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Mutating lysine 336 in Msh6 does not appear to affect DNA mismatch repair in Saccharomyces cerevisiae

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Effects of Msh6 Lysine 336 Mutation on MMR of Yeast DNA

Introduction

The study of various mutations is vital in understanding the causes and modes of prevention and treatment for many diseases. One important repair pathway that is central to this idea is DNA mismatch repair (MMR). When DNA replication takes place, DNA polymerase pairs Adenine with Thymine, and Cytosine with Guanine. When two bases are matched outside of this pattern, a mismatch exists. Fortunately, DNA MMR proteins exist such that mismatches not repaired by DNA polymerase can be repaired (Marinus, 2012).

Mitigation of mismatches is crucial because mismatching results in a different DNA sequence from the template, allowing for mutations. Some mutations can occur in vital genes and subsequently result in certain cancers, underscoring the need for DNA MMR and the importance of DNA MMR proteins in living organisms. A mismatch that is not repaired by DNA polymerase is recognized by MutSα, a heterodimeric protein complex comprising polypeptides Msh6 and Msh2. MutLα (Mlh1 and Pms2) is then recruited and cleaves the daughter strand near the mismatch by performing the endonuclease function. Another protein, Proliferating Cell Nuclear Antigen (PCNA), properly orients MutLα so it can correctly cut the daughter strand. Exonuclease 1 then degrades the region of the daughter strand that contains the mismatch. To replace the DNA segment that has been removed, DNA polymerase δ re-synthesizes the DNA where the gap exists. During this process, Rpa binds to the template strand to protect regions not requiring repair (Edelbrock *et al*, 2013). Finally, DNA ligase binds the newly synthesized segment to the unattached end of the daughter strand (Shuman, 2009).

Regarding diseases associated with defects in DNA MMR, mutations in *MSH6* and *MSH2* are often responsible for Constitutional Mismatch Repair Deficiency (CMMRD) and Lynch Syndrome (LS), respectively (Abedalthagafi, 2018). Lynch syndrome is an autosomal dominant disease (Bin Naeem *et al*, 2023). The cancers associated with LS include colon, ovarian, endometrial, urinary tract, stomach, brain, and skin cancers, among others (Biller *et al*, 2022). CMMRD is an autosomal recessive disease (Bin Naeem *et al*, 2023). The cancers associated with CMMRD include brain tumors, digestive tract cancer, hematological malignancies, and Leukemia. The onset of the cancers associated with CMMRD is often in early childhood as opposed to adulthood in LS (AlAli *et al*, 2022).

Characterizing mutations is important to determine if a mutation is harmful or not. Not all mutations result in significant changes in the protein

function, often due to the similarity between the correct amino acid and the amino acid resulting from the mutation. However, when an amino acid is significantly different, interactions of the polypeptide may change such that the protein is no longer functional. If it is found that a mutation does not impact the protein's functionality, then it is known that patients with a disease linked to the gene of interest have an alternative cause of disease. Each missense mutation is therefore unique and should be examined individually for its effects.

The mutation examined in this study is *msh6-K336T* in yeast, which is equivalent to *K431T* in humans. The mutation resulting in *K431T* changes interactions in the mismatch region as threonine is vastly different than lysine in its attributes of being less polar than lysine and non-ionizable. Such a significant structural change in Msh6 may alter mismatch binding or interactions between Msh6 and Msh2 such that MutSα is not functional. This will likely prevent MutSα complex interaction and hinder recognition and subsequent steps for the repair of mismatches. For this study, *Saccharomyces cerevisiae* (yeast), a single-celled eukaryote, is used as the model organism. Yeast is used because the DNA mismatch repair process in humans and yeast is conserved, and because Msh6 in yeast is homologous to Msh6 in humans (Botstein *et al*, 1997). The goal of this investigation is to understand protein functionality and how a single *MSH6* mutation can impact the process of DNA MMR. Specifically, the study is concerned with the initial step of mismatch recognition by MutSα.

Methods

SIFT and CoDP analysis:

Sorting Intolerant from Tolerant (SIFT) was performed by using the yeast and human Msh6 amino acid sequences. For Combination of Different Properties of Msh6 Protein (CoDP), the amino acid position being studied was selected and the desired amino acid change was provided. The programs provided an output on tolerance for the given amino acid change in Msh6. The predictions from both CoDP and SIFT were used to guide formulation of the hypothesis before conducting experiments.

Plasmid Generation and Bacterial Transformation:

The plasmid pRS415, containing wild-type *MSH6*-MYC, was mutagenized by Site-Directed Mutagenesis (GenScript, Piscataway, N.J.). The plasmid was verified by examination of the sequence files and restriction digests. Plasmids obtained from GenScript were resuspended and transformed into competent DH5 α *Escherichia coli* (*E. coli*) cells prepared using the Mix & Go Competent Cells (Zymo Research), according to the manufacturer's instructions. The cells were plated on LB containing ampicillin and incubated at 37°C.

Plasmid Extraction:

The Invitrogen PureLink™ kit was used for extraction. Briefly, a pellet of bacterial cells was resuspended in 250µL of resuspension buffer and mixed. 250µL of lysis buffer was added, the sample was mixed by inversion, and incubated at room temperature for five minutes. 350µL of neutralization buffer was then added followed by ten minutes of centrifugation at 13,000 rpm. Next, 800µL of the supernatant was transferred to a spin column followed by centrifugation, washing with 700µL of wash buffer, and centrifugation. The purified plasmid was eluted with 50µL of elution buffer. The plasmid concentration was measured using a Nanodrop.

Restriction Enzyme Digest and Gel Electrophoresis:

Samples for the restriction enzyme digest were prepared by adding 2µL of green buffer and 5µL of plasmid to three 1.5mL microcentrifuge tubes. 1µL of PacI and 1µL of NotI was then added to the first tube, 1µL of NotI was added to the second tube, and all tubes were filled to 20µL by adding water. The samples were incubated for 30 minutes at 37°C. Then, 10µL of each sample was loaded into a 1% agarose gel along with a marker. Gel electrophoresis was performed for 30 minutes at 135 volts.

Yeast Transformation:

The genotype of the strain transformed is *MATα msh6Δ::kanMX leu2-3,112 ura3-1 trp1-1 his3-11,15 LYS2 CAN1 RAD5*. Competent cells were prepared using Zymo Research Frozen-EZ Yeast Transformation II™ kit, following the manufacturer's protocol. Two tubes of 50µL of competent yeast cells were labelled for the plasmid and the no-plasmid control. To the plasmid tube, 5µL of plasmid DNA was added, followed by 500µL of EZ Yeast solution. To the no-plasmid control tube, 5µL of water was added followed by 500µL of EZ Yeast solution. Both tubes were vigorously vortexed and inverted, then incubated at 30°C for 45 minutes, vortexing every 10 minutes during incubation. For each sample, 100µL of the mixture was added to its respective plate lacking the amino acid leucine (–LEU) and a sterile loop was used to evenly distribute the cells. The plates were incubated at 30°C for 2 days. A single colony was then picked to streak onto a new –LEU plate. The plate was incubated at 30°C for 2 days.

Mating and Diploid Selection:

Two haploid strains were mated. The genotype of the haploid MATα cells is *MATα msh6Δ::kanMX4 leu2-3,112 ura3-1 trp1-1 his3-11,15* containing pRS415 *(LEU2 msh6-K336T).* The genotype of the haploid MATa cells is *MATa*

his3Δ leu2Δ met15Δ ura3Δ. Strains were mated on YEPD and incubated overnight at 30°C. For diploid selection, the YEPD plate was replica plated to a plate lacking leucine and tryptophan (–LEU-TRP). The -LEU-TRP plate was incubated at 30°C for 2 days. A single colony was picked, transferred to a new -LEU-TRP plate, and incubated at 30°C for 2 days.

CAN1 Forward Mutation Assay:

For both the diploid cells and the haploid cells, 3 colonies of the controls (*MSH6*, *msh6Δ*) and the *msh6-K336T* or *msh6-K336T/MSH6* were transferred to YEPD plates. The YEPD plates were incubated at 30°C. The YEPD plates were then replica plated to a plate lacking the amino acids leucine and arginine and containing 60μg/ml canavanine (+CAN) plate and a –LEU plate. The –LEU and +CAN plates were incubated at 30°C for 2 and 3 days respectively.

Results

SIFT and CoDP analysis:

To conduct pre-investigation predictions, SIFT and CoDP were used. Based on SIFT Analysis, which examines sequence homology to predict the effect of a given amino acid substitution, it was predicted that the amino acid change would be tolerated in humans and not tolerated in yeast (Ng and Henikoff, 2003). Using CoDP Analysis, which predicts how any amino acid substitution would impact human Msh6 protein function, it was predicted that the mutation would likely impair molecular function (Terui *et al*, 2013).

Confirmation of *msh6-K336T* allele on the pRS415 plasmid:

The plasmid map (**Figure 1**) shows important regions within the pRS415 plasmid. *LEU2* is the gene for beta-IPM dehydrogenase, a protein essential for leucine biosynthesis. *AmpR* is the gene conferring resistance to the antibiotic ampicillin. Ori is the bacterial origin of replication. *ARSH4/CEN6 (CEN/ARS* as written on the map) are the genes responsible for plasmid replication in yeast and plasmid maintenance in yeast, respectively. *MSH6* with the mutation of interest is *msh6-K336T.* The restriction sites are indicated for PacI and NotI, the restriction enzymes used in this study.

Plasmid extraction is the removal of a plasmid from an organism through lysing, binding, washing, and elution of cells. The purpose of the plasmid extraction was to extract the pRS415 plasmid from *Escherichia coli (E. coli)* to obtain and isolate *msh6-K336T* to then check for *MSH6* presence in the plasmid using gel electrophoresis and for future transformation to yeast. This was

accomplished through lysing of the *E. coli* cells, then binding the plasmid DNA to a column, washing with ethanol, and elution of the pRS415 plasmid. The plasmid was successfully extracted with no shown impurities and found to have a concentration of 58.3 ng/μL.

Restriction digest results in the cleavage of DNA at specific sites by Restriction Endonucleases. Gel electrophoresis uses an electric charge to separate DNA samples by size. The purpose of the restriction digest was to cut the pRS415 plasmid using restriction enzymes NotI and PacI so that the digested plasmid could be examined using gel electrophoresis. This was done to confirm the presence of *msh6-K336T* on the pRS415 plasmid as indicated by fragment size. The gene's presence is important because it allows for the successful extraction of the plasmid from the bacteria to be confirmed. The restriction digests were able to be examined through gel electrophoresis by comparing the resultant sizes. Based on the restriction digest gel results as compared to the marker as shown in Figure 1, it is seen that the plasmid cut by NotI only resulted in one band about level with the 10 kilobases (kb) marker. This is consistent with the linearized plasmid size of ~10.3 kb. The plasmid cut by both NotI and PacI resulted in bands at ~7 kb and ~4 kb. This is consistent with the theoretical sizes of 6.7 kb and 3.6 kb for this restriction digest. Though still about level with the 10 kb marker, the uncut plasmid appears smaller than the linearized plasmid, probably due to supercoiling. In the UC lane, the predominant band present does run lower than expected for circular DNA. Therefore, due to agreement in the resultant sizes, specifically in lanes C1 and C2, the gel electrophoresis results confirm the presence of *msh6-K336T* on the pRS415 plasmid.

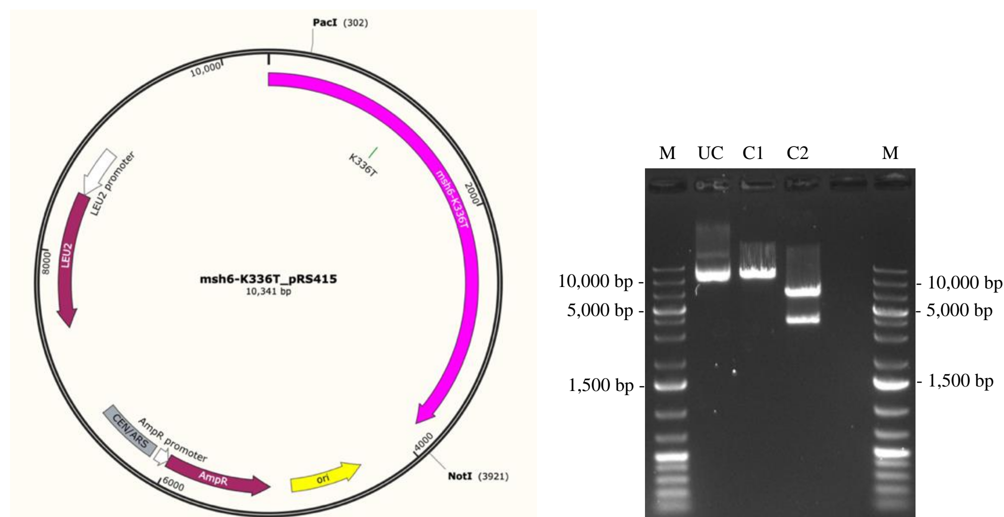

Figure 1. Plasmid map of pRS415 containing *msh6-K336T* with important features indicated (left image). This includes PacI at position 302 and NotI at position 3921. **Restriction digest and Gel Electrophoresis** result for *msh6-K336T*_pRS415 (right image), with abbreviations: M for Marker, UC for uncut plasmid, C1 for plasmid cut by NotI only, and C2 for plasmid cut by both NotI and PacI.

Haploid and Diploid strain construction:

Transformation involves introducing DNA into an organism; in this case the plasmid previously extracted from *E. Coli* was introduced to competent yeast cells. This was done by mixing the plasmid with competent yeast cells and selecting on a –LEU plate. Additionally, from the transformants, a single colony was transferred to a new –LEU plate, isolating colonies. Colony growth confirmed the successful transformation of the pRS415 plasmid into the yeast cells given that the plasmid contains *LEU2*. Thus, isolated colonies can be used for mating haploids and to test MMR function.

Mating of two haploid yeast strains was performed to obtain diploid yeast cells containing the wild-type *MSH6* allele and the pRS415 plasmid with *msh6-K336T*. Mating was accomplished by mixing yeast haploid strains *MATa* (wild-type *MSH6*) and *MATα* (plasmid-containing) and allowing them to mate on a YEPD plate then transferred to a –LEU-TRP double dropout plate to select for the diploid strains. For diploid selection, resultant diploid cells were then transferred onto a new –LEU-TRP double dropout plate to isolate diploid yeast cell colonies. These diploid colonies are of genotype *msh6-K336T/MSH6 TRP1/trp1-1 LEU2/leu2-3,112*.

The *msh6-K336T* variant displays a wild-type MMR phenotype:

To determine the MMR functionality of the *msh6-K336T* allele, the *CAN1* Forward Mutation Assay was performed. This is possible because the *CAN1* gene encodes the arginine permease, and the assay uses a toxic arginine analog, canavanine. For a functional DNA MMR system, mismatches that escape proofreading by the DNA polymerase during DNA replication are efficiently recognized by MutSa and are repaired. This results in a functional *CAN1* gene, encoding a functional arginine permease. Canavanine can enter the cell through the arginine permease, and this results in cell death. Little to no colonies grow when *MSH6* is wildtype. When DNA MMR is not functional, mismatches that occur during DNA replication are not recognized by MutSa. Unrepaired mismatches become permanent mutations. This results in a defective *CAN1* gene, encoding a nonfunctional arginine permease. Though present in the environment, canavanine cannot enter the cells. Numerous colonies are expected to grow when Msh6 is absent or defective. When the mutant is plated, it can be compared to the controls

to determine if DNA mismatch repair is impaired or not. Since the hypothesis was that the mutation to *MSH6* would impair function, the yeast with the *K336T* mutation was expected to behave closer to the *msh6Δ* yeast. The *CAN1* assay was done by growing haploid and diploid *msh6-K336T* containing yeast cells on YEPD plates then replica plating to +CAN and –LEU plates to assess growth and subsequently determine MMR functionality. Diploid yeast was used to test whether a copy of wild-type *MSH6* would rescue the function of mismatch repair, in the presence of a defective allele. If the hypothesis that the *msh6-K336T* mutation would impact mismatch repair was correct, numerous colonies would grow in the haploid strain. However, in the diploid that contains *msh6-K336T* and a wild-type copy of the *MSH6* gene, mismatch repair function would be restored. Little to no colonies are therefore expected in the diploid *MSH6/msh6-K336T* strain. Wild-type *MSH6* and *msh6Δ* were used as the controls in this experiment. After incubation, the haploid +CAN haploid plate showed minimal to no growth in *msh6-K336T* and wild-type *MSH6,* while it showed growth for the *msh6Δ*. This suggests that *msh6-K336T* is similar to wild-type and was able to synthesize a functional arginine permease despite being a mutant. The +CAN diploid plate showed no growth for *MSH6/ msh6-K336T,* indicating synthesis of functional arginine permease in *MSH6*/*msh6-K336T*. The haploid –LEU plate showed growth for all three strains. The diploid –LEU plate showed growth for all three stains as well. The growth seen on both –LEU plates was expected given that all strains were able to synthesize the amino acid leucine. Additionally, uniformed transfer of the cells during replica plating is demonstrated by growth on -LEU. The growth result from the *CAN1* Forward Mutation Assay indicates that in yeast, *msh6-K336T* is a minimally adverse mutation that seems to still have an adequately functioning MMR process as revealed through apparent arginine permease synthesis and resultant cell death. This experiment was repeated three additional times to validate our initial findings. These results are displayed in **Figure 2.**

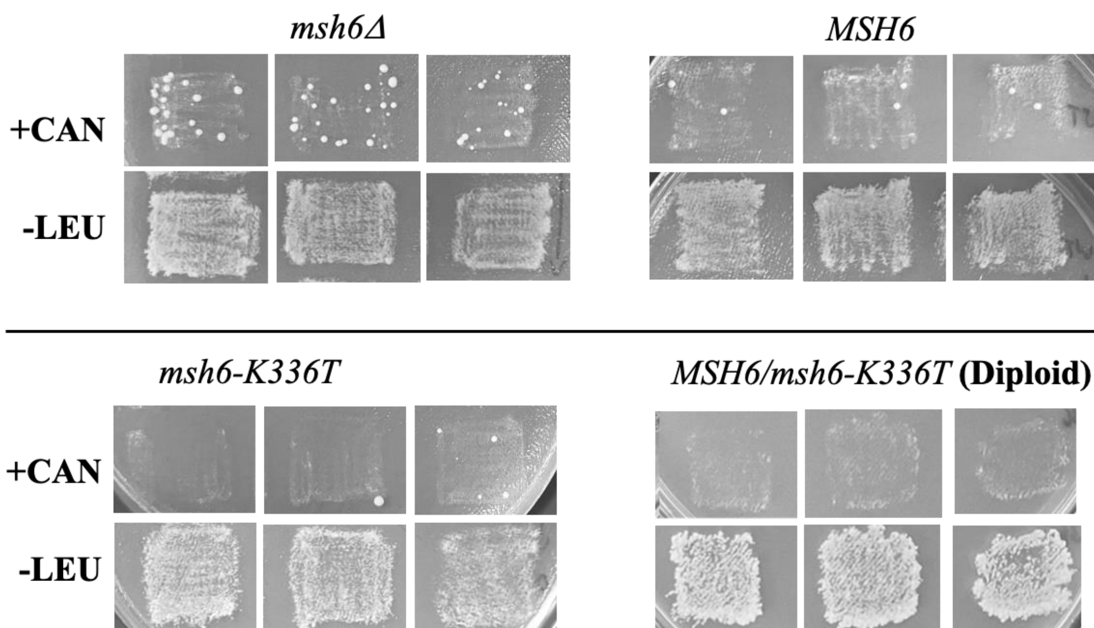

Figure 2. ***CAN1*** **forward mutation assay.** Patches showing the amount of growth after incubation of *msh6D, MSH6*, *msh6-K336T* (haploids), and *MSH6/msh6-K336T* (diploids). Growth on +CAN plates is shown in the top row for each strain and -LEU is shown in the bottom row.

Discussion and Conclusion

This study aimed to understand the effect of a single missense mutation *msh6-K336T* on the function of Msh6, a protein that functions in DNA MMR. The hypothesis was that the mutation would negatively affect mismatch repair function. This was done by first extracting the pRS415 plasmid from *E.coli* and performing a restriction digest to confirm *MSH6* presence on the pRS415 plasmid and to confirm that the DNA extracted was the plasmid. Next, the plasmid was transformed into yeast. This allowed functional analysis of the allele of interest in the model organism. The plasmid-containing yeast strain (*MATα*) was then mated with a *MATa* haploid strain to produce diploid yeast strains for further analysis. Using both the diploid and haploid yeast strains, the *CAN1* forward mutation assay was performed. From this assay, it was observed that no *msh6-K336T* colony growth occurred with the diploid strain, which was consistent with the wild-type behavior. This was expected since the diploid contains a wild-type copy of the *MSH6* gene. Additionally, *msh6-K336T* haploid cells showed no growth from the *CAN1* forward mutation assay, displaying a wild-type phenotype when an

intermediate or *msh6Δ*-like phenotype was expected. This indicated that MMR was functional in the cell such that *CAN1* was properly encoded, allowing arginine permease to be synthesized correctly and canavanine to enter the cell. This second observation is more telling of the nature of the allele given that the strain is haploid and therefore only contains *msh6-K336T*. The *CAN1* forward mutation assay results indicated that *msh6-K336T* is not a deleterious mutation and behaves in a pseudo-wild-type manner. Overall, through analysis of the *CAN1* forward mutation assay, specifically regarding *msh6-K336T* growth as compared to positive and negative controls, it can be concluded that *msh6-K336T* is MMR proficient. Translating this to human health, human *msh6-K431T* is indicated not to be deleterious. This is important in guidance for genetic counseling given that *MSH6* is important in Lynch Syndrome. Patients with *msh6-K431T* who have LS should be further tested for the causative agent of their disease given that *msh6-K431T* is not deleterious. This result suggests that patients with the *msh6-K431T* mutation may not need to be screened as frequently for LS as compared to patients with more adverse MMR gene mutations.

This work gives insight into the strength of allele characterization and its implications in clinical treatment. The importance of understanding disease states on a genetic level is clarified in such a study as this and can be applied in further studies. One further study could be quantitatively analyzing the *msh6-K431T* variant. The analysis performed in this study is qualitative. Quantifying the mutation rate of the allele would provide the mutation rate relative to the wild-type *MSH6* strain. It is possible that the mutation is not entirely equivalent to wild-type and quantitative analysis would reveal the mutation rates of the allele, providing more information than a qualitative assay. Additionally, the effect of mutagenesis on the Msh6 protein levels can be assessed. Previous studies have shown that missense mutations in Msh2 (the binding partner of Msh6) can result in reduced Msh2 levels (Gammie *et al*., 2007). Wild-type MMR function does not always correspond to normal protein levels. It would be interesting to determine if there are any changes in the protein level of the Msh6 variant examined in this study.

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References

Abedalthagafi M. (2018). Constitutional mismatch repair-deficiency: current problems and emerging therapeutic strategies. *Oncotarget*, *9*(83), 35458–35469. https://doi.org/10.18632/oncotarget.26249

AlAli, M. N., Zikry, A. H., AlShammari, S. A., Zayed, M. A., Alswayyed, M., & AlObeed, O. A. (2022). A Constitutional Mismatch Repair Deficiency Syndrome Presented With an Advanced Rectal Cancer in a Juvenile Female: A Case Report and Literature Review. *Cureus*, *14*(4), e24615. https://doi.org/10.7759/cureus.24615

Biller, L. H., Creedon, S. A., Klehm, M., & Yurgelun, M. B. (2022). Lynch Syndrome-Associated Cancers Beyond Colorectal Cancer. *Gastrointestinal endoscopy clinics of North America*, *32*(1), 75–93. https://doi.org/10.1016/j.giec.2021.08.002

Bin Naeem, S., Ullah, N., Jhatial, M. A., Muzaffar, S., Abbas, M., Iftikhar, I., Jameel, A., & Masood Sheikh, R. (2023). Constitutional Mismatch Repair Deficiency (CMMRD) Syndrome: A Case Report of a Patient With Multiple Metachronous Malignancies. *Cureus*, *15*(7), e41870. https://doi.org/10.7759/cureus.41870

Botstein, D., Chervitz, S. A., & Cherry, J. M. (1997). Yeast as a model organism. *Science (New York, N.Y.)*, *277*(5330), 1259–1260. https://doi.org/10.1126/science.277.5330.1259

Edelbrock, M. A., Kaliyaperumal, S., & Williams, K. J. (2013). Structural, molecular and cellular functions of MSH2 and MSH6 during DNA mismatch repair, damage signaling and other noncanonical activities. *Mutation research*, *743-744*, 53–66. https://doi.org/10.1016/j.mrfmmm.2012.12.008

Gammie AE, Erdeniz N, Beaver J, Devlin B, Nanji A, Rose MD. Functional characterization of pathogenic human MSH2 missense mutations in Saccharomyces cerevisiae. Genetics. 2007 Oct;177(2):707-21. doi: 10.1534/genetics.107.071084. Epub 2007 Aug 24. PMID: 17720936; PMCID: PMC2034637.

Marinus M. G. (2012). DNA Mismatch Repair. *EcoSal Plus*, *5*(1), 10.1128/ecosalplus.7.2.5. https://doi.org/10.1128/ecosalplus.7.2.5

Ng, P. C., & Henikoff, S. (2003). SIFT: Predicting amino acid changes that affect protein function. *Nucleic acids research*, *31*(13), 3812–3814. https://doi.org/10.1093/nar/gkg509

Oakley A. J. (2019). A structural view of bacterial DNA replication. *Protein science : a publication of the Protein Society*, *28*(6), 990–1004. https://doi.org/10.1002/pro.3615

Shuman S. (2009). DNA ligases: progress and prospects. *The Journal of biological chemistry*, *284*(26), 17365–17369. https://doi.org/10.1074/jbc.R900017200

Terui, H., Akagi, K., Kawame, H., & Yura, K. (2013). CoDP: predicting the impact of unclassified genetic variants in MSH6 by the combination of different properties of the protein. *Journal of biomedical science*, *20*(1), 25. https://doi.org/10.1186/1423-0127-20-25