reactions could be monitored. However, the complete MMR process was reconstituted in the laboratories of Gou-Min Li[72] and Paul Modrich[74] from purified recombinant constituents by supplementing the MutSα–MutLα–RPA–EXO1–PCNA–RFC–(HMGB1) system with DNA polymerase δ and DNA ligase I. The reconstituted systems used by both groups catalysed the repair of a heteroduplex substrate that contained a G–T mismatch and a 5′ strand break. In addition, the Zhang *et al.* system could efficiently repair an IDL of three extrahelical nucleotides, provided that MutSα was replaced with MutSβ. No 3′→5′ MMR was detected in the Zhang *et al.* system[72]. By contrast, the Constantin *et al.* system supported bidirectional MMR[74]. The reasons that underlie this difference are unclear at present, but might be linked to the varying purity of RFC preparations.

Molecular mechanisms of mismatch repair

On the basis of the available biochemical evidence, three working models of mammalian MMR have been proposed. The molecular-switch model[33] favours the stochastic bidirectional diffusion of multiple MutSα (possibly with MutLα) sliding clamps from the mismatch. Clamps that encounter a strand break that is tagged with other MMR factors, possibly including PCNA, would then trigger the repair process. The active-translocation model[31,41] posits that the MutSα (possibly with MutLα), once released from the

Table 2 | **Substances with cytotoxicities that are dependent on the mismatch-repair status**

Substance type	Substance	Cytotoxic metabolite	Mode of action
S_N1-type methylating agents	*N*-methyl-*N*-nitrosourea (MNU), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), temozolomide, procarbazine, dacarbazine, streptozotocin	6-*O*-methyl-2′-deoxyguanosine 	^{Me}G base pairs with T during replication. These lesions are unsuccessfully addressed by the MMR system, which results in a cell-cycle arrest in G2
Antimetabolites	6-Thioguanine	6-*S*-methylthio-2′-deoxyguanosine 	As above, but two replication cycles are required, because 6-thioguanine has to be incorporated into DNA before being methylated
Intrastrand crosslinking agents	*Cis*-diammine-dichloroplatinum (II) (CDDP; also known as cisplatin)		This lesion is repaired primarily by the NER pathway, but if it persists until replication, it would arrest the replication fork, involving MMR proteins in the process
Intrastrand crosslinking agents	1-(2-chloroethyl)-3-cyclohexyl- nitrosourea (lomustine, CCNU), mitomycin-C (MMC)	(DNA adducts of MMC are not well characterized) 	The chloroethyl group can react with the opposite DNA strand to generate a covalent crosslink. Some MMR-deficient cells are more sensitive to these substances than MMR-proficient ones. The mechanism of MMR involvement is unknown

^{Me}G, methylguanine; MMR, mismatch repair; NER, nucleotide-excision repair; S_N1, unimolecular nucleophilic substitution.

mismatch, will translocate along the DNA in a controlled manner that is dependent on ATP hydrolysis. The DNA bending/verification model proposes that MSH proteins remain in the vicinity of the mismatch and that the communication between the mismatch and the strand-discrimination signal involves DNA bending rather than protein movement along the DNA[54,55]. This last model is based on studies in *E. coli*, where it was shown that MutH can be activated by mismatch-bound MutS–MutL in *trans*[75]. This has so far not been demonstrated in a mammalian system. Moreover, as MMR excision tracts initiate at the strand-discrimination signal and proceed ~150 nucleotides past the mismatch site, the mismatch position must become free during the excision step. This requires that the mismatch recognition complex leaves the mismatch site at some point. Indeed, although mismatch-dependent excision was activated in the experimental system that was developed by Wang and Hays, the repair process failed to proceed to completion[54,55].

The available evidence, therefore, seems to favour the first and second models. As the difference between them lies essentially only in the mode of translation of MutSα from the mismatch, which does not affect the principle mechanisms of the process, they will be discussed as one (FIG. 3). An ATP- and mismatch-activated MutSα clamp (with or without MutLα) might travel away from the mismatch in either direction. Clamps that encounter a nick that is 5′ from the mismatch would first contact an RFC molecule that is bound at the 5′ side of the nick. EXO1 could be loaded here, presumably following the displacement of RFC, and start the strand degradation in the 5′→3′ direction, towards and past the mismatch (FIG. 3a).

Diffusion of the activated clamp in the opposite direction would lead to an encounter with a PCNA molecule bound at the 3′ terminus of the strand break. The RFC molecule that is bound at the 5′ side of the break would not be displaced by MutSα, and therefore no EXO1-mediated degradation in the direction away

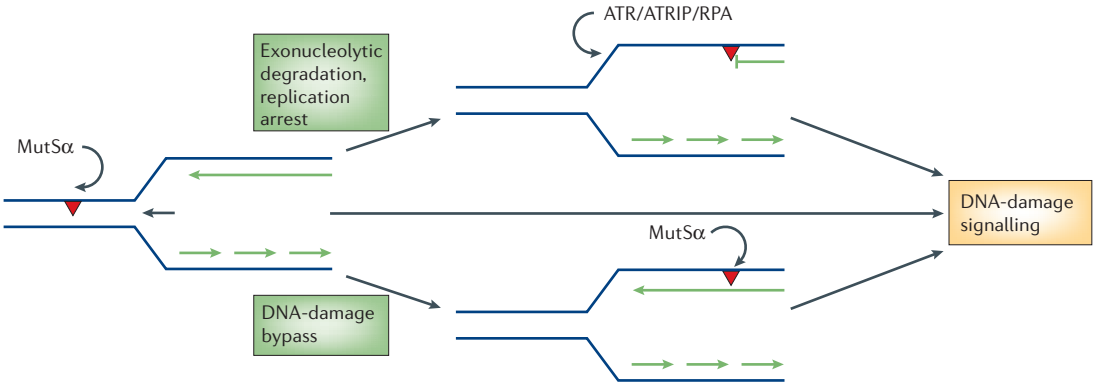


Figure 4 | **Mismatch repair in DNA-damage signalling.** According to the current models, mismatch-repair proteins (as represented by MutSα) either recognize DNA damage (red triangle) and signal to the cell-cycle checkpoint machinery directly, which can bring about cell-cycle arrest and apoptosis (central black arrow), or unsuccessful attempts to process the damage cause replication-fork arrest (top pathway). In the case of an unsuccessful repair attempt, single-stranded DNA regions recruit replication protein A (RPA) and ATR-interacting protein (ATRIP), and activate the ATR kinase, which subsequently phosphorylates the effector checkpoint kinase CHK1. Alternatively, the damage could be bypassed by an error-prone DNA polymerase and the signalling could be triggered by the repair of the secondary lesions created in this way (bottom pathway).

from the mismatch would take place. Instead, the complex that contains MutSα, MutLα, EXO1, PCNA and RFC (and possibly HMGB1) would assemble at the 3′ terminus of the nick and mediate degradation towards the mismatch (FIG. 3b). The single-stranded DNA would, in both cases, be coated by RPA, which also would inhibit further degradation once the mismatch was removed. DNA polymerase δ could then load and fill the gap. Finally, DNA ligase I would seal the remaining nick, thereby completing the repair process.

MMR deficiency and drug resistance

Above we discussed the mechanism of MMR-catalysed repair of biosynthetic errors. However, the MMR system is also involved in the signalling and/or the inappropriate processing of different types of DNA damage, because MMR-deficient cell lines are more resistant to death that is induced by several different types of chemical than matched MMR-proficient ones (TABLE 2 and REF. 76).

MMR and DNA-damage tolerance. MMR-deficient cells are up to 100-fold more resistant than matched MMR-proficient cells to death that is induced by methylating agents of the unimolecular nucleophilic substitution (S_N1) type. The primary cytotoxic lesion that is generated by these substances is O^6-methylguanine (^{Me}G), which pairs with C or T during replication. In wild-type cells, ^{Me}G–C and ^{Me}G–T mispairs might be recognized by MutSα[77], which could activate MMR. As the modified base is in the template strand, and as the repair polymerase will regenerate ^{Me}G–C and ^{Me}G–T mispairs during the repair synthesis, the MMR process was proposed to be triggered repeatedly until the replication fork arrests (FIG. 4, top). Because MMR-deficient cells do not attempt to process ^{Me}G–C and ^{Me}G–T mispairs, they survive at the cost of extensive mutagenesis. They are therefore DNA-damage tolerant rather than DNA-damage resistant[78].

The cell-cycle arrest that is activated by clinically relevant doses of methylating agents was shown to require the ataxia telangiectasia and Rad3 related (ATR) kinase, which phosphorylates its effector checkpoint kinase CHK1 (REF. 79). As this kinase cascade is activated in response to replication blocks, it was inferred that the cell-cycle arrest was triggered by the unsuccessful processing of ^{Me}G–C and ^{Me}G–T mispairs during S phase. A puzzling, and so far unexplained, observation is that the checkpoint is activated only during the second cell cycle after treatment[80–82]. Our data indicate that DNA recombination might help rescue the cells during the first S phase, but that this leaves behind structures that are cytotoxic in the subsequent cell cycle[83].

MMR-deficient cells are also more tolerant to 6-thioguanine treatment than MMR-proficient cells, albeit only ~10-fold. This drug is incorporated into DNA, where it can be methylated by *S*-adenosylmethionine to 6-methylthioguanine (^{Me}6-thioguanine), which has similar miscoding properties to ^{Me}G (REFS 84,85). ^{Me}6-thioguanine can only be generated after the first S phase, so ^{Me}6-thioguanine–T mispairs can only arise during the second S phase. Correspondingly, the cell-cycle arrest that is induced by 6-thioguanine is substantially delayed. Furthermore, 6-thioguanine treatment activates the ATR/CHK1-dependent checkpoint[76,86,87], which indicates that this cell-cycle arrest is triggered also by DNA-damage processing at the replication fork[88].

An MMR defect is associated with an ~2-fold resistance to death that is induced by *cis*-diammine-dichloroplatinum (II) (CDDP; also known as cisplatin)[89–91]. The lesion that is believed to be responsible for much of the biological activity of CDDP is a 1,2-intrastrand crosslink between the N^7 atoms of two adjacent purines. Interestingly, the 1,2-G–G adduct could be shown to bind purified MutSα[77], especially when one of the

Unimolecular nucleophilic substitution
(S_N1). Methylating agents of the S_N1 type transfer the carbonium ion CH_3^+ to electron-rich centres such as the exocyclic oxygen or nitrogen atoms of DNA bases. These reagents give rise to ~10-fold higher levels of O^6-methylguanine in DNA than S_N2-type (bimolecular) agents.

Nucleotide-excision repair
(NER). A DNA-repair process in which a small region of the DNA strand that surrounds a bulky DNA lesion, such as a UV-induced pyrimidine dimer, is recognized, removed and replaced. NER has two branches: transcription-coupled repair efficiently removes damage from transcribed strands of active genes, and global repair removes damage from the rest of the genome.

Homeologous
In this context, the term 'homeologous' describes DNA sequences that are similar, such as those that encode the same protein in different organisms, but that are too far diverged to recombine with each other under normal circumstances. Certain homeologous sequences have been shown to recombine when MMR is inactive.

Non-homologous end joining
(NHEJ). The main pathway that is used, predominantly in the G1 phase of the cell cycle, to repair chromosomal DNA double-strand breaks in somatic cells. NHEJ is error prone because it leads to the joining of random ends and to the loss of genetic information through end resection and trimming.

Synaptonemal complex
A structure that holds paired chromosomes together during prophase I of meiosis and that promotes genetic recombination.

Class-switch recombination
After immune cells have undergone V(D)J (variable (diversity) joining) recombination, they can change the constant region of their antibodies in response to stimulation by antigen. This results in changes in the effector functions of the antibodies. The process is initiated by the generation of double-stranded breaks in a region-specific manner, and some components of the non-homologous end joining (NHEJ) machinery, including the Ku heterodimer, have been implicated in their subsequent repair.

Somatic hypermutation
A mechanism for creating extra variability in antibody genes that occurs after V(D)J (variable (diversity) joining) recombination, by introducing point mutations, small insertions and small deletions into the V(D)J coding sequences.

D-loop
When single-stranded DNA invades a duplex and anneals with its complementary sequence, it displaces the other strand of the duplex, which forms a single-stranded loop resembling the letter D.

purines was mispaired with a T (REF. 92). However, unlike ^{Me}G or ^{Me}6-thioguanine, CDDP adducts in DNA present a physical barrier to replicative DNA polymerases. Correspondingly, the treatment of mammalian cells with CDDP triggers a rapid S-phase cell-cycle arrest, which is probably activated by stalled replication forks (FIG. 4, top). Alternatively, the damage could be bypassed by one of several specialized polymerases and the cell-cycle arrest could be activated by the repair process that is taking place behind the replication fork (FIG. 4, bottom). It is also possible that more than one mechanism operates in this instance, as CDDP-treated mammalian cells activate many stress-response pathways (see REFS 76,93 for recent reviews), at least one of which involves MMR[94].

Some CDDP adducts are structurally similar to pyrimidine photodimers that have been introduced into DNA by ultraviolet (UV) radiation. Indeed, CDDP damage is primarily addressed by the nucleotide-excision repair (NER) pathway. Recent evidence from mouse-model systems indicates that an MMR defect increases the resistance of ES cells and fibroblasts to UVB radiation[95], particularly in combination with the inactivation of global NER[96]. The indications are that the MMR is involved in the activation of an S-phase cell-cycle arrest, which might be triggered by aberrant nucleotide pairings that are generated during damage-bypass synthesis.

In contrast to the above reagents, MMR-deficient cells are more sensitive to death that is induced by interstrand crosslinking (ICL) agents such as 1-(2-chloroethyl)-3-cyclohexyl-nitrosourea (CCNU) than MMR-proficient cells (see REF. 97 and references therein). This difference is not large and has not been observed in all experimental systems[91], but it deserves a close examination, given that ICL-inducing chemicals are so far the only reagents to which MMR-deficient cells are more sensitive than MMR-proficient cells.

MMR proteins in apoptosis. In the above instances, the detection of DNA damage by the MMR system is believed to trigger a process of DNA metabolism that activates a cell-cycle checkpoint. However, MMR proteins have also been proposed to have a role in direct apoptotic signalling[98]. In this scenario, DNA-damage recognition by MMR factors would suffice to trigger cell-cycle arrest and apoptosis without the need to process the damage (FIG. 4, middle).

It is conceivable that such signalling cascades might be activated through the direct interaction of the damage-bound MMR proteins with signalling kinases such as ATR, CHK1 and CHK2, all of which have been reported to form complexes with MSH2 (REFS 98,99). It is also possible that the signalling cascade involves the ATM/ATR-substrate CHK2-interacting zinc-finger (ASCIZ) phosphoprotein, which forms MMR-dependent nuclear foci in cells that have been treated with methylating agents that give rise principally to *N*-modified purines and/or abasic sites[100]. However, these hypotheses remain to be substantiated.

The concept of direct signalling seems to be supported also by reports of dominant-negative mouse *Msh2* (REF. 101) and *Msh6* (REF. 102) alleles, which carry mutations in the ATP-binding sites of MSH2 or MSH6, respectively. The proteins that are encoded by these alleles retain the ability to bind mismatches, but fail to activate the downstream steps of MMR. Surprisingly, cells that express these alleles show sensitivity to methylating agents, 6-thioguanine and CDDP that is comparable to that observed in wild-type cell lines, which indicates that signalling pathways are activated directly in the absence of DNA processing.

MMR and other pathways of DNA metabolism

It has been known for many years that MMR affects the efficiency and fidelity of both mitotic[103,104] and meiotic[105] DNA recombination. During mitotic recombination, MMR proteins were proposed to prevent strand exchange between similar, but non-identical (homeologous), sequences, probably by blocking branch migration on mismatch detection. Consequently, MMR inactivation leads to an increased frequency of homeologous recombination, both in *E. coli*[106] and in mammalian cells[20]. The finding that gene-conversion tracts are longer in the absence of MMR indicates that the detection of mismatches by the MMR system aborts the strand-exchange process either before or after mismatch processing. When the strand exchange is aborted before mismatch processing, it would be given another chance to seek a perfectly homologous fragment but, failing that, the double-strand break would have to be repaired by an alternative mechanism, such as non-homologous end joining (NHEJ). When the strand exchange is aborted after mismatch processing, a sequence change would be introduced into the repaired duplex (FIG. 5).

Although mitotic recombination involves the same set of MMR proteins as that involved in the repair of replication errors, data from yeast studies and knockout mouse models indicated that meiotic recombination is mediated by different combinations of MMR factors. The process requires the MSH4–MSH5 heterodimer rather than MutSα or MutSβ[107,109]. Correspondingly, *Msh2*$^{-/-}$ or *Msh6*$^{-/-}$ animals exhibit no notable meiotic defects. MLH1 seems to function preferentially in complex with MLH3, as both male and female mice that lack these proteins are sterile[43,110]. PMS2 is also thought to be required, but as only the males are sterile, the MLH1–PMS2 heterodimer might be involved only in a subset of events such as synaptonemal-complex formation during meiosis I[111].

Class-switch recombination and somatic hypermutation (SHM) of the variable regions of immunoglobulin genes, which take place during specific stages of antigen-stimulated B-cell differentiation, function differently in the absence of MMR[112–115], as observed in mouse knockout models. SHM affects specific transcribed regions of the genes, where the activation-induced deaminase (AID) enzyme converts C residues to U. Processing of the U residues, possibly in the context of the AID-induced G–U mismatches, was postulated to trigger a mutagenic process that involves an error-prone DNA polymerase such as DNA polymerase η[116,117]. Mutations at G–C base pairs arise predominantly within the

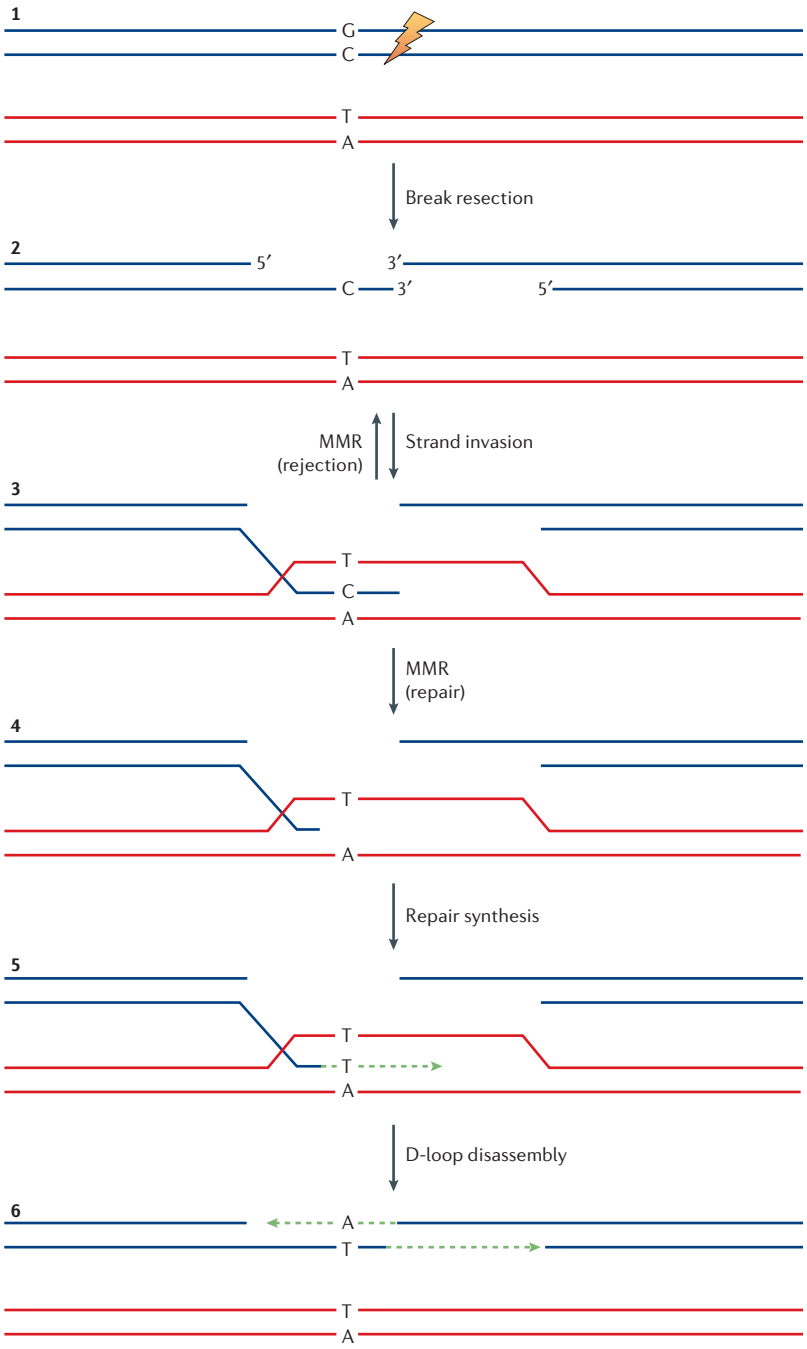


Figure 5 | **Mismatch repair in gene conversion.** During the repair of a double-strand break by homologous recombination (**1**), the 5′ termini on either side of the break in the blue duplex are resected to generate long single-stranded 3′ overhangs (**2**), one of which invades the homologous region of the red duplex. If the sequences of the invading and recipient strands are not identical (**3**), mismatches that arise during the strand invasion are detected by the mismatch repair (MMR) system, which could either reject the invading strand, or repair the mismatch. In the repair scenario (**4**), repair synthesis (green) would be templated by the recipient sequence (red) (**5**). D-loop disassembly and annealing with the original blue complementary strand, followed by primer extension of the other blue 3′ terminus, will therefore introduce the sequence change (**6**). Note that this reaction does not lead to a crossover product. If, however, following the generation of the 3′ overhangs (**2**), the sequence of the invading strand is identical to that of the recipient, the blue 3′ terminus will be extended (**5**), the D-loop will be disassembled and the extended 3′ overhang (green) will re-anneal with its original blue complementary strand (**6**). Extension of the other blue 3′ terminus and ligation (not shown) will complete the repair process.

consensus sequence RGY$^{A}/_{T}$ (R = purine, Y = pyrimidine), whereas consensus sequences $^{T}/_{A}$A are mutated in A–T base pairs. Interestingly, MMR defects reduce the incidence of only the A–T base-pair mutations. Moreover, the SHM phenotypes of animals that lack the different MMR genes differ — *Msh2*-, *Msh6*- and *Exo1*-knockout mice exhibit more noticeable phenotypes than mice with inactivated *Mlh1* and *Pms2* genes. So, the presence of MutSα seems to promote mutagenesis rather than prevent it.

MMR proteins also seem to affect the process of aberrant triplet-repeat expansion, as seen in neurodegenerative disorders such as Huntington's disease, myotonic dystrophy and fragile X syndrome. The expansions that occur in these $[CTG]_n$, $[CGG]_n$ or $[GAA]_n$ sequences are very large, and it is unlikely that the looped-out DNA will be processed by MMR. However, these structures were shown to be stabilized in *E. coli* strains[118] and mice[119] that are deficient in MMR. Although the mechanism of this process is unknown, the available evidence indicates that MutSβ might bind these loops and interfere, rather than assist, with the correct processing of these structures[120].

Conclusions

The MMR system has received a considerable amount of attention during the past decade, primarily thanks to its link with hereditary non-polyposis colon cancer (HNPCC), one of the most common inherited cancer-predisposition syndromes. Understandably, the initial investigations into MMR focused primarily on its role in mutation avoidance. Although the MMR system could be reconstituted from eight recombinant proteins, there are undoubtedly other redundant or non-essential factors that participate in the process *in vivo*, and these remain to be identified.

It is now clear that the MMR system is multifaceted and that it participates in several different pathways of DNA metabolism, namely those that involve recombination. Given the importance of these processes in the maintenance of genomic stability, it would be of substantial interest to learn how MMR proteins affect the outcome of meiotic and mitotic recombination events. Most of our insights come from yeast studies, and it is desirable to understand these processes in mammalian cells.

The study of MutL homologues deserves particular attention, as the biological roles of these proteins remain enigmatic and as the malfunction of different heterodimeric combinations of MLH proteins gives rise to very diverse phenotypes, which range from genomic instability to sterility.

The involvement of MMR proteins in DNA-damage signalling also requires further study, as this process could have an important role in spontaneous cell transformation and cancer. Moreover, the MMR status affects, in some cases by several orders of magnitude, the response of cells to certain classes of therapeutics. In my view, one of the greatest challenges of modern molecular-cancer research is to understand how one and the same system guards genomic stability on the one hand, while contributing to cell death on the other.

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