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Functional Residues on the Surface of the N-terminal domain of Yeast Pms1

Mercedes E. Arana^{a,b}, Shannon F. Holmes^{a,b,1}, John M. Fortune^{a,b,2}, Andrea F. Moon^b, Lars C. Pedersen^b, and Thomas A. Kunkel^{a,b,*}

^aLaboratory of Molecular Genetics, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina, 27709

^bLaboratory of Structural Biology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina, 27709

Abstract

Saccharomyces cerevisiae MutL α is a heterodimer of Mlh1 and Pms1 that participates in DNA mismatch repair (MMR). Both proteins have weakly conserved C-terminal regions (CTD), with the CTD of Pms1 harboring an essential endonuclease activity. These proteins also have conserved N-terminal domains (NTD) that bind and hydrolyze ATP and bind to DNA. To better understand Pms1 functions and potential interactions with DNA and/or other proteins, we solved the 2.5 Å crystal structure of yeast Pms1 (yPms1) NTD. The structure is similar to the homologous NTDs of *E. coli* MutL and human PMS2, including the site involved in ATP binding and hydrolysis. The structure reveals a number of conserved, positively charged surface residues that do not interact with other residues in the NTD and are therefore candidates for interactions with DNA, with the CTD and/or with other proteins. When these were replaced with glutamate, several replacements resulted in yeast strains with elevated mutation rates. Two replacements also resulted in NTDs with decreased DNA binding affinity *in vitro*, suggesting that these residues contribute to DNA binding that is important for mismatch repair. Elevated mutation rates also resulted from surface residue replacements that did not affect DNA binding, suggesting that these conserved residues serve other functions, possibly involving interactions with other MMR proteins.

Keywords

Mismatch repair; MutL α ; DNA binding; mutator; mutation rate

1. Introduction

Eukaryotes encode multiple homologs of the *E. coli* MutL protein that are essential for mismatch repair (MMR) of DNA replication errors [1–4]. These MutL homologs form different heterodimers [4,5], including the MutL α complex comprised of Mlh1-Pms1 in

*Corresponding author: Tel. 919-541-2644, Fax 919-541-7613, kunkel@niehs.nih.gov.

¹Current address: Synthron Pharmaceuticals, Inc, 9000 Development Drive, P.O. Box 110487, Research Triangle Park, NC 27410

²Current address: Science and Technology Directorate, US Department of Homeland Security, Washington, DC 20528

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

yeast and MLH1-PMS2 in humans [6,7]. In addition to MMR, MutL α participates in cellular responses to DNA damage [3,8–10] and in meiotic recombination [11–14], such that defects in MutL α can have profound effects on cancer susceptibility and fertility [1,4,14–20]. MutL α has a highly conserved N-terminal domain (NTD, yellow in Fig. 1A) in both Mlh1 and Pms1 that binds and hydrolyzes ATP [7,21–24], and less well-conserved C-terminal domains (CTD) that are required for dimerization [25,26]. MutL α also interacts with other MMR proteins, including MutS α [21,27–29], exonuclease 1 [30–32], PCNA [30,31,33–35] and other proteins [36] and it binds avidly to DNA [37,38]. DNA binding is required for the endonucleolytic activity of MutL α [39], whose active site in the C-terminal region of yPms1 (Fig. 1A) incises the nascent strand containing the replication error. This endonucleolytic cleavage serves as an essential step in MMR [39–41].

Crystal structures of the NTDs of *E. coli* MutL [42,43] and human PMS2 [22] have provided valuable insights into how these proteins bind and hydrolyze ATP and how the ATP catalytic cycle may modulate conformational changes that are important for protein-protein and protein-DNA interactions. For example, in the *E. coli* MutL NTD [42], ATP binding results in homodimerization of two NTDs creating a positively charged cleft. This cleft is partly comprised of helix H, which contains Arg266. The mutation R266E reduces DNA binding affinity, suggesting that DNA binds within the cleft formed by LN40 dimerization [43]. This possibility is consistent with several observations of DNA binding by the yeast MutL α heterodimer. Like *E. coli* MutL [44–46], yeast MutL α binds to DNA with high affinity [37,38]. This binding is salt sensitive, indicating that DNA binding involves electrostatic interactions. DNA binding was suggested to be functionally important because concomitant glutamate substitutions of two arginines in helix H (Arg273 and Arg274) of yeast Mlh1 reduced DNA binding by MutL α and by Mlh1 alone [38]. This reduced DNA binding correlated with a loss of MMR activity [38] and a loss of crossing over during meiosis [11,47].

The possibility that *E. coli* MutL binds DNA within a cleft formed by the NTD dimerization does not exclude the possibility that amino acids in addition to those in helix H of MutL and Mlh1 may contribute to DNA binding. For example, both LN40 with the R266E substitution [43] and yeast MutL α with the R273E/R274E substitutions in Mlh1 retain partial DNA binding capacity [38]. In addition, substituting glutamate for Lys328 in helix H of yPms1, which structurally aligns with Arg274 in Mlh1, does not reduce the ability of the Pms1 NTD to bind to DNA, nor does it elevate the spontaneous mutation rate of a haploid yeast strain [38]. Moreover, the NTDs of Mlh1 and Pms1 can bind to DNA independently of each other, and in the absence of detectable dimerization [38], suggesting that formation of a cleft is not a prerequisite for DNA binding by yeast Mlh1 or Pms1. Given these observations, and knowing that yPms1 must interact with DNA to allow its essential endonuclease to function, the present study was undertaken to better understand how *S. cerevisiae* Pms1 binds to DNA. The results implicate two conserved positively charged surface residues in DNA binding, and identify other surface residues that appear to be important for mismatch repair *in vivo*.

2. Materials and Methods

2.1 Protein Expression and Purification

To obtain the yPms1 NTD for crystallization, DNA encoding residues 32 to 396 of yPms1 was amplified from full length cDNA of yPms1 using the forward primer 5'-CCGCGTGGATCCATGACACAAATTCATCAGATAAAC and reverse primer 5'-CGGCCGCTCGAGTTTATTTGGGAAGAGCTAATTCTTG, followed by insertion into the pGEX4T3 (GE Healthcare) vector using the *Bam*HI and *Xho*I restriction sites. GST-tagged yPms1 NTD was expressed in BL21(DE3) cells (Stratagene). The cells were grown

in 2× YT broth at 37 °C in the presence of 100 µg/mL ampicillin until OD₆₀₀ reached 0.5, at which time the temperature was decreased to 22 °C. After 25 minutes, IPTG was added to each flask to a final concentration of 200 µM and expression continued overnight at 22 °C. The cells were pelleted at 6,000 rpm (9,000 RCF) for 10 min and resuspended in 150 mL of sonication buffer 1× PBS (1× phosphate buffered saline, pH 7.2 with an additional 600 mM NaCl). The cells were lysed by sonication in an ice water bath 3 times for 20 sec each time. After centrifugation at 48,000 RCF for 35 min, 15 mL of glutathione sepharose 4B resin (GE Healthcare) was added to the supernatant and placed on a slow rocker at 4°C for 1 hr. The resin was then washed with sonication buffer, followed by two washes with sonication buffer containing 10 mM ATP and 10 mM MgCl₂, and then two additional washes in 1× PBS buffer. The yPms1 NTD was cleaved from the GST fusion tag by batch thrombin (Sigma) digestion at 4°C overnight. The supernatant was collected by centrifugation at 500 RCF. The protein was concentrated, then dialyzed against 20 mM Tris pH 7.0, 100 mM NaCl and 1 mM DTT. AMPPNP (Sigma) and MgCl₂ were added to the protein sample to a concentration of 5 mM each. The final protein concentration was 9.3 mg/mL. Mutations in yeast NTD were generated with the QuickChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. Yeast Pms1 NTD and the K197E and R198E mutant derivatives were expressed and purified as described previously (Hall et al., 2002). The K218E and R243E/K244E mutants were expressed and purified in a similar manner, except that proteins were dialyzed against 25 mM Tris pH 8.0, 10% glycerol, 50 mM NaCl, 4 mM MgCl₂ and 1 mM DTT.

2.2 Protein crystallization, data collection and refinement

Crystals of the NTD yPms1 were grown in batch at 4°C by mixing 10 µl of the protein solution containing 5 mM AMPPNP and MgCl₂ with 4 to 6 µl of mother liquor consisting of 10% PEG4000, 0.1 M Tris-HCl (pH 8.5) and 0.2 M sodium acetate. For data collection, a crystal was transferred in four steps from the well, increasing the concentrations of PEG and ethylene glycol, to a cryo-protectant consisting of 18% PEG 4000, 0.1 M Tris-HCl (pH 8.5), 0.2 M sodium acetate, 5 mM AMPPNP, 5 mM MgCl₂, and 12.5% ethylene glycol. The crystal was frozen in a stream of nitrogen gas cooled at −170 °C and data were collected on a RUH3R Rigaku generator equipped with Osmic mirrors and a RaxisIV area detector. Data were processed and scaled using HKL2000 [48]. A solution was obtained from the molecular replacement program MOLREP in the CCP4 Suite [49] using a search model of human PMS2 obtained from PDB coordinates 1H7S [22]. The model of the yPMS1 NTD was refined using iterative cycles of model building in O [50] and refinement in CNS [51]. The final model is comprised of two molecules of the yPms1 NTD in the asymmetric unit, each with a bound AMPPNP molecule. Molecule A consists of residues D42-T109, A119-M274, and L287-P395. Molecule B consists of residues Q37-L275 and L285-A393. The geometry of the final model (Table 1) was analyzed by Molprobit [52].

2.3 Yeast strain and plasmids

S. cerevisiae haploid strain E134 (MATa *ade5 lys2::InsE_{A14} trp1-289 his7-2 leu2-3, 112 ura3-52*) and pms1Δ derivatives of E134 have been previously described [53,54]. The URA3-based yeast integrative plasmid YIpPMS1 has also been described previously [23,55–57]. For genetic studies described below, mutations encoding the changes were introduced into the *Pms1* gene in the yeast integrative plasmid YIpPms1 using the QuikChange Site-directed Mutagenesis kit (Stratagene).

2.4 Yeast strain construction

The chromosomal *Pms1* gene in strain E134 was replaced with the mutant alleles using mutant derivatives of plasmid YIpPms1 cut with *Hpa1*, as previously described [23]. Twelve isolates from each mutation type were replica-plated onto selective media to screen

for mutators via patch test. Genomic DNA was then isolated from the highest mutators and the *Pms1* gene was PCR amplified using the following primers: 5'-ATT TTT CAC CAC ATC GAA ACC-3' and 5'-TCA TAT TTC GA ATC CTT CG-3'. The resulting PCR product was then sequenced to confirm that the correct change, and no additional change was present. At least two different clones for each of the desired mutations were isolated and used to measure spontaneous mutation rates. The *pms1*Δ derivative of E134 was created by disrupting *Pms1* with an open reading frame conferring resistance to hygromycin B (hphMX4), as described in [58].

2.5 Mutation rate determinations

The spontaneous rates for reversion of *lys2::InsE_{A14}* and *his7-2* were measured in haploid yeast strains by fluctuation analysis as described in [53,59]. At least twelve yeast cultures per strain were started from single colonies and grown to stationary phase in YPDA. After appropriate dilutions, cells were plated onto selective medium lacking either lysine or histidine for revertant count, complete medium with canavanine and lacking arginine for Can^r mutant count, and complete medium for total viable count. Mutation rates and 95% confidence intervals were then calculated as previously described [53,60].

2.6 Immunoblot Analysis of Yeast Extracts

We previously reported that the amount of Pms1p present in strain E134 is too low to be detected by Western blot analysis [54], but that Pms1p can be detected when the gene is expressed from a plasmid, pMH8. This was confirmed here, and on that basis, the K197E, R198E, K218E and R243E/K244E mutations were introduced into this plasmid by site-directed mutagenesis, as previously described [53]. All mutations were confirmed by sequencing the entire *PMS1* gene. Strain E134 was then transformed with pMH8 containing wild-type Pms1 or Pms1 mutant derivative plasmids, and Western blot analysis was then performed as previously described [54].

2.7 Filter binding assay

Nitrocellulose filter binding assays using a radiolabelled circular duplex pGBT9 have been previously described [23]. Briefly, 1 μM of NTD WT and NTD yPms1 mutant derivatives were incubated with excess [³H]-pGBT9 substrate in 1× binding buffer (20 mM Tris-HCl, pH 8.0, 8% glycerol, 15 mM NaCl, 3.2 mM MgCl₂ and 80 μg/mL BSA). Reactions were incubated at room temperature for 10 minutes, diluted with excess 1× binding buffer and applied to the filter. Samples were counted in a Beckman Scintillation Counter.

2.8 DNA mobility shift assays

Binding to either a 41-mer duplex DNA or a 41-mer ssDNA substrate was examined. A 10 nM [³²P] 5'-end-labelled single-stranded 41-mer annealed to its complementary strand, or a [³²P] 5'-end-labelled single-stranded 41-mer, was incubated in a 20 μl volume with 10, 25, 50, 125, 250, 500, 1000 and 2500 nM yPMS1 NTD (wild-type or mutant) in 1× binding buffer (20 mM Tris-HCl, pH 8.0, 8% glycerol, 15 mM NaCl, 3.2 mM MgCl₂ and 80 μg/mL BSA) on ice for 30 minutes. Five μL of native binding buffer (5× binding buffer and 50% glycerol) was added and the samples were then analyzed by electrophoresis using a 5% DNA retardation gel (Bio-Rad) run in 0.5× TBE buffer at 100V for 1.5 hrs. Band intensities were quantified using a Molecular Dynamics Typhoon 9400 and ImageQuant software. The percent of bound DNA was calculated from $I_b/(I_b + I_{ub}) \times 100$, where I_b is the intensity of the DNA-protein complex and I_{ub} is the intensity of free DNA. The apparent K_d for the protein-DNA complex was determined by fitting the titrated yPms1 to % protein-DNA = $([\text{protein}] \times [\text{DNA-protein}]_{\text{max}})/([\text{protein}] + K_d)/[\text{DNA}] \times 100$, where [protein] is the

concentration of the yPms1, $[\text{DNA-protein}]_{\text{max}}$ is the concentration of DNA that will bind protein and the K_d is the apparent dissociation constant for the protein-DNA complex.

3. Results

3.1 The structure of the yPms1 NTD

The X ray crystal structure of the yPms1NTD was determined at 2.5 Å resolution (Table 1). This domain, which encompasses residues M32-K396 (Fig. 1B), is comprised of two α/β domains (Fig. 2A). The first domain (D42-S240) contains the four ATP binding motifs (I, II, III and IV) characteristic of the GHF superfamily of ATPases to which MutL proteins belong [61]. This domain consists of an 8-stranded anti-parallel β -sheet (strands 1–8) with one face exposed to the solvent and the other flanked by 6 helices (A',A,B,D,E,E'). The second domain (G257-K396) consists of a 4-stranded mixed β -sheet (strands 9,10,13,11) flanked on one side by helix E from the first domain and helix F from the linker region (S241–R256). Helices H and I of the second domain flank the other side of the sheet. Also present is a small 2-stranded sheet consisting of β -strands 14 and 15 (Fig. 2A).

The yPms1 NTD crystallized with two molecules in the asymmetric unit (Fig. 2B). Both molecules have AMPPNP bound in the ATPase site. The crystal packing between molecules A and B is asymmetric and likely non-physiological. As per the superimposition, molecules A and B are very similar, with an RMSD of 1.2 Å across 313 C α atoms (Fig. 2C). In addition, residues in the loop between β -strands 9 and 10 of both molecules are not observed in the structure. Molecules A and B differ with respect to the position of the ATP lid loop, which in the *E. coli* MutL LN40 dimer covers the ATP molecule [43]. In molecule A, lid residues T110-V118 are disordered, and residues T122-E128 form a β -strand that packs in a parallel orientation against β -strand 8 of molecule B in the asymmetric unit (Fig. 2B). In molecule B, the entire lid loop is ordered and appears in an open position (Figs. 2B and 2C), so that the AMPPNP in the ATPase site is solvent exposed. Rather than containing a β -strand, the lid from molecule B consists of two helices (Y108-K114 and D117-Q122). Residues T122-E128 do not form a β -strand, but are defined as a coil. The position of AMPPNP binding in both ATPase sites is similar, with each AMPPNP analog binding one magnesium ion. Although the different positions of the lid loop in molecules A and B reflect different packing environments, they suggest that different conformational states are obtainable by this region of the structure.

3.2 Comparing the NTDs of yPms1, hPMS2 and *E. coli* MutL

The structures of all three NTDs are very similar to one another, with the main difference being in loop regions. For example, the NTD of yPms1 shares 40% sequence identity with that of hPMS2 (Fig. 1B). Their structures are similar (Fig. 3A), displaying an RMSD of 1.4 Å over 273 C α atoms. The NTD of yPms1 contains an additional helix at the N-terminus (A', Fig. 1B, and Fig 2A) that is not ordered in hPMS2. In addition, the ATP lid is completely disordered in the NTD of hPMS2 when ATP γ S is bound. The biggest structural difference lies in the loop region between β -strands 9 and 10 (Fig. 3A), the region of greatest sequence divergence in the alignment (Fig. 1B). In hPMS2, strand 9 is much shorter and the loop region connecting the two strands is ordered and contains a helix (α H') not found in yPms1. The NTD of yPms1 shares 23% sequence identity to LN40 and the structures superimpose well (Fig. 3B), with an RMSD over 234 C α atoms of 1.8 Å, a value similar to that mentioned above for the yPms1 to hPMS2 comparison. In the crystal structure, LN40 homodimerizes (Fig. 3B) upon binding ATP, resulting in a closed ATP lid with the N-terminus of the other molecule in the dimer extending across the lid and locking it in position. In addition, helix A' becomes ordered in LN40 and is involved in forming the dimerization interface. In both molecules A and B of the NTD of yPms1, A' is ordered and

superimposes relatively well with respect to A' of LN40 with ADPNP bound. The conformation of molecule A in the yPms1 structure most closely resembles that of the LN40 molecules, with the lid partially closed (Fig. 3C). Interestingly, the second helix in the open lid (Fig. 3C) of molecule B (D117-Q122) overlaps in a sequence alignment with residues D85-D88 of LN40 that form helix C of the ATP lid. This overlap suggests that upon ATP binding, the lid domain could occupy a similar position as seen in LN40.

3.3 Mutator effects of reversing the charge of conserved, positively charged surface residues

Based on the yPms1 NTD structure, we identified numerous lysine and arginine residues on the surface that were not interacting with other residues, suggesting that they might not have a structural role and thus be available to interact with DNA and/or other protein partners. Some of these residues are not conserved in MutL homologs and were not investigated further here. However, several others are conserved (underlined in Fig. 1B), in some cases among both yPms1 and yMlh1 homologs (Table 2), and in other cases only among yPms1 homologs (Table 2). These residues were targeted for replacement with glutamate. Twelve *pms1* mutants encoding these changes (10 contained single mutants, two contained closely spaced double changes) were introduced into the natural chromosomal location of *Pms1* in haploid yeast strains. We then measured spontaneous mutation rates for single base insertions and deletions in the *his7-2* and *lys2::InsEA14* alleles, because these are particularly sensitive indicators of loss of mismatch repair *in vivo*. Complete disruption of *Pms1* (*pms1Δ*) resulted in a 12,000-fold increase in the rate of single base deletions from a run of 14 A-T base pairs in the *lys2* gene. Four of 12 *pms1* mutant strains, K190E, K194E, K229E and R256E (the latter serving as a non-conserved “control”) had very little effect on the spontaneous *lys2* reversion rate (Table 2). Modest mutator effects were observed for R306E, R311E and K328E (the latter from [38]). The most substantial mutator effects were observed for R170/K172E (5700-fold), K197E (890-fold), R198E (290-fold), K218E (2900-fold), and R243E/K244E (1000-fold) (Table 2). These *pms1* mutant strains that were mutators at the *lysA14* locus were also mutators for reversion at the *his7-2* locus monitoring single base additions to a run of seven A-T base pairs. These surface residues are therefore candidates to test for interactions with mismatch repair proteins or DNA.

3.4 DNA binding

Four of the mutants displaying the strongest *in vivo* mutator effects (K197E, R198E, K218E, R243E/K244E) reside along a shallow positively charged groove (highlighted by the line in Fig. 4) spanning from Lys197 and Arg198 on one side of the yPms1 NTD to Lys218 and Arg243/Lys244 on the other side. All four mutants can be expressed as full-length Pms1p, as determined by Western blot analysis (Fig. 5), and all four *pms1* mutants retain partial MMR function (Table 2). These results and the fact that the residues that were replaced are on the surface of the yPms1 NTD, argue against gross structural defects, and indicate that the mutant Pms1 proteins do heterodimerize with Mlh1. To determine if the mutator effects observed *in vivo* correlate with reduced DNA binding *in vitro*, we purified yPms1 NTDs with the K197E, R198E, K218E and R243E/K244E replacements and examined their ability to bind DNA. The K218E and R243E/K244E substitutions bound relatively normally to both double- and single-stranded DNA oligonucleotides in an electrophoretic mobility shift assay (Figs. 6A, 6B and Table 3) and bound normally to longer duplex plasmid DNA in a filter-binding assay (Fig. 6C). In contrast, the K197E and R198E proteins bound with reduced affinities to both double- and single-stranded DNA oligonucleotides (Figs. 6A, 6B and Table 3) and to longer duplex plasmid DNA (Fig. 6C).

4. Discussion

The structure of the yeast NTD Pms1 with AMPPNP bound is similar to the structures of NTDs of *E. coli* MutL [42,43] and human PMS2 [22], and it is consistent with our current understanding of the ATP catalytic cycle of MutL proteins that emerged from those earlier studies. This cycle of ATP binding and hydrolysis is essential for MMR [21,23,24], at least partly by promoting conformational changes that modulate ATP-dependent interactions with other macromolecules [1,4]. In addition to reinforcing the earlier studies, the present study provides new information on yPms1 by identifying conserved, positively charged lysine and arginine residues on the surface (Fig. 4) that are not interacting with other residues in the NTD and are potentially available to interact with DNA or other proteins. We then implicate several of these residues in MMR *in vivo* (Table 2), and finally implicate two of them in DNA binding *in vitro* (Table 3).

When substituting, several different basic residues with glutamate resulted in elevated spontaneous mutation rates characteristic of reduced MMR (Table 2). One of these residues, Lys328, was identified earlier [38]. All the others are conserved among Pms1 homologs, but interestingly, none of them is conserved among Mlh1 homologs. This observation is yet another manifestation of a central theme for eukaryotic MMR heterodimers, including MutLα [38], i.e., they are structurally and functionally asymmetric. Curiously, at least some conserved, positively charged surface Pms1 residues do not appear to be important for MMR. For example, Lys190 and Lys194 in yPms1 are conserved in both Pms1 and Mlh1 proteins across several species, yet changing these residues to glutamate in yPms1 has almost no effect on spontaneous mutation rates in yeast (Table 2). Although we cannot completely exclude a small role for these residues in MMR of replication errors, it may be that such residues are conserved for other functions of MutL proteins, e.g., cellular responses to DNA damage or recombination.

Among several possible explanations for partial loss of MMR *in vivo* conferred by reversing the charge on a surface residue is a defect in functional interaction with another macromolecule. The mutator variants identified here could be defective in interacting with Mlh1 when the MutLα heterodimer conformation compacts in response to ATP binding [62]. Alternatively, the mutator variants could be defective in interacting with other proteins, such as MutS heterodimers. Initial candidates that could be used to investigate defective protein-protein interactions include K218E and R243E/K244E, which are robust mutators (Table 2) whose NTDs do not exhibit reduced DNA binding *in vitro* (Figs. 6A–C and Table 3). Another such candidate is R170E/K172E, whose mutator effect approaches that of a null mutant (Table 2). Interestingly, Arg170 and Lys172 are conserved only in Pms1 homologs and are on a surface adjacent to the ATPase active site (Fig. 2A) rather than near the residues implicated in DNA binding.

An earlier study of the *E. coli* MutL NTD suggested that DNA binds within a cleft formed when the NTD dimerizes. The yPms1 NTD also binds to DNA, and does so in a salt sensitive manner that likely involves electrostatic interactions [37]. However, yPms1 NTD binding is seen in the absence of detectable homodimerization or heterodimerization with the Mlh1 NTD [38]. Previous studies demonstrated that substituting glutamate for arginine in helix H of either *E. coli* MutL [43] or yMlh1 [38] reduced (but did not eliminate) DNA binding. In contrast, substituting glutamate for Lys328 in the NTD yPms1, the only conserved positively charged lysine or arginine in helix H, did not reduce DNA binding [38]. Thus, one goal of the present study was to investigate whether conserved lysines and arginines on the surface other than in helix H of the yPms1 NTD might contribute to DNA binding. A candidate DNA binding surface was suggested by the structure of the yPms1 NTD, which revealed a shallow cleft with a high positive charge potential (Fig. 4). This

surface spans Lys197 and Arg198 on one side of the yPms1 NTD to Lys218 and Arg243/Lys244 on the other side, with the intervening surface containing a groove that theoretically could bind DNA (Fig. 4). On that basis, and given the mutator effects conferred by reversing the charges on these residues (Table 2), we examined the DNA binding affinities of yPms1 NTDs harboring the K218E, R243E/K244E, K197E and R198E substitutions. The idea that DNA binds within the positively charged groove between, and directly interacts with, all these residues is not supported by the fact that the K218E and R243E/K244E mutants had no discernable effect on DNA binding. However, the K197E and R198E changes did result in reduced DNA binding affinity (Figs. 6A–C, Table 3), and these reductions correlated with the observed mutator phenotypes, thereby implicating both Lys197 and Arg198 of yPms1 in DNA binding that is important for MMR function. Because the C-terminal domain of yPms1 harbors the active site for an endonuclease activity that is essential for MMR [39–41,58], it will be interesting to see if the K197E and R198E substitutions reduce the activity of this endonuclease *in vivo*.

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Fig. 1A

Fig. 1B

Figure 1. MutL homologs

A. Schematic diagram of *E. coli* MutL and *S. cerevisiae* Mlh1 and Pms1 proteins. Diagram depicts the location of the MutL ATP binding domain (yellow) and the location of the Mlh1 and Pms1 ATP binding domains by homology to MutL (yellow) in the highly conserved N-terminal domain. The conserved ATP binding motifs I, II, III, and IV are also represented (blue bars in N-terminal domain) [42,63]. The endonuclease active site at the C-terminal end of Pms1 is depicted (blue bar) [39]. The Pms1 interaction region in Mlh1 and the Mlh1 interaction region in the less well-conserved C-terminal domain of Pms1 are also represented (red) [26]. Figure was adapted from [23]. **B.** Structural alignment of the N-terminal domains of yPms1 and hPMS2. Secondary structure elements are labeled according to that of hPMS2 [22]. Conserved residues between the two proteins are starred and the lid region is underlined in red. Regions with structural differences have tan background. Residues in orange are disordered, residues in pink were mutated in this study, and side chains for the residues colored blue interact with the bound nucleotide. Individual residues that were mutated and are conserved in yMlh1 are underlined in pink.

Fig. 2A

Fig. 2B

Fig. 2C

Figure 2. Ribbon diagrams of the N-terminal domain of yPms1

A. Ribbon diagram of the crystal structure of the N-terminal domain of yPms1, depicted in *orange*. Amino acids that lie on the surface of the domain are *tan* and numbered, AMPNPP is *magenta* and the magnesium ion is *green*. Secondary structure elements are labeled according to that of hPMS2 [22]. Italicized *black* numbers represent β -strands, and italicized *black* letters represent helices. *Nt* and *Ct* refer to N- and C- termini. The image was created with Molscript and Raster3D [64,65]. **B.** Ribbon diagram of the two molecules A (orange) and B (cyan) of the N-terminal domain of yPms1, with bound AMPNPP (*green*), in the asymmetric unit. The lid domains of A and B molecules are shown in *red* and *blue*, respectively. Selected amino acid residues are in *black*. *Nt* and *Ct* refer to N- and C- termini.

C. Superposition of molecules A and B reveals differences in the conformation of the lid region. The lid domains of A and B molecules are shown in *red* and *blue*, respectively. Selected amino acid residues are represented in *black*. Ct refers to C-terminus.

Fig. 3A

Fig. 3B

Figure 3. Ribbon diagrams of the NTDs of yPms1, hPMS2 and E. coli LN40

A. Superposition of molecule A of N-terminal domain yPms1 (*orange*) onto the structure of hPMS2 (*red*). Selected amino acid residues are represented in *black* while *italicized black* numbers represent β -strands. *Ct* refers to C-terminus. **B.** Superposition of molecule A of yPms1 (*orange*) onto the dimer structure of the LN40 domain of MutL. Figures created with Molscript and Raster3D [64,65]. Positively charged residues are shown in *black*. *Ct* refers to C-terminus. **C.** Superposition of the lid region from molecules A (*orange* with lid colored *red*) and B (*cyan* with lid colored *blue*) of yPms1 with that of LN40 (*grey*). Secondary structural elements are labeled as in Figure 1B and Figure 2A. AMPPNP for each molecule is colored according to chain color.

Figure 4. Electrostatic surface potential of the yPms1 NTD

Electrostatic surface rendering of NTD yPms1 in a similar orientation to that of Figure 3A. The surface potential plot was calculated using the Adaptive Poisson Boltzmann Solver Tool in Pymol [66]. The potential ranges from -8 kTe^{-1} (red) to 8 kTe^{-1} (blue). A groove found on the surface is highlighted with a *dashed yellow line*. Numbers represent positively charged amino acid residues.

Figure 5. Western blot analysis of wild type and mutant Pms1 proteins

The analysis was performed as previously described [54], using extracts of the E134 strain containing the empty pMH8 vector or pMH8 encoding wild type or the designated mutants of yPms1.

Fig. 6A

Fig. 6B

Fig. 6C

Figure 6. DNA binding by the yPms1 NTDs

Proteins examined included wild-type NTD yPms1 and mutant derivatives K197E, R198E, K218E and R243E/K244E. Electrophoretic mobility shift assays were employed to examine binding to **A.** 41-mer duplex DNA and **B.** 41-mer ssDNA. These assays used increasing concentrations of yPms1 and a constant substrate DNA concentration (10 nM). % DNA bound was calculated as described in Materials and Methods. In graphs A and B, wild-type NTD yPms1 (*open circle*), K197E (*open square*), R198E (*open diamond*), K218E (*dashed line and x*) and R243E/K244E (*dashed line, closed triangle*). **C.** DNA binding of NTD yPms1 proteins to double-stranded [^3H] pGBT9. Proteins examined were wild-type NTD Pms1 and mutants K197E, R198E, K218E and R243E/K244E. Binding assays were performed with a constant protein concentration of 1 μM and excess DNA. Binding reactions were performed in the presence of MgCl_2 . Protein–DNA binding is presented as percent DNA bound (percentage of total DNA substrate in a given sample that is protein bound). Error bars represent standard errors of the mean for three independent experiments. Error bars are included for all data.

Table 1**Crystallographic Parameters**

Data Set	yPms1 NTD (32–396) + AMPPNP
Unit Cell Dimensions	a=75.59Å, b=103.29Å, c=117.17Å; $\alpha=\beta=\gamma=90^\circ$
Space Group	P2 ₁ 2 ₁ 2 ₁
Resolution (Å)	50.0 - 2.5
# of observations	107,749
# of unique reflections	31,852
R _{sym} (%)(last shell) ¹	9.7 (45.2)
I/σ I (last shell)	17.0 (2.2)
Mosaicity	0.48
completeness(%) (last shell)	97.7 (99.4)
<u>Refinement statistics</u>	
R _{cryst} (%) ²	23.5
R _{free} (%) ³	28.6
# of waters	141
Mean B value (Å ²)	49.0
<u>r.m.s. deviation from ideal values</u>	
bond length (Å)	0.007
bond angle (°)	1.3
dihedral angle (°)	23.4
improper angle (°)	0.88
<u>Ramachandran Statistics</u> ⁴	
residues in:	
favored (98%) regions (%)	94.0
allowed (>99.8%) regions (%)	99.7

¹) $R_{sym} = \sum (|I_i - \langle I \rangle|) / \sum I_i$ where I_i is the intensity of the i th observation and $\langle I \rangle$ is the mean intensity of the reflection.

²) $R_{cryst} = \sum ||F_o| - |F_c|| / \sum |F_o|$ calculated from working data set.

³) R_{free} was calculated from 5% of data randomly chosen not to be included in refinement.

⁴) Ramachandran results were determined by MolProbity [52].

These coordinates have been deposited with PDB code 3H4L.

Table 2

Spontaneous reversion rates of yeast strains with *pms1* mutations

Pms1 Genotype	Lys ⁺ Reversion Rate ($\times 10^{-6}$)		His ⁺ Reversion Rate ($\times 10^{-6}$)		Conservation among 38 organisms*	
	Rate (95% CI)	Relative Rate	Rate (95% CI)	Relative Rate	Pms1 homologs	Mlh1 homologs
WT	0.093 (0.064–0.13)	1	0.7 (0.5–0.9)	1	-	-
Δ	1100 (840–2200)	12,000	43 (33–62)	61	-	-
R170E/K172E	530 (430–650)	5,700	22 (21–30)	31	21/26	0/0
K190E	0.15 (0.12–0.22)	2	0.7 (0.4–0.9)	1	38	35
K194E	0.58 (0.39–0.73)	6	1.4 (0.9–1.7)	2	37	35
K197E	83 (59–110)	890	4.1 (2.3–4.8)	6	38	0
R198E	27 (23–31)	290	4.6 (3.1–5.7)	7	38	0
K218E	270 (220–410)	2900	12 (11–20)	17	38	0
K229E	1.1 (0.82–2.0)	12	0.9 (0.6–2.3)	1	29	38
R243E/K244E	93 (75–130)	1,000	5.9 (4.3–7.0)	8	30/6	1/0
R256E	0.22 (0.18–0.34)	2	0.9 (0.7–1.2)	1	5	18
R306E	4.1 (2.0–6.5)	44	1.5 (0.9–2.1)	2	38	0
R311E	5.9 (5.0–13)	63	2.1 (1.4–3.1)	3	37	0
K328E**	11 (8.3–19)	60	3.4 (3.0–7.9)	3	37	37

* Organisms and NCBI reference sequence numbers for Pms1 and Mlh1 homologs:

Saccharomyces cerevisiae (NP_014317, NP_013890), Vandewaltomyces polyporus (XP_001644784, XP_001645690), Candida glabrata (XP_447944, XP_446304), Ashbya gossypii (NP_985276, NP_985351), Kluyveromyces fragilis (XP_452705, XP_453504), Penicillium marneffei (XP_002144802), Podospora anserina (XP_001910360, XP_001910200), Aspergillus fumigatus (XP_755674, XP_753480), Aspergillus clavatus (XP_0001275615, XP_001274465), Coccidioides immitis (XP_0012145606, XP_0012239552), Neosartorya fischeri (XP_001260801, XP_001259525), Aspergillus terreus (XP_001208682, XP_001211278), Magnaporthe oryzae (XP_382105, XP_389904), Aspergillus oryzae (XP_001821792, XP_001824017), Tribolium castaneum (XP_974934, XP_972898), Sclerotinia sclerotiorum (XP_001589152, XP_001399369, XP_001398763), Strongylocentrotus purpuratus (XP_786592, XP_793318), Bos Taurus (XP_605426, XP_001790604), Populus trichocarpa (XP_002321013, XP_002325965), Mus musculus (NP_032912, NP_002400), Aspergillus nidulans (XP_663920, XP_657730), Canis familiaris (XP_536879, XP_534219), Homo sapiens (NP_000526, NP_000240), Danio rerio (XP_693648, NP_002165510, XP_002156705), Trichoplax adhaerens (XP_002110081, XP_002111226), Pan troglodytes (XP_001141671, XP_001170390), Equus caballus (XP_001494013, XP_001489268), Acyrthosiphon pisum (XP_001947410, XP_001945011), Oryza sativa (NP_001047292, NP_001045457), Monodelphis domestica (XP_001377577, XP_001362893), Arabidopsis thaliana (NP_567236, NP_567345), Anopheles gambiae (XP_320342), Schizosaccharomyces pombe (NP_594417, NP_596199) and Rattus norvegicus (NP_001099378, NP_112315).

** From [38].

Mutation rates were measured as described in Materials and Methods. Rates are the median for 12 independent cultures.

Table 3Apparent K_d for yPms1 NTDs

NTD yPms1 protein	Apparent K_d (nM)	
	Duplex DNA	ssDNA
WT	160 ± 20	1000 ± 70
K197E	9,500 ± 2000	10,000 ± 2000
R198E	7,200 ± 1300	11,000 ± 1700
K218E	180 ± 20	1200 ± 120
R243E/K244E	250 ± 40	1600 ± 140