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Structural, molecular and cellular functions of MSH2 and MSH6 during DNA mismatch repair, damage signaling and other noncanonical activities

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Abstract

The field of DNA mismatch repair (MMR) has rapidly expanded after the discovery of the MutHLS repair system in bacteria. By the mid 1990s yeast and human homologues to bacterial MutL and MutS had been identified and their contribution to hereditary non-polyposis colorectal cancer (HNPCC; Lynch Syndrome) was under intense investigation. The human MutS homologue 6 protein (hMSH6), was first reported in 1995 as a G:T binding partner (GTBP) of hMSH2, forming the hMutSα mismatch-binding complex. Signal transduction from each DNA-bound hMutSα complex is accomplished by the hMutLα heterodimer (hMLH1 and hPMS2). Molecular mechanisms and cellular regulation of individual MMR proteins are now areas of intensive research. This review will focus on molecular mechanisms associated with mismatch binding, as well as emerging evidence that MutSα and in particular, MSH6, is a key protein in MMR-dependent DNA damage response and communication with other DNA repair pathways within the cell. MSH6 is unstable in the absence of MSH2, however it is the DNA lesion-binding partner of this heterodimer. MSH6, but not MSH2, has a conserved Phe-X-Glu motif that recognizes and binds several different DNA structural distortions, initiating different cellular responses. hMSH6 also contains the nuclear localization sequences required to shuttle hMutSα into the nucleus. For example, upon binding to O^6meG:T, MSH6 triggers a DNA damage response that involves altered phosphorylation within the N-terminal disordered domain of this unique protein. While many investigations have focused on MMR as a post-replication DNA repair mechanism, MMR proteins are expressed and active in all phases of the cell cycle. There is much more to be discovered about regulatory cellular roles that require the presence of MutSα and, in particular, MSH6.

Keywords

DNA mismatch repair; MSH2; MSH6; DNA damage signaling; N-terminal disordered domain

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1. Introduction

The DNA mismatch repair system is conserved from bacteria to humans, indicative of the vital role of this pathway in all living cells. To date, the best understood DNA mismatch repair (MMR) pathway is the methyl-directed MutHLS system in *E. coli*, essentially a post-replication genomic maintenance mechanism. This model has provided the basic framework for understanding eukaryotic MMR. The essential proteins required for MMR in *E. coli* have been purified to homogeneity, cloned, and the entire repair reaction has been reconstituted *in vitro*, for review [1–6]. Several eukaryotic homologues of bacterial MutS and MutL proteins have now been identified [7–13]. The MutS homologous genes identified in yeast include; *msh*1 through *msh*6, MutL homologues are *mlh1*, *pms1*, *mlh2* and *mlh3* (Wang 1998). Homologous human genes that play instrumental roles in MMR include *MSH2*, *MSH6*, *MSH3*, *MLH1* and *PMS2* [7,13–15]. Notable differences exist between bacterial and eukaryotic MMR [16]. Whereas bacterial MutS and MutL function as homodimeric proteins, eukaryotic homologues have evolved as heterodimers composed of three related, yet distinct protein subunits, MutSα (MSH2+MSH6), MutSα (MSH2+MSH3) and MutLα (MLH1+PMS2). Bacterial MMR requires a unique MMR protein - MutH - for strand discrimination by hemi-methyladenine d(GATC) sequence recognition. MutH initiates strand-directed gap repair by endonuclease activity 5' of the unmethylated daughter strand sequence. Eukaryotes do not have hemimethylated adenines, nor an equivalent sequence-specific MutH endonuclease. Excellent reviews of the origin, evolution and diversification of the MMR gene families have been published [17, 18].

The eukaryotic MutSα complex is the evolutionary product of gene duplication and divergence of homodimeric MutS. This process has resulted in two distinct proteins required for initiation of MMR, as well as for additional functions that are not required of the bacterial MMR system. MSH2 and MSH6 share five similar domains, but with sufficient differences to give MSH6 several distinct functions. MSH6 also has a unique N-terminal disordered domain that is absent in its MSH2 partner. The human MSH6 protein was first reported in 1995 as G/T mismatch Binding Protein (GTBP), binding partner of hMSH2 to form the MutSα complex [7, 11,19]. The human *MSH6* gene includes 10 exons that encompass a total genomic sequence of 24 kilobases, and is located on the petite arm of chromosome 2 (2p16.3), within one megabase of *hMSH2*. The hMSH6 gene product is a 160 kDa protein that is unstable without heterodimerization with hMSH2, and consequently utilizes 80%–90% of available hMSH2 [20].

The hMutSα heterodimer binds to DNA mispairs and short insertion deletion loops (IDLs) [7, 15, 21] and hMutSα binds larger IDLs. hMutLα is a mismatch-specific endonuclease, and is the intermediary for activation of the downstream mismatch gap repair process. MMR has been reconstituted *in vitro* using extracts from mammalian cells [22–26] as well as purified proteins [16, 27, 28]. The least complex *in vitro* system to initiate 5' directed mismatch excision requires MutSα, RPA, and EXO1 together with ATP. 3' directed mismatch excision also requires MutLα, PCNA and RFC, indicating that MutLα is required to nick 5' of the mismatch to allow efficient repair when a pre-existing nick is not present [20, 27]. Therefore, to achieve bidirectional mismatch repair from a strand break located either 3' or 5' of a mismatch, PCNA, RFC and DNA polymerase δ are required in addition to MutSα, MutLα, RPA, and EXO1 [16]. A metal binding site on the C-terminal of PMS2 invokes a latent endonuclease property of MutLα that is essential for 3' nick-directed repair[29]. Genetic and cellular evidence bolstering the requirement of MutLα in 3' repair is that cells deficient in MutLα expression are able to initiate 5' but not 3' nick-directed MMR [30]. Additional studies using purified protein extracts have also identified the participation of a high mobility group DNA binding protein (HMGB1) in MutSα - activated EXO1 excision. HMGB1 increases the processivity of MMR-dependent excision [31]. These *in*

vitro studies have contributed much to our understanding of this complex DNA repair system. Nonetheless, there are still unanswered questions, as purified proteins used for *in vitro* MMR biochemical assays do not yet perfectly mimic the complexity of MMR within the human cell.

This review will be focused primarily on MutSα, with particular emphasis on MSH6 and its functional and biochemical contributions as part of the MutSα complex. The literature in the DNA MMR field is large and illuminates a myriad of cellular pathways in which MutSα activity has been implicated. Clearly, there are still many unanswered questions left to investigate.

2. Structural Insight Into MSH2 + MSH6 Mismatch Binding

Crystallography studies reveal that eukaryotic MutSα contain several structural regions similar to the bacterial MutS complex, with the exception of the N-terminal region of MSH6 [32,33].

MSH2 and MSH6 are divided into five conserved domains (1–5) comparable to E. *coli* MutS, excluding the N-terminal disordered domain of MSH6 (Figure 1). MutSα dimerizes from Domains 1–5 as an asymmetric mirror image with each domain juxtaposed. Domain 1 is the DNA mismatch binding domain. Domain 2 represents the connector between mismatch binding and the levers comprising Domain 3. Domain 3 folds in 2 distinct areas that, together, form a lever to Domain 5. Domain 4, the clamp region, allows for nonspecific DNA contact, while Domain 5 confers adenosine binding and hydrolysis (ATPase) (Figure 1) [32–34]. The preponderance of biochemical discovery of specific MSH6 functions has been driven based upon sequence differences from MSH2 within these five domains.

Crystal structures of human MutSα bound to a 15 basepair oligomer containing either a G:T, single base T insert, O^6- meG:T, or G:U mismatch have recently been determined [32]. Full length MSH2 and a major fragment of MSH6, containing all five domains except the first 340 amino acids comprising the N-terminal unstructured fragment, were used to obtain these protein-DNA crystal structures. The DNA binding structure of MutSα was not altered appreciably when bound to each DNA substrate, regardless that each DNA lesion is known to elicit different biological pathways. This may indicate that the hMutSα -DNA binding structure is not directly signaling different downstream pathways. Alternatively, the missing N-terminal unstructured domain of hMSH6 may play a significant role in pathway signaling [35]. In all cases, the DNA structure bound by hMutSα undergoes a bent conformation. Atomic force microscopy studies further demonstrate that DNA confers an initial bent conformation when interacting with MutS. In the presence of a mispair the DNA becomes kinked by the MutS interaction (initial recognition complex; IRC) and further transitions to an unbent DNA:MutS interaction (ultimate recognition complex; URC). The URC is believed to initiate downstream mismatch repair events [36]. It is not known if the URC transition occurs with other types of MutS-bound lesions, however it has been suggested that unrepaired mispairs and complex DNA mispaired lesions initially recognized by MutSα cannot transition to the unbent URC believed to trigger downstream MMR processes [36].

The structural asymmetry of the homodimeric *E. coli* MutS is a key aspect of detection, signal transduction, and 3’ or 5’-directed repair of a DNA mismatch. Likewise, MutSα binds asymmetrically to mismatched bases, [10, 34, 37, 38]. Domain 1 (mismatch binding) contains conserved residues that contact the DNA duplex (Figure 1&2). Amino acids 362–518 of hMSH6 allow for a much more intimate DNA contact than the corresponding region of hMSH2 (amino acids 1–124). DNA contact by this region of hMSH6 is highly specific to the mismatched nucleotide. A specific Phe-X-Glu motif that confers mismatch binding affinity in bacterial MutS is conserved in eukaryotic MSH6, but not in MSH2 or MSH3 [3].

The Phe-X-Glu motif of MutS and MSH6 is biochemically well-suited for lesion detection. The aromatic ring contained in phenylalanine recognizes the stereo-chemical distortion induced in DNA at locations such as mismatch sites or modifications such as cisplatin crosslinks [32, 34, 39]. Together with phenylalanine interaction, a hydroxyl side chain of glutamate hydrogen bonds to the mispaired nucleotide [32, 33, 38]. Very recently, single-molecule multiparameter fluorescence spectroscopy has provided evidence that the asymmetric *E. coli* MutS binds mismatches with a strong directional bias, such that the conserved phenylalanine of the Phe-X-Glu motif is stacked on the incorrect base of each mismatch, affecting the directionality of MMR [40]. Site-directed mutations of the Phe337 site in yeast Msh6 or Phe432 in human MSH6 demonstrate complete disruption of mismatch repair [41, 42]. However, studies involving a Glu339Ala mutant yeast Msh6 demonstrate only a modest increase in mutation rates. G:C to T:A transversion mutations are most likely to occur in yeast Msh6 Glu339Ala mutants, indicating a reduced ability to repair 8oxoG:A mispairs [43, 44]. Thus, it is possible that the Glu339 in yeast Msh6 has less of a role in mismatch repair of undamaged bases than in complex mismatches involving base alteration compounded by DNA replication errors.

DNase footprinting, crystallography, and single-molecule multiparameter fluorescence spectroscopy studies demonstrate that MutS affixed to a mismatch requires a 12–20 basepair length of DNA [33, 40, 45]. A duplex at least 60 basepairs in length is required to bind the MutSα -MutLα ternary complex onto the mismatch [46–48]. The DNA footprint is lengthened to 143 basepairs when MutS, MutL and ATP are present, although MutL does not appear to directly interact with the DNA structure [46]. A molar ratio of 4:1 (MutSα to DNA) has been reported for efficient mismatch repair [28]. However, another report indicates that only one molecule of MutSα is required per DNA substrate [49]. Results from our lab demonstrate that more hMSH6 is bound when hMutSα is interacting with an O^6meG:T mismatch than to an undamaged G:T mismatch, perhaps indicating a greater role for multiple MutSα loading in a DNA damage-sensing context as compared to mismatch repair [35].

3. Mutsα Adenosine Binding And ATP Hydrolysis

Mutations in the adenine nucleotide binding sites in Domain 5 of MSH2 and MSH6 inhibit MMR *in vivo* and *in vitro* [41, 50, 51]. Active MutSα requires dimerization of ATPase domains that are conserved helix-turn-helix motifs within the C-terminal of both MSH2 and MSH6. Residues from each protein interact to form two ATPase sites, which stabilizes the ATPase interface even without ATP binding [15, 52]. ATP binding and hydrolysis play key regulatory roles during MMR although there is still debate over the number and type of adenosines in the two binding sites within MutSα. Nine binding combinations (ADP and/or ATP and/or vacant) are possible within the combined domains, allowing for differing structural conformations and functionality of MutSα [1, 53]. The two adenine nucleotide binding sites differ in binding affinity, with MSH2 having a higher affinity for ADP and MSH6 having a high affinity for ATP in the DNA unbound state, perhaps due to increased dynamics of hMSH6 domain 5 [32, 53–57]. In addition to ATP binding, all MutS complexes have intrinsic ATPase activity conferred by an Adenine nucleotide binding cassette (ABC) motif, as confirmed by both biochemical and genetic studies [58–60]. Binding of the MutSα complex to a mismatched DNA substrate can be disrupted by the addition of excess ATP, however MutSα -driven hydrolysis of ATP is necessary for *in vitro* MMR [50, 60, 61]. Additional evidence has been provided by crystallization studies revealing ADP bound by both hMSH2 and hMSH6 in complex with mismatch-containing DNA [32]. Mutations that render deficient ATPase activity in MutS homologues usually display dominant-negative effects as mismatch binding activity is not affected [1]. In agreement with this, the presence of DNA is required to stimulate ATP hydrolysis by MutS homologues, and mismatch-

containing DNA enhances this hydrolysis [1, 50, 60, 62–64]. There is also evidence that hMutSα ATP hydrolysis is required for a sliding clamp mismatch surveillance activity, however an alternate molecular switch-sliding clamp model has been proposed that does not involve ATPase activity [for review 1]. The precise mechanistic outcomes from ATP binding and hydrolysis during MMR are not yet understood, but likely involve conformational changes in the MutSα -heteroduplex DNA recognition complex that allow motility [32, 59, 60, 64]. The ternary MutSα •MutLα •heterduplex DNA complex has been studied by several different methods, but no consensus has been reached in regard to the specific biochemical or structural nature of this complex, except that ATP is required [1]. In yeast, Msh6-ATP binding is required for Msh2-ATP binding to occur [65]. Conversely Msh2-ATP binding is not required for Msh6-ATP binding, suggesting that at least Msh6-ATP and perhaps Msh2-ATP binding are required for initial ternary complex formation.

Studies involving both a hMutSα single (hMSH2 K675R or hMSH6 K1140R) and a double (hMSH2 K675R- hMSH6 K1140R) ATPase mutant demonstrate that ATPase activity of both hMSH2 and hMSH6 are required for mismatch correction, although a lack of ATPase activity does not inhibit mismatch binding by hMutSα [50]. Conversely, a phenylalanine mutation at the Phe-X-Glu mismatch binding motif in yeast and human MSH6 abolishes ATPase activity along with mismatch binding [41, 42]. These results suggest that MSH6 interaction with a mismatch stimulates ATP hydrolysis [41]. Additionally, MEFs containing mutations in either the Msh2 or Msh6 ATP binding site do not release a mismatched DNA structure in the presence of ATP. Mice with these same mutation have increased tumor burden similar to humans carrying mutated alleles at these sites [51]. Thus, MSH6-MSH2 DNA binding activity and structural conformation are likely altered depending on the number and ratio of ATP/ADP bound. The ATP/ADP binding state and hydrolysis rate of MSH6-MSH2 can alter DNA binding affinity that, in turn, may affect DNA damage response signaling, as well as regulation of additional cellular repair and signaling pathways. Variables such as type of mismatch, surrounding sequence, location within the chromatin, cellular stress levels, and phase of the cell cycle undoubtedly play significant roles for the orchestration of MutSα -induced functions.

4.1 MSH6 And Chromatin Interactions

MutSα interacts directly with undamaged chromatin and other proteins associated with chromatin structure. Electrostatic attractions to DNA are conferred by locations within the N-terminus of MSH6 that are rich in basic amino acids. These loose attractions are postulated to confer mobility of MutSα for sliding clamp activity. Yeast mutants altered to abolish basic amino acid attractions to DNA results in increased mutations and resistance to MNNG. [66]. Mutations in the yeast Msh6 N-terminal DNA binding sequence, in combination with mutations in the PCNA interacting protein (PIP) motif, result in mutation rates similar to that of an *msh6Δ* mutant, indicating that both PCNA binding and non-specific DNA binding conferred by N-terminal Msh6 amino acids are important for efficient MMR. As well, MNNG resistance can be induced by either deletion or substitution mutations in the N-terminal DNA binding region, despite that the deleted residues do not have any higher affinity for O^6meG than for homoduplex DNA [66]. These results illustrate the importance of Msh6 N-terminal nonspecific DNA interactions to MMR and DNA damage response, and perhaps damage surveillance mechanisms as well.

MutSα also interacts with chromatin associating proteins. A PWWP sequence is present within the N-terminus of human MSH6 (amino acid residues 104–107), but not within the N-terminal region of yeast Msh6 [66]. PWWP sequences provide non-specific protein-DNA interaction and are characteristic of proteins that associate with chromatin [67, 68]. Recently, the PWWP domain of hMSH6 was implicated, though not directly verified, as an

interactor with a trimethyl modification at lysine 36 of histone 3 (H3K36me3) in a large scale pull down assay [69]. Trimethylation at lysine 36 of histone 3 is associated with nucleosome loading and chromosome compaction, which greatly hampers metabolic processes requiring DNA access, such as mismatch repair [70, 71]. The hMutSα sliding clamp disassembles nucleosomes in the presence of a mismatch, as well as inhibits chromatin assembly factor −1 (CAF-1) dependent histone deposition in a mismatch-dependent manner [72, 73]. In addition, a recent report by Jiricny describes increased interaction of the N-terminus of hMSH6 with CAF-1, inhibiting nucleosome assembly in the presence of alkylation damaged DNA. Further, MSH6 has higher affinity for underphosphorylated CAF-1, reducing the amount of CAF-1 available for binding to PCNA, which is required for chromatin assembly [74]. Taken together, the above evidence indicates that MSH6 plays an important role in inhibiting or removing chromosomal compaction. Further investigation will be required to determine if the hMSH6 PWWP domain plays a direct role in these chromatin interactions.

Co-immunoprecipitation experiments demonstrate an interaction between MSH6 and HMGB1 [31]. A conserved HMGB1 interacting sequence corresponds to amino acids 631–637 within the connector region (Domain 2) of hMSH6, although interaction at this location has not been experimentally verified [31, 75]. Interestingly, this conserved sequence was originally identified during the discovery of MSH6 by Palombo et al [14]. HMGB1 yeast homologues (Nhp6 A & B) are more actively recruited to duplex DNA in the presence of MutSα. Yeast Nhp6 proteins compete for homoduplex DNA binding, thus perhaps directing access of MutSα to only the DNA mismatch [76]. It is not yet clear if this function is related to activities of HMGB1 during MMR within the cell. HMG proteins can also mediate DNA damage signals to downstream effectors such as p53 to coordinate apoptotic responses [77, 78], and thus may play more than one role within the MMR pathway. Overall, it appears that direct interaction of MSH6 with chromatin and chromatin-associated proteins ensures that DNA packaging is either removed, or prevented, until a DNA repair response can be effected. To date, MMR activities have been almost exclusively studied using synthetic duplex DNA constructs. MMR chromatin-associated activities within the cell remain sparse in the literature [71, 74].

4.2 N-Terminal Domain Of MSH6 Is Disordered

The N-terminal region of eukaryotic MSH6 contains several hundred amino acids upstream of Domain 1 (1–389 in human and 1–295 in yeast, by BLAST alignment to hMSH2). Several activities requiring the N-terminus unique to MSH6 homologues have now been identified. The N-terminal region of yeast Msh6 appears to have somewhat different functionalities than hMSH6, as will be discussed below [66, 79–81]. Computational modeling indicates that MSH6 has a highly disordered N-terminal domain from amino acid 125 (down-stream of PIP) to amino acid 400, after which MSH6 homology to MSH2 is high (Figure 1). Intrinsically disordered domains within specialized proteins have evolved to be flexible to ensure adaptability to environmental stimuli [82]. For example, p53 and the Cdk2 kinase inhibitor p21 regulate cell cycle arrest and DNA damage signaling, requiring structural modifications of each N-terminal disordered domain [83, 84]. These domains are phosphorylated at specific sites that, in turn, modify protein activity [85]. Importantly, interactions of disordered domains with a specific binding partner require both high specificity and low affinity to allow rapid functional response in a dynamic cellular environment. Both physical shape-changing and posttranslational modifications to these disordered domains provide the diversity required for a multitude of functions attributed to these proteins.

4.3 N-Terminal MSH6 And PCNA Interactions

PCNA is a trimeric DNA replication sliding clamp that is essential for various DNA-associated metabolic processes and interacts with many replication and repair proteins, including hMutSα, hMutSα, hMutLα, and EXO1 [86, 87]. The PIP motif (QXX(L/I)XXFF) is conserved at the extreme N-terminal domains of both hMSH6 (aa 4–11) and hMSH3 (aa 21–28) [88].

PCNA is required for MMR during two separate steps, a pre-excision step and during DNA resynthesis [12]. The mechanistic basis for the first MMR step requiring PCNA has now been elucidated. PCNA is a co-activator (with hMutSα and RFC) for MutLα endonuclease to initiate excisional activity of a strand that does not already contain a 5' nick. PCNA is not required for 5'-excisional activity of a strand already containing a 5' nick, as this does not require MutLα endonuclease activity [29]. Additional mechanistic experiments with purified proteins demonstrate that inhibition of MutSα -PCNA interaction, by removal of the PIP motif from hMSH6, has virtually no effect on the hMutLα endonuclease excision step, provided that free PCNA is available [29]. Conversely, the 5'- but not 3'-directed repair synthesis step is partially diminished in the presence of the mutant hMSH6, indicating that this second step requires PCNA for 5'- but not 3'-repair synthesis [32, 89]. As well, hMutSα binds to a mismatch with comparable affinity with or without the presence of PCNA, and mismatch-bound or free hMutSα have similar affinities for PCNA. Biochemical studies have determined a 1:1 stoichiometry of PCNA and hMutSα, with SAX modeling revealing an end-to-end association of the two proteins in an elongated conformation in which the DNA channels are non-aligned [89].

Conversely, yeast PCNA can bind up to three Msh6 N-terminal regions at a time, unlike the human 1:1 stoichiometry of PCNA and MSH6. SAXS analyses demonstrate that the N-terminal region of yeast Msh6 forms an extended tether between Msh6 and PCNA [81]. The N-terminal region of Msh6 remains highly sensitive to proteases, when bound to PCNA, indicating that binding to PCNA does not confer significant order to this domain. This flexible tether is hypothesized to aid yeast MutSα in its interaction with PCNA by providing space to assemble the subsequent MMR proteins for transfer to mismatched bases at regions of newly replicated DNA. This notion is further strengthened by the observation that yeast MutSα -PCNA can bind nonspecifically to homoduplex DNA, but PCNA is disrupted from this complex when Mutsα is bound to a mispair [90].

An investigation using intact human cells indicates that the hMSH6 PIP box is required to coordinate MMR at the replication fork, as also demonstrated by chromatin co-localization studies during active DNA replication [88, 91]. A hMSH6 lacking the N-terminal first 77 amino acids, including the PIP sequence, does not colocalize with PCNA to replication foci, and MMR is significantly decreased in extracts containing this hMSH6 construct [88]. Competitive inhibition of PCNA using peptides containing the $p21^{Cip1/WAF1}$ PIP motif inhibits hMutSα recruitment to replicating DNA [92]. A modest increase in mutations has been observed after nonconservative alteration or removal of the PIP sequence in yeast Msh6 and Msh3 [66, 80]. Interestingly, a mutation in the PIP motif of yeast Mlh1 results in a strong mutator phenotype, despite that PCNA has a higher affinity for Msh6 than Mlh1 [87]. Overall, it appears that within the cell, PCNA initiates MMR by coordinating the MutSα directionality at the replication fork. PCNA may be needed for MMR strand discrimination by coordination of MutSα mismatch recognition to the subsequent endolytic activity of MutLα and/or exonucleolytic activity of EXO1 [3, 29, 87–89, 92, 93].

4.4 N-Terminal MSH6 And Mutsα Nuclear Localization

Three nuclear localization sequences (NLSs) have been identified in the N-terminal region of hMSH6 [94]. Recently, a conserved Ser-Pro-Ser sequence in the N-terminal region of hMSH6 (amino acids 41–43) has been reported that could serve as an alternate nuclear localization sequence [95]. Non-classical nuclear import has been described in signaling proteins containing phosphorylated Ser-Pro-Ser (pSPS) sequences [96]. This alternate SPS nuclear targeting sequence in hMSH6 does contain phosphorylated serines [35]. However, whether post-translational phosphorylation at this sequence contributes to nuclear import of the hMutSα complex has not been experimentally verified. Transport of MSH6 through the nuclear pore assembly is believed to occur only after heterodimerization with MSH2 in the cytosol, as hMSH2 does not contain an NLS and nuclear levels of hMSH2 are decreased in cells lacking hMSH6 [97]. However, hMSH2 can exist independently in the cytosol, and has been reported to enter the nucleus in the absence of hMSH6 via importinα 3 [91, 95, 98]. These studies demonstrate that independent localization of hMSH2 may occur in undamaged cells, although the meaning of this remains unclear, as hMSH2 does not have any known independent cellular function. It is more likely that the hMSH2 not bound with hMSH6 is in the form of hMutSα. The NLS sequence is present in yeast Msh2 rather than Msh6, however a cooperative import of Msh2 and Msh6 into the nucleus also occurs [99]. A similar synergistic cytoplasmic heterodimerization and nuclear import has been reported for MutLα, despite that both MLH1 & PMS2 contain an NLS [100]

4.5 N-Terminal MSH6 Phosphorylation

Regulation of different MMR activities within the cell is poorly understood [91, 101–[101,102]. Phosphorylation of unstable domains has been found to either confer a more stable protein structure, or more disorder and flexibility [103]. Phosphorylation also allows for a large diversity of interactions and functions required by alterations within the cellular environment. Multiple phosphorylation sites on the same protein can function not just as an “on-off” switch, but can confer fine-tuning capabilities similar to a “brightener-dimmer” switch [104]. Information on post-translational regulation of hMutSα is very limited, despite that mutations within N-terminal phosphorylation residues identified in hMSH6 have been discovered in human tumors [105]. There are now 22 distinct phosphorylation sites within hMSH6 identified from several independent phospho-proteome mass spectrophotometric studies. Twenty of these sites are clustered into six regions within the unstructured N-terminus of hMSH6. Fourteen phosphorylation sites are located in four clustered regions of the N-terminal disordered domain and are excellent candidate sites for contribution to post-translational cellular activities of MSH6 (Figure 3) [35, 106–113]. The majority of phosphorylation sites are CK2, CDK, MAPK, and Aurora kinase recognition motifs. Serine 309 is the only PKC recognition motif, while a single ATM/ATR recognition motif is located at serine 348 and this site has been reported to undergo phosphorylation after gamma irradiation [113]. The presence of both CDK and ATM/ATR recognition motifs within this region argue that phosphorylation of different residues of MSH6 are associated with cell cycle and/or DNA damage responses [35]. CK2, the recognition motif for eight of the twenty sites, is frequently referred to as the master regulator of cellular function. CK2 has over 300 substrates and is constitutively active in human cells [114, 115]. We do not yet understand the functional significance of MSH6 phosphorylation or dephosphorylation at each of the identified sites. Experimental limitations of such studies include instability of hMSH6 without dimerization with hMSH2, technical difficulties of expressing exogenous hMSH6 in mammalian cells, and the N-terminal disordered region is the most proteolytically-sensitive domain of this fragile molecule [81, 88].

Phosphorylation of both hMSH2 and hMSH6 *in vitro* by PKC and CK2 and *in vivo* (by labeling with [^{32}P]H_3PO_4, has been reported by others, although our group was able to detect only phosphorylation of hMSH6 [35, 116]. Phosphorylation of hMSH6 protein is significantly increased in the presence of a PKC activator, and hMSH6 phosphorylation levels decrease when PKC activity is blocked [35]. Increased MMR, nuclear translocation, and chromosomal binding activity occurs after kinase activation or by alkylation treatment [101, 102, 116]. Endogenous hMSH6 undergoes decreased phosphorylation, and decreased MMR protein expression occurs, after exposure to different kinase inhibitors [116, 117]. Decreased ubiquitin-dependent proteosomal degradation has also been described after hMutSα phosphorylation by PKCζ [118]. To more closely examine effects of specific hMSH6 N-terminus phosphorylation sites, we have created a recombinant hMutSα that contains S→A mutations in four serine residues comprising the fifth cluster of phosphorylated sites (S252–261). This alteration results in a dramatic decrease in binding affinity to G:T as compared to O^{6}meG:T. These results agree with our finding that increased phosphorylation of hMSH6 correlates with more binding to a G:T mismatch, but not to O^{6}meG:T. This serine cluster contains only CK2 recognition motifs, suggesting a role for CK2-induced phosphorylation and DNA mismatch-specific activity. Indeed, mismatched DNA is a normal endogenous event during DNA replication, and therefore an appropriate activity to be orchestrated by the CK2 'master cellular regulator'. The overall mechanistic model generated from these results is that MMR pathway signaling and MMR-dependent alkylation damage signaling are differentiated within the cell by elegant tandem mechanisms that include amount of hMSH6 N-terminal phosphorylation (more for G:T than O^{6}meG:T) and binding stoichiometry of hMutSα to a specific lesion (higher for O^{6}meG:T than G:T) [35].

Based on current evidence, it is likely that MutSα downstream signaling for MMR and DNA damage signaling is regulated, in part, by alterations in phosphorylation patterns of the N-terminal domain of hMSH6. Challenging experimental designs will be required to further investigate the role(s) of the N-terminal disordered domain of hMSH6.

5. Regulation of hMSH6 and hMSH2 Expression Within the Cell

Investigations of MSH6 regulation and protein expression during the cell cycle are somewhat limited, due to the instability of MSH6 without MSH2 heterodimerization. Many early studies have used chemical treatment or serum starvation to induce cell cycle arrest, which would likely influence MSH6 expression, stability, or nuclear translocation. The most compelling evidence is that MSH6 and the other key MMR proteins (MSH2, MLH1, MSH3, PMS2) are constitutively expressed in G1 phase, with increased expression during S and G2 phase [91,119–121]. Further, cell cycle synchronization studies by our lab, accomplished using centrifugal elutriation and without chemicals or nutrient deprivation, demonstrate that all four MMR proteins are at highest concentration within the nucleus during S and G_2 phases of the cell cycle within both mouse and human cells. Mismatch binding, as well as repair fidelity and efficiency, are all higher in S phase despite equally high MutSα protein levels during G_2 phase [91]. One likely reason for this is because hMutSα is located within replication factories, therefore physically close to the replication fork during DNA synthesis [91]. Functions other than MMR requiring increased levels of hMutSα during G_1 and G_2 have been identified, such as DNA alkylation damage signaling. [91, 97, 101, 122].

MSH6 transcriptional regulation studies indicate constitutive mRNA expression Constitutive expression of housekeeping genes is often regulated by the SP1 transcription factor. Multiple GC-rich binding sites within the *MSH6* promoter region strongly indicate regulation by SP1, and functional studies using a luciferase reporter construct reveal that all seven SP1 binding sites are capable of inducing MSH6 gene expression [123]. However,

E2F transcription factors regulate gene expression during and after entry into S phase [124–127]. MSH2 and MSH6 expression, along with several other DNA repair genes, increase in the presence of the E2F1 and E2F3 transcription factors [124, 125]. Also, E2F siRNA knocks down MSH2, but not MSH6 expression, in cells over-expressing Bcl-2, an anti-apoptotic protein [128]. Additionally, the E2F7 promoter element, known to repress transcription at the end of S phase, is present in both MSH2 and MSH6 promoter regions [126].

Both E2F1 and E2F3 have been found to regulate expression of MSH2, MSH6, and other genes directing the activation of G_2 arrest, DNA damage signaling, and apoptosis [129–131]. Cell cycle specific MSH2 and MSH6 gene and/or protein regulation by these transcription factors has yet to be investigated. An increase in MSH6 protein driven solely by stabilization with MSH2 cannot be ruled out. However, co-regulation of gene expression is also likely to occur due to close proximity of *hMSH2* and *hMSH6* genes on chromosome 2. Evidence of both SP-1 and E2F transcriptional regulation additionally argue for direct up-regulation of MSH6 expression during S-phase. MutSα is thought to be degraded after G2 phase by the proteosome complex. The ubiquitin-proteosome pathway is responsible for degradation of several proteins within DNA repair pathways [132], including MSH6 and MSH2. Inhibition of proteosomal degradation results in accumulation of MutSα proteins with diminished activity [94]. Additional studies by these investigators demonstrate that phosphorylation of hMutSα by PKCζ significantly inhibits proteosomal degradation [118], indicating that post-translational phosphorylation of MSH6 stabilizes the MutSα complex and prevents degradation. Clearly, more information is needed to better understand cellular regulation of hMutSα gene transcription, as well as protein translation and degradation. Results from such studies would likely illustrate the complexity of cross-talk in gene regulation within cells containing damaged DNA.

6.1 MMR – Noncanonical Damage Signaling, Other DNA Repair Pathways, and Other Mutational Scenarios

In addition to mismatch repair, MutSα is also associated with other types of base lesions, base excision repair, transcription-coupled repair, and double strand break repair [2, 133]. MutSα recognizes specific lesions including O^6meG, complex pyrimidine dimers, halogenated pyrimidines, bulky adducts such as benzo[*c*]phenanthrene dihydrodiol epoxide, and cisplatin adducts [134–137]. The MutSα complex has different binding affinity and repair efficiency, depending on the specific DNA lesion and sequence context [3, 21, 61, 133, 135,136]. MutSα has higher binding affinity to specifically modified bases that are also mismatched, particularly O^6meG:T, indicating increased lesion recognition after DNA replication or an error-prone repair attempt. Indeed, it has now been well documented that therapeutic levels of monofunctional alkylating agents do not affect the cell cycle until the second replication phase [102, 138].

MutSα can also bind damaged bases that arise from normal cellular metabolism, such as 8-oxoGuanine [139–142]. Processing of 8-oxoG:G and 8-oxoG:T mispairs by the MMR pathway is similar to the high repair efficiency of G:G and G:T mispairs. [22, 142]. In comparison, repair of G:A and 8-oxoG:A is very inefficient [22, 61, 142]. Binding of MutSα to an 8-oxoG:A mismatch induces ATP hydrolysis and ADP→ ATP exchange, indicating that MutSα recognizes, binds, and is at least partially activated by this lesion despite that it is poorly repaired by MMR [141]. A Glu→Ala substitution in the Phe-X-Glu motif of yeast Msh6 results in increased G→T transversion mutations, indicating that recognition by the MMR pathway may be required for correct repair of 8-oxoG:A [43]. MutY homologue (MYH) activity, within the base excision repair (BER) pathway, is significantly more efficient than MutSα for actual excisional repair of adenine frequently

misincorporated opposite an unrepaired 8-oxoG [142]. In addition, individual *Myh* or *Msh2* knock-out mouse embryonic fibroblasts (MEFs) exhibit increased 8-oxoG levels within chromosomal DNA, but no further increase of 8-oxoG occurs in double knock-out MEFs, indicating that MYH and MutSα are working within the same repair pathway, or have overlapping functions, for 8-oxoG:A [143]. Conversely, a synergistic accumulation of 8-oxoG within some tissues has been observed within *Msh2/Myh* double knock-out mice, albeit with a delay in lymphomagenesis in the double knock-out mice as compared to *Msh2* knock-out only [140, 144]. Both MutSα and MYH are at highest concentrations within the nucleus during S-phase and are associated with replication factories [91, 145]. Further, hMSH6 interacts directly with hMYH and enhances the binding of hMYH to 8-oxG:A [146]. Mutations near the hMSH6 binding domain of hMYH are associated with hMYH polyposis and cause defective glycosylase activity, despite unaltered binding of the mutated hMYH to hMSH6 [147]. Taken together, this evidence suggests differing degrees of competition, cooperation, and substrate exchange between MSH6 and MYH, and merits further investigation.

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and other monofunctional alkylators produce several alkylated DNA adducts, the majority of which undergo efficient repair by the BER pathway [148]. BER does not repair O^6meG, instead this lesion is directly repaired by methylguanine methyltransferase (MGMT) [149]. Repair is a covalent transfer of the methyl group from the O^6 position on guanine to a cysteine residue on MGMT, quenching all further enzymatic activity, hence the term "suicide enzyme" [150, 151]. Mammalian cells that do not express MGMT and have proficient MMR are highly sensitive to monofunctional alkylating agents, as these cells undergo MMR-dependent damage signaling, G_2 checkpoint arrest and apoptosis rather than repair [152–155]. Cells deficient for both MMR and MGMT tolerate high levels of O^6meG and have increased mutation frequencies, as these cells neither recognize nor repair O^6meG [156–158]. Increased G→A transition mutations occur in MMR deficient cells exposed to monofunctional alkylators because DNA polymerases frequently misinsert thymine opposite O^6meG during replication bypass [159].

There is now strong evidence that the specific activity of MutSα during DNA damage response to O^6meG lesions is regulated differently than during MMR. Human MutSα undergoes rapid nuclear localization and increased chromatin binding in response to alkylating agents [97,101, 160]. Two different mechanistic models leading to MMR-dependent cell death have been proposed. The signaling model proposes direct activation of the ataxia-telangiectasia mutated and Rad3-related (ATR) DNA damage signaling pathway by MSH2, substantiated by several reports of direct interactions between MSH2 and ATR [161–164]. Direct interaction between Msh6 and the yeast counterpart to ATR, Mek1p, has also been reported [165]. This model has been further validated through separation-of-function mutations in *Msh2* and *Msh6* knock-in mice [51, 166]. In these studies, knock-in mutations in both Msh2 and Msh6 ATPase domains inhibit MMR activities but not DNA damage signaling response to alkylating agents. Mice harboring these knock-in mutations have increased incidence of cancer, although requiring longer onset than MMR knock-out mice. There are also indications that the direct interaction between MSH2 and ATR after MNNG treatment is independent of RPA and the Rad9-Rad1-Hus1 (9-1-1) complex, both of which are involved with classical ATR-dependent pathway activation [161, 164, 167]. In contrast however, MSH6, MSH2, and MSH3 have been shown to interact directly with the 9-1-1 complex in the presence of MNNG. In fact, Rad9 interaction with hMSH6 is important for nuclear localization of this MMR protein in response to alkylation damage [168]. Additionally, after treatment with MNNG, hyper-phosphorylated RPA, along with MutSα, MutLα and PCNA, remain chromatin-bound throughout the prolonged 2^{nd} cell cycle [101]. At this time, the consensus is that down-stream activation of the ATR-Chk1 pathway by MMR-dependent DNA damage response to monofunctional alkylators does

occur, the complexity of signaling events and specific proteins that play a role are still under debate.

The alternate futile repair model of MMR-dependent DNA damage signaling suggests that iterative rounds of MMR-provoked gap excision of the daughter strand (containing a thymine misinserted opposite the O^6meG during the first replication cycle) occurs during the second replication cycle, causing replication fork collapse. Both *in vitro* evidence of abortive MMR opposite an O^6meG in the parent strand, and *in vivo* evidence of persistent gaps in DNA within human and yeast cells has been published [134, 152]. This indirect model of MMR-induced ATR activation indicates that the MMR-induced iterative repair causes a lethal accumulation of single-strand gaps and/or stalled replication forks that then signal the ATR pathway. These two alternative models are not mutually exclusive and may provide redundant mechanisms to ensure apoptosis of genetically damaged cells. Given these intriguing but sometimes conflicting data, further investigations to better define the MMR-dependent damage signaling pathway are warranted.

6.2 MMR And DNA Double-Strand Breaks (DSB)

The Mre11/Rad50/Nbs1 (MRN) complex plays an upstream role in detection and repair of DNA DSBs. Homologous recombination (HR) is directly responsible for DSB repair in S and G_2/M phases of the cell cycle. Upon strand excision, a strand invasion step occurs via sister chromatid hydrogen bonding with homologous sequences. Inappropriate base pairing of partial complementary strands (homeologous regions) are prevented by MutSα binding to the mismatches. Thus inappropriate double-strand break repair of homeologous strands is suppressed in favor of homologous repair. The exact mechanism by which homeologous sequences are rejected by MutSα activity is still unclear [169–172]. More recent work describes a more complex relationship between MMR and HR. Unrepaired O^6meG leads to G_2 M cell cycle arrest during the second cell cycle, DSBs, and sister chromatid exchanges, only in MMR and HR proficient surviving cells [173]. MRN foci formation is dependent on MMR processing of O^6meG, and is inhibited in cells that are MMR deficient. Suppression of MRN function decreases both temozolomide (TMZ)-induced G_2 arrest and cytotoxicity, indicating that this complex is required for MMR processing of O^6meG:T [174]. Recent studies posit a direct link between O^6meG-induced MMR activation and HR repair activities, as MMR proficient cells that lack HR (*Xrcc2, Brca2,* or *rad51d* mutants) undergo cell cycle arrest during the 1st cell cycle, rather than the 2nd cell cycle, after exposure to low concentrations of MNNG. These cells also exhibit increased γH2AX signaling, decreased sister chromatid exchange, and increased cell death. This HR deficient phenotype is eradicated by removal of O^6meG or deficient MMR, indicating that MutSα-dependent processing of O^6meG:T lesions lead to DSBs and increased cytotoxicity [152, 175, 176]. These results also indicate that HR is required for resolution of secondary nicks or gaps resulting from hMutSα recognition of O^6meG:T. The interdependence of MMR and HR for resolution of O^6meG:T lesions promote speculation that tumors with intact MMR, but deficient HR, would exhibit hypersensitivity to TMZ. MSH6 interaction with Ku70, a DSB repair protein within the non-homologous end-joining (NHEJ) pathway has also been observed [177]. The Ku70-MSH6 interaction is increased when cells are treated with neocarzinostatin to induce DSBs. Evidence also exists for functional cross talk between the Bloom syndrome helicase (BLM) and MSH6. Bloom Syndrome is a rare inherited disorder characterized by genetic instability. BLM, the BS gene product, is a RecQ family DNA helicase that plays an important role in HR. MSH6 interacts directly with BLM both *in vitro* and within cells. This interaction has been found to occur at both the N-terminus and C-terminus of MSH6. It has been reported that MutSα can stimulate the helicase function of this protein [178, 179]. In addition, BLM and MSH6 are part of the BRCA1 associated surveillance complex (BASC) [180].

6.3. MMR And DNA Interstrand Crosslinks (ICL)

ICLs are formed from several different chemical agents, such as bifunctional alkylating agents (Bis(2-chloroethyl) nitorosurea; BCNU), nitrogen mustards, mitomycin C, cisplatinum, cylcophosphamide, melphalan, and photoactivated psoralen. ICLs are potent inhibitors of DNA replication, recombination and transcription, often leading to cell death or increased mutation frequency in surviving cells. Despite significant clinical use and intense investigations, the mechanism of ICL removal within human cells is still largely unresolved. It has been determined that DNA must undergo an endogenously induced double-strand break during repair of an ICL, although it is not clear at what step of the repair process that this occurs [181]. Several different pathways appear to cooperate or compete at various stages of ICL repair. The use of cell lines that have specific genetic deletions has identified several proteins within different DNA repair pathways that appear to play critical roles in crosslink repair [182–185]. There is growing evidence that initial recognition and incision steps are limited to a unique role of specific proteins, perhaps for all ICLs, regardless of subsequent repair pathways. Initial incisional events appear to require ERCC1-XPF activity that, unlike during nucleotide excision repair (NER), cleaves the DNA strand on both sides of an ICL to “unhook” the adduct from one strand of the DNA [186]. The MutSα heterodimer was found by the Legerski group to be essential for the incisional activity of ERCC1-XPF as well. MutSα binds to psoralen ICL-containing DNA with high affinity, and this binding is further stimulated by the presence of PCNA [185]. Unlike MMR or DNA damage signaling for other lesions, MutLα does not appear to play a role in psoralen ICL repair. However there is disagreement as to whether the presence of MutSα increases resistance or sensitivity to cisplatinum, presumably due to either competitive inhibition or cooperative repair of ICLs by ERCC1-XPF and MutSα [187–189]. MSH2 and MLH1 have both been found to interact with FANCD2, and MMR deficient cells exhibit inhibition of the FA-BRCA pathway and ICL repair, therefore suggesting another role for MMR proteins for repair of ICLs in the activation of the FA pathway [190]. Clearly, much work remains unfinished to sort out the biochemical contribution of MutSα during the repair of ICLs.

6.4. MMR - Somatic Hypermutation and Class Switch Recombination

A functional MMR system is the basis for preventing mutational events through correction of mismatched bases. Paradoxically, in lymphocytes the MMR system functions in a very noncanonical manner to ensure that antigen-dependent mutations within the variable (V), diversity (D), and join (J) regions of immunoglobin (Ig) genes occurs at elevated frequency (somatic hypermutation) and that double-strand breaks are generated to allow for class switch recombination, see reviews [191, 192]. The maturation of B cells to produce high affinity antibodies with different effector functions is a combination of somatic hypermutation (SHM) to create antigen specificity, and class switch recombination to create different antibody effector isotypes, combined with increased cell proliferation (clonal expansion). Thus while the majority of the genome is protected from mismatch-induced mutations by the MMR pathway during chromosomal replication, V(D)J Ig loci undergo hypermutation with the help of MutSα. These mutations (10^{-2} to 10^{-3} per bp or 10^{6} times higher than genomic replication) allow for successful maturation in B cell germinal centers that ultimately produce different classes of antibodies with high affinity toward each new antigen presented to the B cell. See review [192]. How does MutSα switch between these two very opposite genomic outcomes? SHM in B lymphocytes is initiated by activation-induced deaminase (AID), an enzyme capable of deaminating deoxycytosine (dC) into deoxyuracil (dU) at very high frequency on single-stranded DNA. Localized generation of multiple dU at the variable regions of Ig genetic sequences is achieved through coordination with transcription bubbles at these loci. How AID so precisely targets the V(D)J Ig loci, while the rest of the genome is protected from this mutagenic enzyme, is not entirely clear,

but likely involves chromatin epigenetic modifications, *cis* DNA regions, and protein cofactors [191]. The very high frequency of C and G mutations at Ig loci are similar, indicating that both DNA strands are equally deaminated [193]. What role does MMR play in error-prone repair of this plethora of U:G mismatches? Based on many different investigations, the current model is that some of the U:G mismatches are replicated without repair to produce C→T transitions, some U:G mismatches recruit uracil N-glycosylase (UNG) and APE1 of the BER pathway to create abasic sites and single-strand breaks that are mis-repaired by low fidelity polymerases, and some U:G mismatches are recognized by MutSα, leading to single-strand gaps that undergo error-prone re-synthesis by polymerase eta (Pol η). Pol η is particularly suited to MMR gap resynthesis as this polymerase most commonly initiates mutations at A:T basepairs, which are also highly mutated at Ig loci [191, 194–199]. Pol η has been shown to interact with MSH2, and MutSα enhances the processivity of Pol η [200]. Mono-ubiquitylated PCNA is required to load Pol η, or other low fidelity polymerases, at the MMR gaps. It is not entirely clear how this highly error-prone MMR activity is restricted to IG loci. The germinal B cell microenvironment, local chromatin or epigenetic alterations, MMR posttranslational modifications, AID activity, and either competition or cooperation with BER proteins has been suggested. The increased number of U:G mismatches within the local genomic environment may also play a role in MMR targeted activity. We have previously observed mono-ubiquitylated PCNA co-immunoprecipitate with MSH6 from chromatin after MNNG exposure to cells lacking MGMT, and more recently the Jiricny group has observed PCNA mono-ubiquitylation and recruitment of Pol η to O^6meG:C lesions as well as U:G mismatches [101, 201].

Class switch recombination (CSR), similar to SHM, involves both AID and MMR proteins. CSR requires DNA double-strand breaks in switch (S) regions upstream of constant gene segments containing different isotopes of antibody effector functions, and then recombination to bring each constant antibody effector close to the antigen-specific variable region that has undergone SHM. AID deaminates several dC residues to dU in S regions similar to SHM, but the next steps differ in that DNA double-strand breaks are produced rather than point mutations. It is still unclear exactly how double-strand breaks are accomplished, although UNG, APE1 and MMR are required, and single-strand nicks or gaps occur during this process. Unlike SHM, which appears to require only MutSα, CSR requires both MutSα and MutLα interactions to create the double-strand breaks at S regions [202, 203]}. CSR also requires mono-ubiquitylated PCNA, but not Pol η, again unlike SHM. Chromatin assembly and histone modification proteins have also been implicated in the interplay of AID, MMR, UNG, mono-ubiquitylated PCNA and double-strand break formation during CSR [191]. The blunt, or very short microhomology, double-strand breaks generated in S regions of Ig loci are repaired by NHEJ-initiated long distance recombination of different constant gene segments into close proximity with the antigen-specific V region to generate different antibody isotypes for each antigen specific antibody. The exact role of MutSα or MutLα in coordination with the NHEJ pathway is not clear, although MSH6 has been found to interact with Ku70, and MutLα interacts with DNA-PK-cs, to enhance NHEJ [204, 205].

6.5. MMR and Trinucleotide Repeat Expansion

Expansion of trinucleotide repeats (TNR) within genomic DNA, both intergenerationaly and within somatic cells, are the cause of many hereditary degenerative diseases, reviewed in [206, 207]. Pathogenic TNR occur beyond a crucial threshold length. The exact mechanisms that cause mutational TNR in each of these diseases are not known, but are believed to arise from three primary sources that are not mutually exclusive; aberrant DNA replication, gene conversion, and error-prone DNA repair. MMR fails when extrahelical loops, sometimes forming hairpin structures with mismatched central bases, are left unrepaired and

incorporated into duplex DNA. While the MutSα is responsible for initiating repair of extrahelical loops, evidence suggests that this heteroduplex may also be causative in TNR expansion and lead to disease manifestation or progression. Several mouse models of different TNR expansion diseases that have been crossed with MMR deficient mouse models (Msh2, Msh3, Pms2) have demonstrated suppression of TNR expansion [208–211]. There is now supporting evidence that Mutsα is involved with the lack of repair of extrahelical loops and also with incorporation of these unrepaired loops into duplex DNA [207].

Paradoxically, while TNR expansion is dependent on MutSα, MutSα prevents expansion, thus MSH3 plays a very different role from MSH6 during the progression of these TNR diseases. The exact mechanism(s) of MMR contribution to TNR expansion is unknown but likely involves chromatin structure, the nature and stability of DNA extrahelical loops, and downstream repair signaling events.

7. Summary

The MutSα complex has evolved from a simple prokaryotic post-replication proof reading process to a eukaryotic genome guardian with multiple functions. The most basic function, MMR correction of misincorporated bases during DNA synthesis, contributes to overall replication accuracy in all species. Current evidence suggests that MSH2 and MSH6 are constitutively expressed during all phases of the cell cycle via SP1 transcription factor binding, and are up-regulated in a cell cycle-dependent manner via E2F. It is not yet clear if there is additional independent regulation of MSH6 expression. This ‘dynamic duo” is capable of movement along the contours of homoduplex DNA, although it is not clear if this is used for constitutive surveillance or only during gap repair, nor is it clear if this movement requires ATP hydrolysis. A question not yet answered is whether constant genomic surveillance, or whether the location of MutSα in replication factories during DNA synthesis, contributes more significantly towards mismatch repair and/or other DNA damage signaling activities. The MSH6 Phe-X-Glu motif, conserved from the *E. coli* MutS homodimer, interacts with mismatched bases, to signal appropriate repair of the incorrect base and, if necessary, to align MutLα for endonuclease activity 5’ of the mismatch. Through the course of evolution, hundreds of amino acids have been added to the N-terminal region of MSH6, an additional distinction from MSH2 in higher eukaryotes. Evidence is emerging that one function of this highly disordered domain is to maintain the flexibility needed to interact with proteins and chromatin in a context specific manner. Phosphorylation of the MSH6 N-terminal domain regulates MutSα stability, nuclear import, and MutSα response to alkylation damage. MutSα interacts with many downstream proteins involved in DNA-damage induced cell cycle arrest, as well as several other DNA repair pathways (Figure 2). Interaction of MutSα, MSH6 specifically, with several different repair proteins within MYH, NER, DSB, and ICL repair pathways have been well documented. However, specific functions of MutSα within each DNA repair process are still in debate, and could even be detrimental to repair in some circumstances. During HR repair, MutSα suppresses recombination with mismatched DNA to allow for accurate strand invasion. Specific damage, such as O^6meG, initiates increased MutSα translocation to the nucleus and signals ATR, directly or indirectly, resulting in cell cycle arrest and/or cell death. Additionally there are many unanswered questions in the mechanistic role of MutSα in promoting targeted mutations and structural rearrangements during the maturation of antibody isotype diversity and antigenic specificity. As well, the roles of MSH2 and MSH3 during TNR expansion is puzzling. Future studies to elucidate all of the physiological roles of MutSα, and specific contributions by MSH2 and MSH6, will require careful experimental designs that investigate effects of chromatin alterations, post-translational modifications, cell cycle phase, and the cellular environment.

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Abbreviations

MMR	DNA mismatch repair
HMGB1	high mobility group DNA binding protein
IRC	initial recognition complex
URC	ultimate recognition complex
CAF-1	chromatin assembly factor-1
PIP	PCNA interacting protein
NLS	nuclear localization signal
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
TMZ	temozolomide
ATR	ataxia-telangiectasia mutated and Rad3-related
MRN	Mre11/Rad50/Nbs1 complex
HR	homologous recombination
DSB	double-strand break
BLM	Bloom syndrome helicase
ICL	interstrand crosslink

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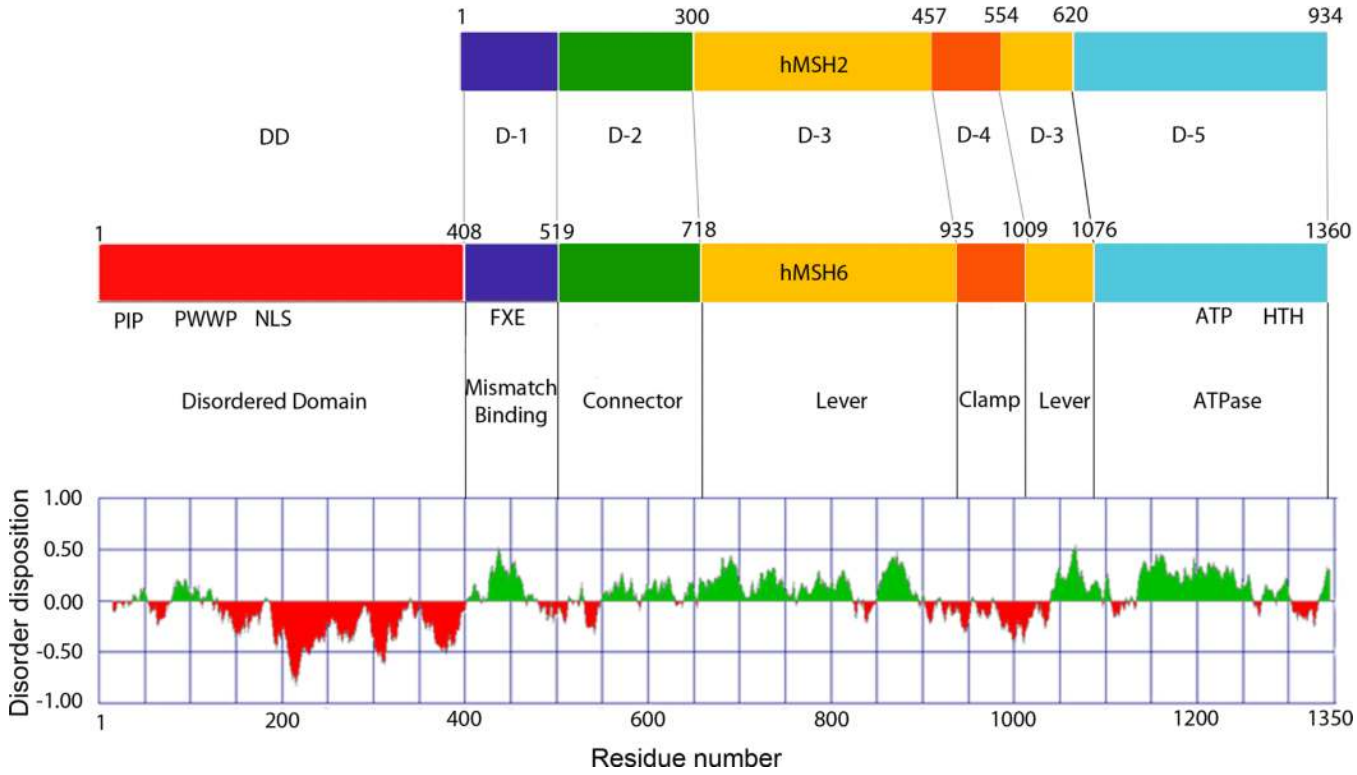

Figure 1.
MSH2 + MSH6 domains in a comparative linear array, including amino acid disorder disposition as depicted in lowest graph [212].

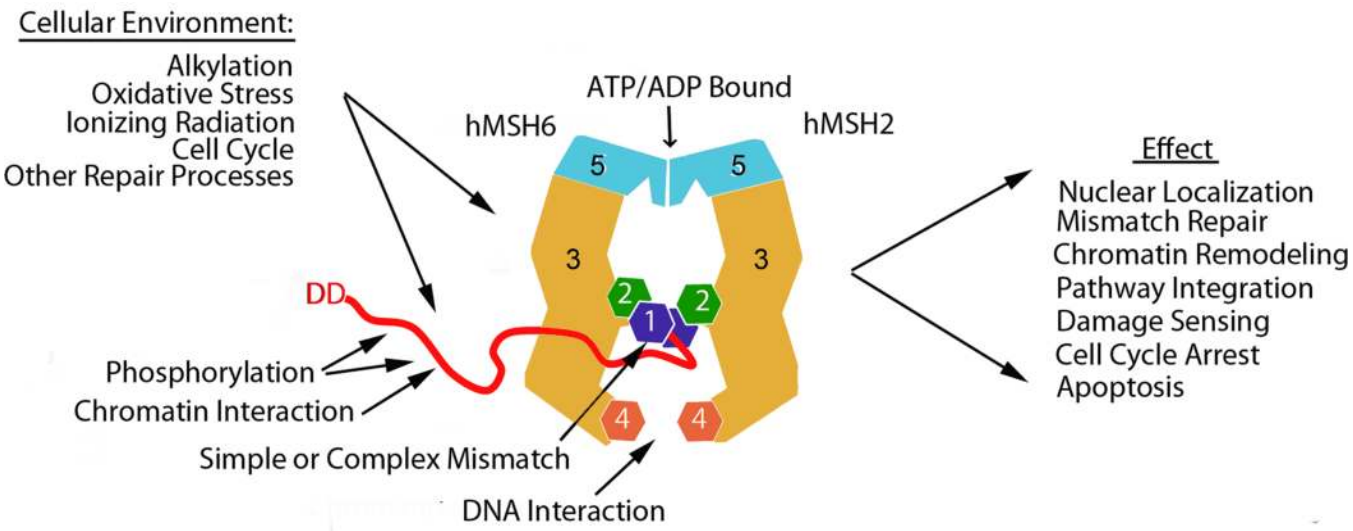

Figure 2.
hMutSα in DNA binding configuration. Environmental influences (Cellular Environment) and subsequent effects on hMutSα are briefly listed (hMutsα figure based on [32]).

hMSH6 Phosphorylation Sites[1]

	P-site #	aa type[1] & #	kinase family	sequence motif
1	1	S14	CDK2	QSTLYSFFPKSPALSDANKAS
1	2	S41	MAPK	GGRAAAAPGASPSPGGDAAWS
1	3	S43	MAPK	RAAAAPGASPSGGDAAWSEA
2	4	S79	DMPK, PAKB	LNGGLRRSVAPAAPT
2	5	T86	AuroraC, AuroraB	SVAPAAPTSCDFSPG
2	6	S91	CDK2, CDK3, p38	APTSCDFSPGDLVWA
3	7	S137	CDK2, CDK3	VRVHVQFFDDSPTRGWVSKRL
3	8	T139	DNAPK	QFFDDSPTRGWVSKR
4	9	S200	CK2	LAVCDEPSEPEEEEE
4	10	T213	CK2	EEMEVGTTYVTDKSE
4	11	Y214	InsR	EMEVGTTYVTDKSEE
4	12	S219	CK2	EVGTTYVTDKSEEDNEIESEE
4	13	S227	CK2	DKSEEDNEIESEEEVQPKTQG
5	14	S252	CK2	SRQIKKRRVISDSESDIGGSD
5	15	S254	CK2	QIKKRRVISDSESDIGGSDVE
5	16	S256	CK2	KKRRVISDSESDIGGSDVEFK
5	17	S261	CK2	ISDSESDIGGSDVEFKPDTKQ
6	18	T305	Pim2, p70S6K, Pim3, Pim1	RKRKRMVTGNGSLKR
6	19	S309	PKC (δ,ι,θ,ζ,α,γ)	RMVTGNGSLKRKSSR
6	20	S348	ATM/ATR	SAPQNSESQAHVSGG
6	21	S830	CDK2	RLLSKIHNVGSPLKSQNHPDS
6	22	T924	AuroraC, AuroraB, AuroraA	TAFDHEKARKTGLITPKAGFD

[1]S = serine; T = threonine; Y = tyrosine

{ = Indicates clustered groups (1-6) of phosphorylated amino acids

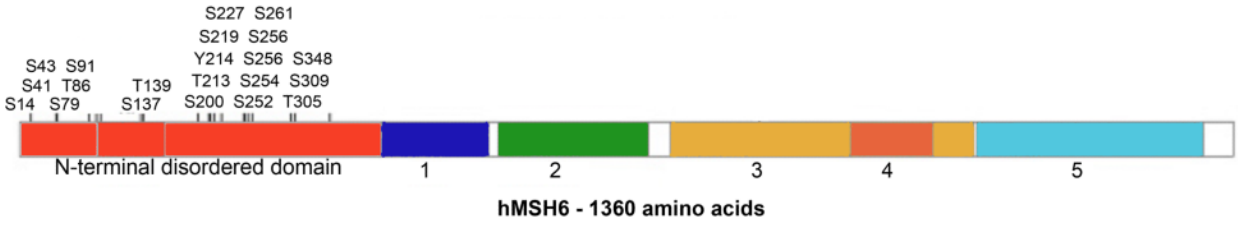

Figure 3.
hMSH6 N-terminal phosphorylation sites [35].