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When you're strange: Unusual features of the MUTYH glycosylase and implications in cancer

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Abstract

MUTYH is a base-excision repair glycosylase that removes adenine opposite 8-oxoguanine (OG). Variants of MUTYH defective in functional activity lead to MUTYH-associated polyposis (MAP), which progresses to cancer with very high penetrance. Whole genome and whole exome sequencing studies have found MUTYH deficiencies in an increasing number of cancer types. While the canonical OG A repair activity of MUTYH is well characterized and similar to bacterial MutY, here we review more recent evidence that MUTYH has activities independent of OG:A repair and appear centered on the interdomain connector (IDC) region of MUTYH. We summarize evidence that MUTYH is involved in rapid DNA damage response (DDR) signaling, including PARP activation, 9-1-1 and ATR signaling, and SIRT6 activity. MUTYH alters survival and DDR to a wide variety of DNA damaging agents in a time course that is not consistent with the formation of OG:A mispairs. Studies that suggest MUTYH inhibits the repair of alkyl-DNA damage and cyclopyrimidine dimers (CPDs) is reviewed, and evidence of a synthetic lethal interaction with mismatch repair (MMR) is summarized. Based on these studies we suggest that MUTYH has evolved from an OG:A mispair glycosylase to a multifunctional scaffold for DNA damage response signaling.

Graphical Abstract

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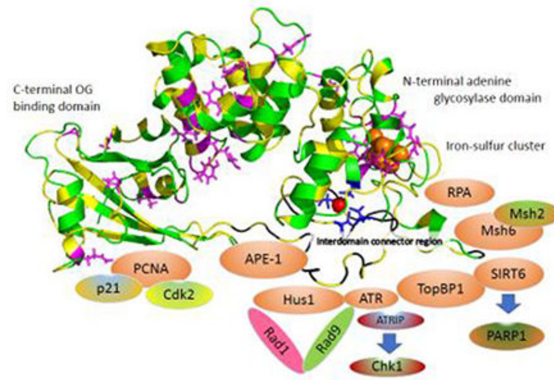

Keywords

MUTYH; MutY; base excision repair; DNA damage response; glycosylase; oxidative DNA damage

1. Introduction

The integrity of our DNA is constantly being challenged by the deleterious effects of oxidation. Reactive oxygen species (ROS) are generated by endogenous factors such as cellular respiration and inflammatory responses, as well as by exogenous factors such as exposure to chemicals, ionizing radiation and ultraviolet light [1]. Human cells have evolved a myriad of DNA repair enzymes and pathways to repair this damage, including numerous base excision repair (BER) glycosylases [2]. The low redox potential of guanine makes it particularly susceptible to oxidation [3], and a common form of ROS damage is 8-oxo-7,8-dihydroguanine (OG). Due to the ability of OG to mimic T when it adopts the *syn* conformation, OG is highly mutagenic during DNA replication, leading to the incorporation of adenine in the newly synthesized strand, instead of cytosine, forming OG:A mispairs [4], as shown in Figure 1. Further replication of the A template strand [5] or aberrant removal of OG opposite A by the OG DNA glycosylase 1 (OGG1) [6] results in formation on an incorrect T:A bp. Cells have evolved the BER glycosylase MUTYH to recognize the pre-mutagenic OG:A mispairs and remove the undamaged adenine opposite OG. Adenine excision is catalyzed by a proposed double-displacement mechanism based on structural and experimental evidence [7]. The resulting AP site product is then a substrate for the human AP endonuclease APE1, which interacts with MUTYH [8–10]. The specialized repair polymerase lambda (POLΛ) is able to insert the proper cytosine base, enabling full repair to the original G:C base pair via the long-patch base excision repair pathway [11]. Inheritance of MUTYH variants with altered activity leads to an accumulation of G:C to T:A transversion mutations in somatic tissue over time, altering the function of key oncogenes and tumor suppressors, leading to cancer [12–16].

Here we selectively explore new and unusual MUTYH results and ponder some unanswered questions regarding MUTYH relevant to cancer. Readers can refer to previous reviews and references therein for a more general overview [17, 18]. We propose that earlier data together with recent studies have created an intricate portrait of MUTYH involvement in

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DNA damage response signaling, not just to oxidative DNA damage, but to many forms of damage. These data no longer easily fit the model of MUTYH as merely an adenine glycosylase. While removal of undamaged adenine bases may seem strange, many experimental findings point to an even more cryptic involvement of MUTYH in DNA damage response. We summarize data that is emerging on MUTYH and present several hypotheses that may tie these disparate findings together in the hope this may stimulate and guide further research. Given the role of MUTYH in cancer progression, further work to understand MUTYH will lead to improvements in our ability to predict, diagnose and treat cancer.

2. The Role of MUTYH in Cancer

The slow accumulation of somatic DNA mutations in cells is a widely-accepted model of cancer development [19]. The critical role of MUTYH in preventing OG-induced G:C to T:A transversions is highlighted by the association of germline mutations in the MUTYH gene with colorectal adenomas and carcinomas [20]. This was discovered in the analysis of siblings with multiple adenomatous polyps in the colon, which can be due to germline mutations in the *APC* gene and leads to Familial Adenomatous Polyposis (FAP) [20]. Despite a pattern of inheritance that suggested a germline mutation, the germline *APC* gene was normal in these siblings. However, DNA from their adenomas were found to have an unusually high level of G:C to T:A mutations in *APC*, suggesting that germline DNA repair enzymes involved in oxidative DNA damage repair were involved. Sequencing of BER genes found biallelic MUTYH protein missense mutations, either Tyr179Cys and Gly396Asp, which were not biallelic in control samples or in unaffected family members. Work in our laboratory found that these missense variants in the highly homologous *E. coli* MutY protein lead to compromised adenine excision activity, evidence that they underlie the abundance of G:C to T:A mutations in *APC* and thus likely initiated adenoma development in these individuals [20–22]. Further clinical evidence has accumulated to support this connection [23, 24], and this is now termed MUTYH-associated polyposis (MAP). Crystal structures of the bacterial homologs have illuminated the structural consequences of these two common MAP variants, as shown in Figure 2. The mutated tyrosine equivalent to the human Y179C variant intercalates 5' of the OG of the OG:A mismatch to disrupt base-stacking interaction, facilitate adenine "base flipping" into the MUTYH active site and stabilize MUTYH on the mismatch [22, 25, 26]. The mutated glycine equivalent to the human G396D is located in a tight turn region of the OG recognition domain that interacts with the DNA backbone near the OG nucleotide phosphodiester, and this region is destabilized by mutation to any residue larger than Gly [22, 25].

Since the discovery of MAP the number of known germline pathogenic variants has grown, as shown in Figures 3 and 4. The Leiden Open Variation Database (LOVD; https://www.lovd.nl) is a repository for reported germline MUTYH variants and their association with disease [27, 28]. Based upon this curated sample, there are 27 initially reported as pathogenic protein missense variants (at 21 locations), of which 17 (in 14 locations) are considered confirmed upon independent review by the LOVD curators. Additionally, 33 single nucleotide changes that lead to truncated proteins have been observed in the combined LOVD [28] and the ClinVar [29] databases, as well as 21 frameshift and 8 splice site

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mutations confirmed as pathogenic in LOVD [28]. Interestingly, truncating mutations in the distal extended C-terminal region not homologous to the bacterial MutY appear to have clinical associations to MAP. This is supported by the presence of the suspected pathogenic missense variants V493F and P534Q, the later which has reduced binding affinity to PCNA [30], but wild-type mutation-suppression activity when expressed in bacterial cells [31]. Taken together, this is evidence that MUTYH interactions with PCNA are important to MUTYH function in human cells.

The Broad Institute has made available a pooled whole genome and whole exome sequence database in clinical and control populations from over 131,000 individuals (http://gnomad.broadinstitute.org/about; Ref. [32]). The mapping and abundance of 261 MUTYH protein missense variants from this population is shown in Figure 3 (top) and are mapped to the MUTYH structural model in Figure 4, highlighted in yellow. Additionally, there were 133 individual protein truncations observed in 28 positions. Interestingly, 95 of 133 were in 5 'hotspot' locations Y104X, W156X, Q338X, Q414X, and E480X. There were also 81 predicted splice site variants. The two most common cancer-associated variants Y179C and G396D are also two of the most common missense variants in this sample, with allele frequencies of 0.15% and 0.30%, respectively. These two MUTYH variants are found in individuals of European origin, likely due to a genetic population founder effect [33].

Interestingly, there is a correlation between MAP clinical age of onset and disease severity and MUTYH *in vitro* activity on a defined OG:A substrate for Y179C and G396D [9, 21, 22, 34–36], with the higher adenine glycosylase activity of G396D associated with a later age of onset and less severe phenotype [34, 35], which is evidence that subtle structural differences in MUTYH variants are able to alter lifetime cancer risk. Thus, the simple categorization of MUTYH missense variants as pathogenic or benign is misleading. Careful analysis of the mammalian enzyme suggest that in addition to subtle differences in adenine excision activity [9, 30, 36–38], MAP variants alter protein purification yield [9, 30, 39], DNA binding affinity [9], and protein-protein interactions [9, 10, 36, 40, 41]. Additionally, MUTYH is post-translationally modified [42–44] as well as transcriptionally regulated [45, 46]. Careful *in vitro* characterization including correction for active fraction is able to tease apart the intrinsic reduction in the enzyme activity caused by deleterious amino acid changes [30, 47, 48]. Protein purification and active site concentration determination of MUTYH also provides hints to origin of dysfunction of suspected MAP variants, as the amount of active enzyme relative to total protein is often less than wild-type, evidence of altered protein folding that reduces the ability to effectively engage on the DNA substrate to elicit base excision. Repair activity of MAP variants has also been assessed using bacterial mutation suppression assays of human MAP variants expressed in *E. coli* [31]. Our mammalian cell assay using a synthetic OG:A lesion which activates GFP expression upon repair has revealed interesting differences between *in vitro* and cellular results [36]. While clinical evidence supports the pathogenic potential of the G396D variant, previous *in vitro* measurements of binding and the rate of adenine excision found surprisingly subtle differences versus the wild-type protein [9, 20–22], However, the GFP assay found similar levels of repair in cells expressing G396D and Y179D, suggesting this cellular assay provides a more accurate cancer risk assessment. Interestingly, the Q338H polymorphism in the IDC region of MUTYH also displayed significantly reduced OG:A lesion repair [36].

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Although clinical associations between the Q338H polymorphism and cancer risk has been reported with smaller samples in Japanese populations [49, 50], the association with cancer is not statistically significant when averaged across very large samples [51], thus one interpretation is that this cellular assay can detect reductions in OG:A repair that are so subtle that they do not lead to increased cancer risk. However, there is good evidence that Q338H alters protein-protein interactions with 9-1-1 DNA damage response complex proteins [30, 52], and together with other genetic and environmental factors, Q338H may alter cancer risk for specific individuals.

3. Whole genome studies confirm OG:A repair is central to the role of MUTYH in cancer

Five recent genome-wide sequencing studies firmly establish G:C to T:A mutations as central to the signature of MUTYH-associated polyposis [12–16]. This strongly supports the long-held view that MUTYH acts to suppress tumorigenesis by initiating base-excision repair of OG:A mispairs. We find these studies reassuring given the diversity of MUTYH interactions and studies that implicate MUTYH in other forms of DNA damage, as will be covered later. For example, significantly more somatic mutations are present in early-stage MAP adenomas, which confirms that the MAP mutator phenotype is present early in disease [12].

Notably, two genome-wide studies of pancreatic neuroendocrine [13] and adrenocortical [14] cancers (neither previously associated with MUTYH deficiency) found that tumors with a mutational spectrum dominated by G:C to T:A mutations were from heterozygous carriers of MAP variants, and further analysis confirmed a somatic loss of heterozygosity at the MUTYH gene locus. This pattern was found in nine of 98 sporadic pancreatic neuroendocrine tumors and in four of 136 adrenocortical carcinomas analyzed. These studies have focused on cancer types not previously associated with MAP and are based on relatively small sample sizes, leaving open the possibility that these results may not represent larger populations. It may be that the contribution of monoallelic MUTYH status is more phenotypically diverse than previously recognized and more likely in older individuals, and thus has escaped clinical notice. Despite estimates that heterozygous carriers of confirmed MAP variants have only a slight increase in CRC risk (see [23, 53] and references therein), family history of CRC appears to enhance this risk significantly. For example, in a study of over 2300 MAP monoallelic carriers, lifetime risk of CRC was estimated as 5–7%, but was 10–12% amongst those with a first-degree relative diagnosed with CRC by age 50 [54]. Others have pointed out that the basis of these estimates often relies on using the founder mutations Y179C and G396D as the first criteria for full MUTYH sequencing, and so may be an underestimate [55].

The study of cancer genomes has distilled the complex landscape of mutations into a simplified model of mutational signatures [56], which puts the spotlight on DNA repair pathways as a critical cellular defense against cancer development. Surprisingly, in addition to the primary G:C to T:A signature previously associated with MAP and MUTYH [57] there is a significant secondary G:C to A:T signature in MAP tumors in two of these studies

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[12, 15]. One proposed etiology of this pattern is mutations initiated by the spontaneous deamination of 5-methylcytosine to thymine [58]; however, there is no evidence that MUTYH is involved in the repair of T:G mismatches. An alternative explanation would be that inactivating G:C to T:A mutations in other DNA repair pathways could result in a secondary pattern of mutations not directly caused by MUTYH protein activity. In any case, mutational signatures have revealed G:C to A:T mutations as an interesting trend in MAP tumors and there would be considerable value in identifying the underlying origin of this mutational signature.

4. MUTYH response to oxidative damage occurs before widespread OG:A mispair formation

The canonical activity of MUTYH begins upon the creation of the OG:A mispair substrate after one round of cellular replication. This requires the existence of OG lesions that are not repaired before DNA replication, causing replicative polymerases to insert A in the newly synthesized strand opposite OG [4]. In mammalian cell culture, cells typically require approximately 24 hours to replicate, so that in unsynchronized cells, approximately 1/24th of the cells treated will have undergone replication after one hour. Seemingly contradicting this is evidence that *Mutyh*–/– mouse embryonic fibroblasts (MEFs) and *Mutyh*–/– MEFs expressing MAP variants have significantly increased cellular OG levels at 30 minutes, 1 hour and 2 hours after oxidative damage treatment versus wild-type controls [59, 60]. Although MUTYH has no known OG lesion removal activity on OG:C bps, the binding affinity of the mouse MUTYH on OG:C bps is similar to that of OG:A, which is not true of the *E. coli* MutY [61]. *In vitro* experiments of the mouse homolog of the common G396D MAP variant shows only a slight deficiency in adenine excision activity [9], but this variant has a reduced ability to compete with OGG1 for binding to OG opposite an AP site [6]. MAP variants raise cellular OG levels significantly above that of the *Mutyh*–/– control MEFs [60], consistent with the idea that structurally aberrant MUTYH can act as an inhibitor of OG:C repair. These data suggest the possibility of a regulatory function of MUTYH in OG:C lesion processing or that MUTYH enhances DNA damage response signaling such that OG repair is enhanced.

Rapid MUTYH activation of poly-ADP ribose (PAR) has also been observed [62]. In mammalian cells one hour after treatment with the oxidative damaging agent menadione, MUTYH siRNA knockdown dramatically reduces both PAR accumulation and nuclear translocation of apoptosis inducing factor (AIF), signals of activated DNA damage response [62]. PAR formation under normal conditions leads to chromatin relaxation and recruitment of DNA repair cofactors, but is also a major determinant of apoptotic and necrotic cell death in response to overwhelming DNA damage [63]. More recent research has shown that PARP-1 not only binds singlestrand DNA breaks, but is intimately involved in AP site processing during base excision repair [64, 65]. MUTYH has high affinity for AP site analogs [61], and the binding of MAP variants is more severely inhibited by APE1 competition versus wild-type MUTYH [9]. Thus MUTYH appears to have a role in PAR activation before the significant formation of OG:A mispairs, which possibly could mediate

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involvement in the OG:C repair enhancement observed [59, 60, 66]. Interactions between MUTYH and SIRT6 [67] may be relevant to this, as discussed in Section 4 below.

At least four additional studies support the role of MUTYH in rapid DNA damage response. Reduced Chk1 phosphorylation in MUTYH knockdown cells is seen at 30 minute and 1 hour time points after treatment with either UV radiation [68] and hydroxyurea [68, 69], and at 1 hour after hydrogen peroxide treatment [70]. Both Chk1 protein expression and Chk1 phosphorylation are enhanced by MUTYH overexpression in HeLa cells treated with hydrogen peroxide [70], but the opposite trend is seen in MEFs treated with UVA radiation and 6-thioguanine; this experiment found increased Chk1 phosphorylation in *Mutyh*–/– MEFs versus WT controls [71]. Phosphorylation of Chk1 and H2AX in response to oxidative DNA damage occurs via TopBP1-dependent ATR kinases [72]. It is proposed that recruitment of TopBP1 to DNA damage by MUTYH within one hour of hydroxyurea treatment is essential for Chk1 phosphorylation and checkpoint activation via ATR [69].

An alternate explanation for the rapid onset of MUTYH-mediated cytotoxicity is that oxidized guanine triphosphates in the nucleotide pool during replication leads to OG insertion opposite adenine in the template strand, and adenine excision by MUTYH leads to cytotoxic singe-strand breaks [73]. However, it is thought that replication-associated mismatch repair pathway is the primary pathway for removal of incorporated oxidized dGTPs [74]. In mismatch repair deficient cells, knockdown of MutT homolog MTH1 (which removes oxidized dGTPs) does not lead MUTYH to induce mutagenic and cytotoxic strand breaks in the template strand [73]; indeed, higher MUTYH expression together with MTH1 knockdown was associated with enhanced survival [73]. Additionally, recent whole genome/ whole exome mutational signatures of MAP tumors do not show significant T:A to G:C mutations, which would be the consequence of this activity [15]. Taken together, it does not appear that oxidation of the nucleotide pool can explain involvement of MUTYH in rapid DNA damage response signaling.

A simplified model of MUTYH as an executioner of cell death under oxidative DNA damage is consistent with the above studies and other experimental observations [45, 75], however the molecular mechanism for this activity is still murky. OGG1 directly recognizes and initiates base repair on OG lesions with high efficiency [76], and is bifunctional, with lyase activity that leads to a single strand break intermediate. MUTYH, on the other hand, is a monofunctional glycosylase which has no direct strand scission activity, and binds the secondary OG:A mispairs; thus one would expect OGG1, not MUTYH, to be involved in rapid DDR signaling to oxidative DNA damage. Although one could argue that in asynchronously growing cell culture there will always exist cells undergoing replication, and thus OG:A mispairing could occur immediately, this does not seem consistent with magnitude of the increases in PAR activation [62] and cellular OG levels [60]. Thus the studies highlighted here support a role for MUTYH in DNA damage response to oxidative damage, but because of the rapid timing, there is an open question as to whether adenine excision or OG:A mispair recognition is a key component of this role.

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5. MUTYH interdomain connector (IDC) region is a scaffold for DNA Damage Response (DDR)

MUTYH is the human homolog of MutY, which was originally discovered in *E. coli*, and the core adenine excision and OG recognition domains are highly conserved from bacteria to mammals [17, 25]. Almost all MutY enzymes, with the exception of a few bacterial lineages [77], also contain a $[4Fe–4S]^{2+}$ (Fe-S) cofactor and its associated Fe-S cluster loop (FCL) motif that have been shown to be required for repair activity, and implicated in aiding in lesion location [17, 78]. The striking difference between bacterial MutYs and the MutY homologs in higher eukaryotes is the evolution of a new extended structural motif directly between the catalytic N-terminal domain and the C-terminal OG-recognition domain. This interdomain connector (IDC) region in humans is 40 amino acids longer than the *E. coli* MutY IDC region and contains 3 highly conserved cysteines (Cys332-X_6-Cys339-X_2-Cys342), which suggests it may be a second metal binding site, and indeed appears to coordinate a Zn^{2+} ion [79]. ICP-MS metal analysis of mouse MUTYH protein revealed a Zn^{2+} ion in addition to the known four Fe atoms of the N-terminal Fe-S cluster, and mutagenesis of the Cys- X_2-Cys residues in the IDC significantly reduced Zn and Fe levels, the ability to suppress DNA mutations and active fraction of protein [79, 80]. EXAFS analysis suggests that the Zn^{2+} ion is most likely coordinated to four cysteines [79]. Sequence conservation along with computational modeling of human MUTYH suggested that Cys 244, adjacent to the adenine binding pocket and $[4Fe–4S]^{2+}$ cluster in the N-terminal domain, is a strong candidate for the fourth Cys ligand [80]. Mutagenesis of Cys 244, along with Cys 332, reduced the amounts of Zn^{2+}, and altered activity directly proportional to the amount of protein containing Zinc. This has led us to refer to the Zn^{2+} ion coordinated within the IDC as a “Zinc Linchpin” motif to emphasize its role in coordinating engagement of the two functional domains on the OG:A mismatch to initiate adenine excision. Indeed, predicted folding of the IDC region and interaction of the N-terminal Cys to coordinate the Zn^{2+} ion places the Zinc linchpin near the $[4Fe–4S]^{2+}$ and its associated FCL, which are integral to DNA damage recognition (Figure 5). The model containing the fully coordinated Zn^{2+} ion (Figure 5) is consistent with biochemical results with the purified Q338H variant, a common polymorphism in the IDC region. Adenine excision is altered for this variant in a salt-dependent manner, evidence that the IDC domain interacts with the N-terminal adenine excision domain [36, 52]. A large number of MAP variants are localized near the Zn-linchpin and the $[4Fe–4S]^{2+}$ cluster, further underscoring the critical functions of these two cofactors.

An overview of MUTYH protein-protein interactions is shown in Figure 5. Hus1 of the 9-1-1 complex appears to use the IDC region of MUTYH as a scaffold in a manner that does not exclude the binding of APE1 or SIRT6 [41, 67, 81]. MUTYH interactions with 9-1-1 play a critical role in cellular survival to mitomycin C, which induces both interstrand DNA crosslinks and ROS [82]. Interestingly, APE1 strand scission activity is stimulated by both MUTYH [10] and 9-1-1 [83], and both APE1 and Hus1 bind to the MUTYH IDC region [8, 10], thus one speculative hypothesis is that the enhanced strand scission activity attributed to MUTYH [75] is due to APE1 activity enhancement.

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MUTYH interacts with SIRT6 in the IDC region, and this interaction is stabilized by Hus1 [67]. SIRT6 was shown to be an important factor in the cellular repair of oxidative DNA damage using a plasmid host cell reactivation assay [84]. SIRT6 knockout MEFs have a 40% reduction in oxidative DNA damage repair and SIRT6 overexpression leads to two-fold increase in this repair [84]. Intriguingly, SIRT6 stimulates PARP-1 activity by mono-ADP-ribosylation at lysine residue 521 [85], and PARP-1 knockdown abolishes the pro-repair effect of SIRT6 [84], suggesting that the enhanced oxidative DNA damage repair due to SIRT6 is fully dependent on its role in PARP-1 stimulation. Thus, MUTYH enhancement of PAR accumulation [62, 86] could be due to MUTYH interactions with SIRT6. PARP-1 is thought to enhance BER via binding to AP sites and single-strand breaks (which can be the products of BER glycosylases and APE1 processing of base damage), in particular the 5'-deoxyribose phosphate (5'-dRP) intermediate [87]. Both SIRT6 and PARP-1 also enhance DNA repair via chromatin remodeling ([88] and [63], respectively). If the pro-survival effects of both PARP-1 and SIRT6 to oxidative DNA damage are enhanced by the presence of MUTYH as a scaffold, it follows that PARP-1 overactivation, which leads to both necrosis and apoptosis [62, 63], could be altered by the structural conformation of the MUTYH IDC region. From this model, we would expect wild-type MUTYH expression to enhance PARP-1 activation to oxidative base damage, which is consistent with previous studies [62, 86]. Cancer-associated MAP variants in the IDC region could be defective in this scaffolding function.

A recent clinical study found that MAP adenomas have higher levels of LINE1 hypomethylation than sporadic and FAP colorectal cancer controls [89]. LINE1 hypomethylation status in tumor tissue is considered a surrogate clinical biomarker for global genomic hypomethylation and is associated with chromosomal instability and poor prognosis in cancer [90]. Recent research has isolated SIRT6 as a critical factor in repression of LINE1 hypomethylation and maintaining normal DNA methylation status [85], and both MAP and LINE1 hypomethylation are associated with a microsatellite stable, chromosomally unstable and mismatch repair proficient cancer phenotype [91, 92]. Additionally, chromosomal aberrations are also seen in both *Mutyh*–/– [93], and *Sirt6*–/– [94] MEFs. Given that MUTYH interacts with SIRT6 [67] and SIRT6 appears to have a significant role in maintaining chromosomal stability, one mechanistic explanation that ties these observations together is that full SIRT6 activity, which represses hypomethylation and chromosomal instability, depends upon interactions with wild-type MUTYH, which is altered in individuals with inherited MAP variants.

The OG:A mispair represents a unique cellular signal. It signifies a critical failure of cells to repair DNA correctly before replication. Accordingly, the evolution of MUTYH from a DNA damage sensor to a scaffold to amplify DNA damage response signaling seems reasonable. Whether the above MUTYH IDC protein-protein interactions are altered by the binding of MUTYH to OG:A or OG:C lesions is a central question. Substrate binding by MUTYH may structurally alter the scaffold properties of the IDC region to modulate the activities of Hus1, APE1, SIRT6 and downstream PARP-1 activation. One can imagine that MUTYH binding to OG:A or OG:C leads to enhanced DNA damage response, tipping DDR towards overactivation and cell death under DNA damage treatment. Although purely

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speculation, such models will hopefully inform the design of future experiments that will help determine the precise role of MUTYH in DNA damage response.

6. The microsatellite stability of MAP tumors: a hint toward therapeutic approaches?

Microsatellite unstable (MSI) tumors are attributed to the loss of functional mismatch repair (MMR) which leads to a hypermutator phenotype. In addition to the inherited Lynch syndrome, which is approximately 3% of colorectal cancer cases, MSI occurs in 12% of sporadic colorectal cancers, typically due to epigenetic silencing (hypermethylation) of the MLH1 promoter [95]. The high level of somatic mutations in MSI tumors leads higher production of immunogenic neoantigens, and PD-1 immune checkpoint inhibitors have been shown to be highly effective against some MSI cancers [96]. Given that MAP tumors also display high levels of tumor infiltrating lymphocytes [97], which is a sign of an active immune response, it would follow that CRC due to MAP may also be responsive to PD-1 inhibitors [98]. If true, screening for MAP status in cancer that are not MSI may help determine if PD-1 immunotherapy is a reasonable treatment option. However, many individuals with MSI cancers do not respond to PD-1 immunotherapy [96], thus therapies that address this subtype are still needed.

Despite the immunogenic similarities between MAP and MSI tumors, an interesting contradiction is that MAP tumors are overwhelmingly microsatellite stable (74 of 77 in a recent meta-analysis [23]), suggesting that carcinogenesis in MAP depends on functional mismatch repair. This leads to the speculative hypothesis of a synthetic lethal interaction between mismatch repair and MUTYH [99], which appears reasonable given that they both repair replication-associated oxidative DNA damage and interact with each other at the molecular level [100].

Are MUTYH deficient tumor cells dependent on MMR functionality? Unusual results in *Msh2*–/– mice support this hypothesis. Double knockout *Msh2*–/– *Mutyh*–/– mice have an 87% *increased* median lifespan versus *Msh2*–/– mice (262 days versus 140 days) due to reduced tumor burden [59]. Thus, in the mismatch repair deficient context, loss of MUTYH *reduces* cancer progression (or conversely, MUTYH enhances cancer progression). One explanation is that normal cells with dual *Mutyh/Msh2* deficiency can survive, but cancer proliferation is inhibited in cells lacking both MMR and MUTYH. This result is not easily explained by the overlapping roles of MUTYH and MMR in the repair of oxidative DNA damage, as steady state cellular levels of OG in various tissues of *Msh2*–/– mice were similar with and without MUTYH, and the contribution of MUTYH loss to the overall cellular mutation rate is relatively small [59].

Strikingly, a human case study strongly parallels this mouse study, where biallelic germline MUTYH variants appeared to reduce the onset and severity of combined MUTYH/MMR deficiency [101]. In two siblings carrying the same MAP genotype (Y179C/G396D), the individual heterozygous for a pathogenic MSH6 allele had a significantly attenuated phenotype relative to the sibling who was wild-type for MSH6. Importantly, adenomas in the attenuated case lacked a loss of MSH6 heterozygosity, and were positive for MSH6

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expression and microsatellite stable. Thus, loss of MUTYH function with MMR heterozygosity led to a presentation consistent with a mild MAP phenotype, but with an apparent suppression of the genetic and phenotypic presentation of Lynch syndrome seen in this family. This is human genetic evidence that MUTYH deficiency together with somatic loss of MMR activity inhibits cancer progression. Although Lynch syndrome progression is not significantly altered by MUTYH heterozygosity [102], MUTYH heterozygotes alone have a mild two-fold increase in cancer risk [103]. This certainly leaves open the possibility that a more complete inhibition of MUTYH function may alter the progression of MSI-high cancer, and conversely, cancer progression in MAP patients could be delayed by MMR inhibition.

Further evidence of the functional dependency between MMR and MUTYH has also been seen in cell culture experiments. Consistent with this synthetic lethal hypothesis, T-cell acute lymphoblastic leukemia cell lines that the lack functional MMR depend on MUTYH for survival, especially when combined with MTH1 knockdown [73]. In a separate study, reduced survival of colorectal cancer cell lines caused by either deficiencies in MLH1 and polymerase gamma, or MSH2 and polymerase beta are dramatically enhanced by MUTYH knockdown [104], suggesting MUTYH acts as a key factor to induce cell death when other DNA repair pathways fail. Although superficially these studies contradict each other, they both point to MUTYH as a key factor that decides the fate of cells when MMR is lost.

Since dual germline MUTYH and MMR mutations are rare [102, 105, 106], a related question is whether the frequency of somatic loss (as opposed to germline deficiency) of both MUTYH and MMR in sporadic cancer tissues is lower than would be expected by random chance, which would suggest a functional dependency. This data may already exist in databases of cancer somatic variants, and deserves a closer look using bioinformatic methods. There is a recent report of MUTYH inhibition by the clinically approved anti-diabetes drug acetohexamide and structural analogs to it [107]; if this is indeed true, laboratory testing of MUTYH/MMR synthetic lethality would be greatly expedited.

7. MUTYH is involved in the cellular response to a wide variety of DNA damage

In addition to oxidative DNA damaging agents, MUTYH is implicated in cellular response to a variety of different DNA damage treatments, including alkylating agents [108, 109], DNA crosslinking agents [110], UV radiation [68, 107], hydroxyurea [68, 109] and mitomycin C [82]. One confounding factor is that all of these treatments are known to induce reactive oxygen species (ROS; refs. [111–115], respectively), thus they do not clearly implicate MUTYH in a novel mechanism of action. Here we highlight three studies that represent the most compelling examples of MUTYH activity that appear independent of oxidative DNA damage.

In an unbiased whole genome expression study of immortalized lymphocytes from healthy subjects, Fry, *et al.* [108] investigated differences in gene expression associated with survival to the alkylating agent N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) in an effort to understand the factors underlying response to chemotherapeutic agents. Surprisingly, they

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found that high basal MUTYH expression was more strongly associated with higher cell death to MNNG versus genes known to mediate alkylating agent survival, such as the mismatch repair enzymes MSH2 and MSH6, and the DNA methyltransferase MGMT. This association was experimentally tested in mammalian cells using siRNA knockdown in human cells and in the *Mutyh*–/– MEFs, confirming that reduced MUTYH expression leads to enhanced survival to MNNG. Importantly, the concentrations of MNNG used by *Fry*, *et al.* were seventy-five fold lower than concentrations shown to induce ROS [111]. One hypothesis is that MUTYH could be acting as a glycosylase for MNNG-induced methyl adducts. Interestingly, there is a recent report that MutY deletion in *Corynebacterium glutamicum* reduces MNNG survival to a similar level as loss of the primary alkyl-DNA glycosylase [116], new evidence that the interaction of MUTYH to alkyl-DNA damage survival is conserved. MUTYH expression levels in lymphocytes of normal individuals is highly variable [108], and it would be interesting to see if these levels alter the clinical response to alkylating agent chemotherapy.

A more recent study of the effect of MUTYH on 6-thioguanine and UVA radiation-induced DNA damage touches many themes in this review [71]. Reduced DNA damage response (phosphorylation of Chk1) in *Mutyh*–/– cells was seen within 30 minutes of DNA damage treatment (suggesting OG:A mispair formation does not mediate this signaling). Loss of MUTYH was associated with increased cellular OG levels but enhanced survival to some other form of DNA damage, possibly an oxidized form of 6-thioguanine [117]. Further experiments found that the presence of either wild-type or glycosylase-impaired cancer variants equally stimulated this cytotoxic response. Enhanced cell survival in response to the loss of the mismatch repair protein MSH2 and MUTYH was not additive, suggesting an overlap in functional roles. Loss of MUTYH was associated with reduced cell cycle arrest and reduced levels of chromosomal damage (as measured by micronucleus formation), evidence that MUTYH is an active factor in either inducing toxic DNA lesions or preventing their efficient repair.

More recently Mazouzi, *et al.* implicate MUTYH as a cellular factor that inhibits the repair of UVC-induced cyclobutane pyrimidine dimers (CPDs) [107]. Initial screening of small molecules that enhance the survival of XPA-deficient cells to UV radiation found acetohexamide, which is a clinically approved treatment for type 2 diabetes mellitus. CRISPR knockout of MUTYH functionally mimics the effect of acetohexamide without an additive effect, and acetohexamide appears to downregulate MUTYH in a proteasomal-dependent manner [107]. Importantly, XPA/MUTYH dual deficiency is required to see these effects, and MUTYH deficiency alone has no effect on UV survival. Although UV radiation does lead to oxidative DNA damage [113], the authors directly measure significantly reduced CPD immunostaining with dual loss of XPA and MUTYH versus loss of XPA alone. Computational modeling of MUTYH interactions with a CPD lesion are interpreted as supporting a direct interaction (hypothetically one that would inhibit proper CPD repair rather than enhance it), but *in vitro* measurements of MUTYH binding or glycosylase activity on CPDs are not presented. A recent study found that *E. coli* MutY has no activity on CPD and other UV-induced products or effect upon UV induced mutagenesis [118], but this does not rule out non-productive binding of CPDs by MUTYH.

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Clustered DNA damage formed by UV and other forms of radiation are resolved by NER, BER, and doublestrand break repair pathways in the same region of DNA [119, 120], thus the fact that MUTYH inhibition enhances CPD repair does not necessarily mean MUTYH interacts directly with CPDs. APE1, the downstream partner of MUTYH, inhibits the removal of DNA cisplatin crosslinks by NER [121], and PARP-1 is directly involved with UV lesion repair in NER [122], evidence that the overlapping roles of BER and NER is not unique to MUTYH. Given that MUTYH alters the activity of APE1 [10], and has a role in PAR formation in response to DNA damage [62], these interactions could have a role in MUTYH involvement with CPD repair. There is ample evidence of NER involvement in oxidative DNA damage repair [123], and XPA in particular has been shown to have a significant role [124].

Interestingly, acetohexamide was previously identified as a top hit in a high-throughput chemical screen of clinically-approved compounds that enhance cellular oxidative damage repair in BRCA1-deficient, but not in BRCA1-proficient, breast cancer cell lines [125]. If acetohexamide truly does downregulate MUTYH [107], then enhanced OG repair would be associated with loss of MUTYH in BRCA1-deficient cells, an apparent contradiction, since WT MUTYH has been shown to reduce cellular OG levels [60]. Clearly further work is needed to fully resolve the role of MUTYH in CPD repair.

8. Conclusions

The evidence presented here suggests that the evolution of MUTYH represents a balancing act in cancer avoidance. Structurally and functionally, MUTYH is unique among BER glycosylases in a number of key aspects. It recognizes an extremely rare mispair, and then removes an undamaged DNA base—consider the accuracy required for such an enzyme! The absolute requirement of having an OG opposite the excised base has led to an enzyme with two structurally independent but integrated components, an N-terminal domain which excises adenine, and a C-terminal domain that recognizes OG. MUTYH remains tightly bound to the AP site product formed by adenine excision to prevent OG excision by OGG1 glycosylase in this context [6] which could potentially cause a double-strand break (DSB). This would suggest an incredibly strong evolutionary selection has occurred to promote tight binding to AP site products [61].

Surprisingly, numerous bacterial species, as well as fungi, insects, and single-celled eukaryotes apparently have lost the MutY homolog gene in the course of evolution [77, 126]. With new evidence that MUTYH activity apparently reduces alkyl-DNA damage survival [108] and nucleotide excision repair efficiency [107], we speculate that MUTYH interference with other DNA repair pathways may underlie the loss of MutY homologs in simpler organisms, but in vertebrate animals, the role of MUTYH in cancer avoidance makes it indispensable. One can imagine that an enzyme that has evolved a critical function may acquire off-target effects but if the benefit of the enzyme enhances the long-term evolutionary fitness of the organism, such effects will be tolerated. The molecular details of MUTYH interaction with other DNA repair pathways are currently under investigation.

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The dizzying diversity of MUTYH interactions to DNA damage response proteins implicate it in a number of DDR pathways, and the IDC region of MUTYH in higher eukaryotes appears to be a structural scaffold for many of these interactions. The OG:A mispair is a distinct molecular signature of a cell that has failed to properly repair oxidative DNA damage before DNA replication. These features provide a logical rationale for evolution of MUTYH from a base excision repair glycosylase to a scaffold that triggers enhanced DNA damage response. The evolutionary timing of the appearance of the extended IDC in higher eukaryotes mirrors the need for cancer avoidance in complex organisms, consistent with this role.

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Abbreviations:

MUTYH	MutY homolog
OG	8-oxoguanine
OGG1	OG glycosylase 1
bp	base pair
BER	base excision repair
ROS	reactive oxygen species
MMR	mismatch repair
DDR	DNA damage response
CPD	cyclopyrimidine dimer
IDC	interdomain connector
SSB	single-strand break
AP	apurinic/apyrimidinic
EXAFS	extended X-ray absorption fine structure
ICP-MS	inductively coupled plasma mass spectrometry
FCL	Fe-S cluster loop

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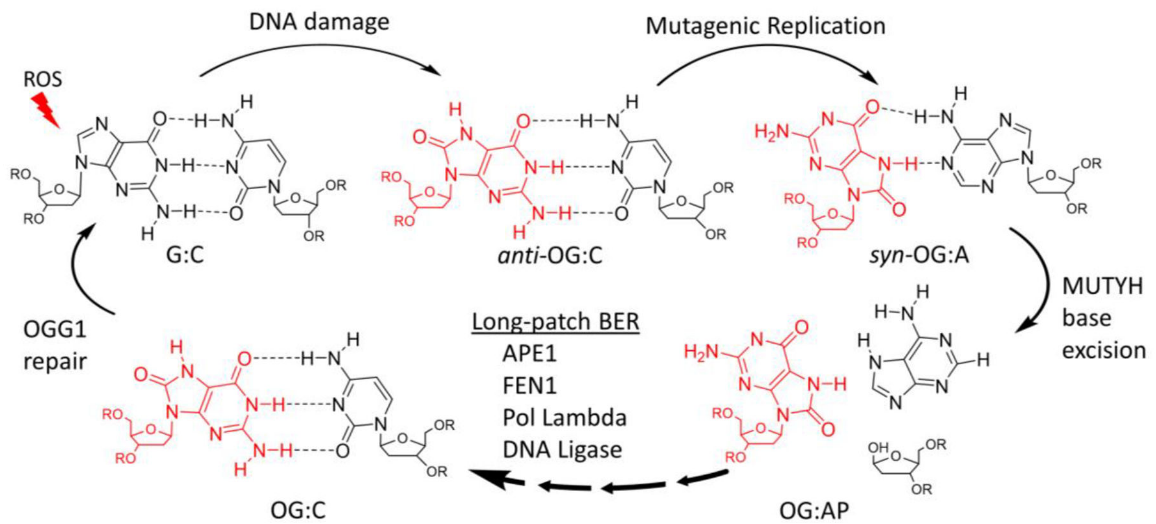

Figure 1.

MUTYH-mediated base excision in response to adenine misincorporation opposite 8-oxoguanine by replication polymerases. Downstream BER enzymes complete repair of A to C, allowing OG:C repair to G:C by the OG glycosylase OGG1. [R] represents the rest of the DNA molecule.

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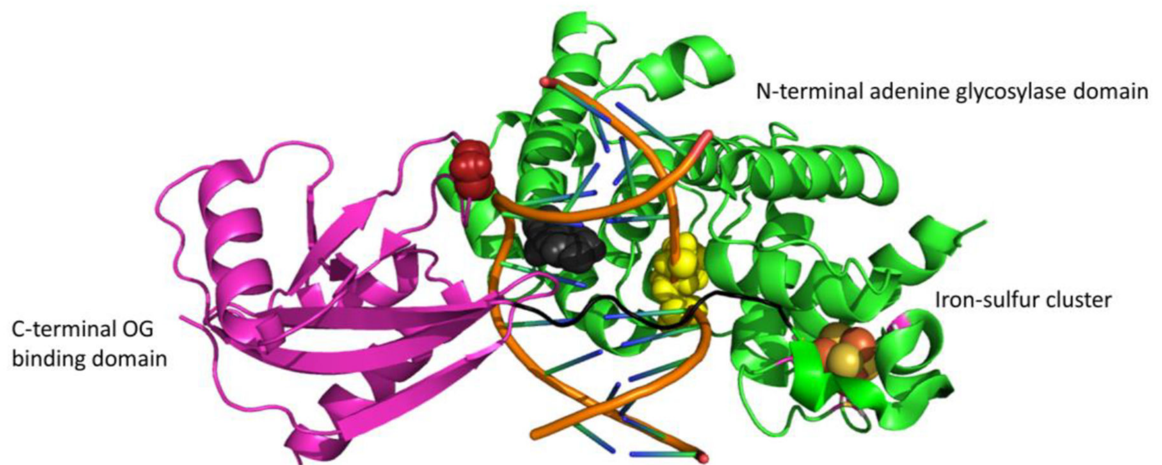

Figure 2.
GsMutY crystal structure obtained with the 1N transition state analog (PDB 5DPK) [7]. N-terminal adenine glycosylase region is shown in green, the interdomain connector (IDC) region is black, the C-terminal region is magenta. Transition state analog 1N shown in yellow. The two common MAP cancer variants G396D (red) and Y179C (grey) map to highly conserved regions of GsMutY. The iron-sulfur cluster is shown in orange/yellow.

Figure 3.

Overview of MUTYH missense variant distribution in a large population (top) and pathogenic MUTYH variants (bottom). Top blue histogram: log scale plot of MUTYH missense variant frequency in over 131,000 individuals from clinical and control populations (http://gnomad.broadinstitute.org/about; ref. 32), with the most common variants labeled. Middle diagram: approximate regions associated with MUTYH protein partners. Black histogram: location of reported pathogenic MAP missense variants based on LOVD (27,28) and a recent report (38). Red histogram: location of pathogenic nonsense (truncated protein) variants based on merged LOVD and ClinVar (29) annotations. Missense and truncating mutations listed at bottom in black and red text, respectively.

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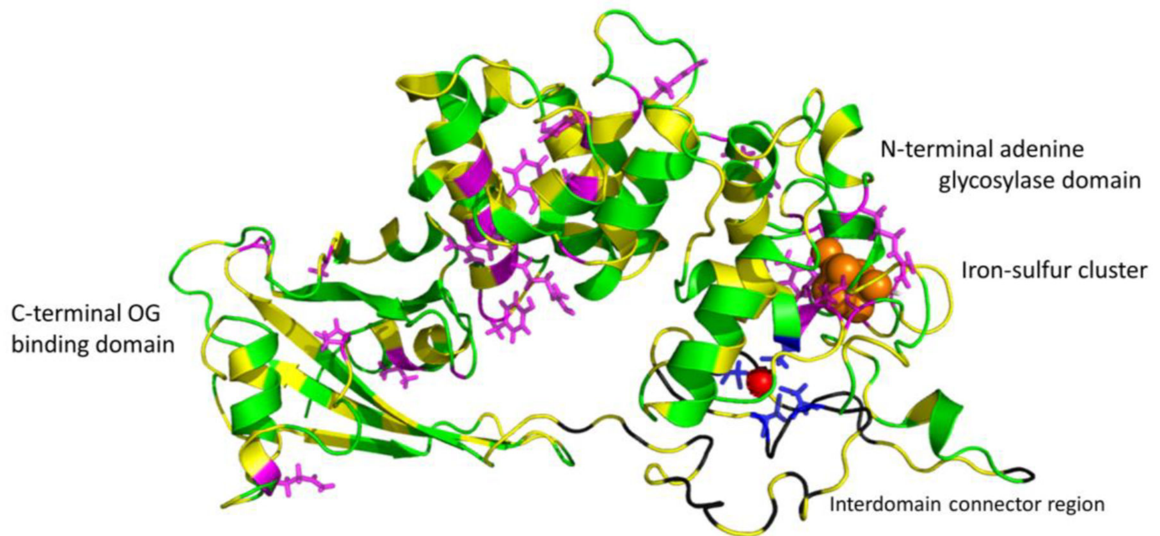

Figure 4.

Structural mapping of 21 pathogenic (magenta) and 261 uncategorized (yellow) protein variants to the human MUTYH model [80], which lacks the unstructured 81 amino acid N-terminus region. The pathogenic cancer-associated MAP variant locations (emphasized with side chains shown) are from LOVD database [27,28]. Yellow regions denote MUTYH missense variants detected in whole genome or whole exome sequencing in over 131,000 individuals from clinical and control populations (gnomAD MUTYH entry: http://gnomad.broadinstitute.org/gene/ENSG00000132781; ref. 32). Wild-type sequence: green; Interdomain connector (IDC): black; Zinc lynchpin motif: red/blue; Iron-sulfur cluster: orange.

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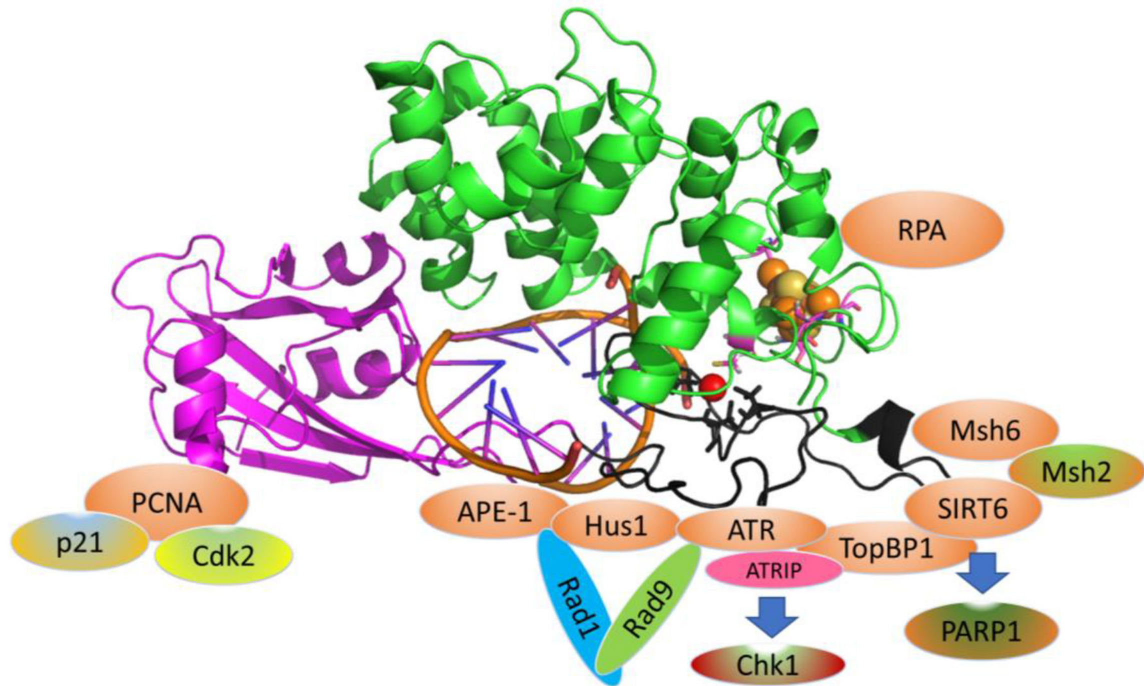

Figure 5.
MUTYH Interactome. Protein partners shown in beige have been reported to directly interact with MUTYH; accessory and downstream partners are shaded. N-terminal adenine glycosylase region is shown in green, the interdomain connector (IDC) region is black, the C-terminal region is magenta. Human MUTYH computation model of Zinc ion coordination (red) with hypothesized 4th ligand Cys 230 [80]. MUTYH interacts with PCNA [8], APE-1 [8–10], Hus1 [30,41], TopBP1 [69], SIRT6 [67], MSH6 [100], and RPA [8].