FSHing for DNA Damage: Key Features of MutY detection of 8-oxoguanine:adenine mismatches

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Conspectus: Base excision repair (BER) enzymes are genomic superheroes that stealthly and accurately identify and remove chemically modified DNA bases. DNA base modifications erode the informational content of DNA and underly many disease phenotypes, most conspicuously, cancer. The "OG" of oxidative base damage, 8-oxo-7,8-dihydroguanine (OG), is particularly insidious due to its miscoding ability that leads to formation of rare pro-mutagenic OG:A mismatches. Thwarting mutagenesis relies on capture of OG:A mismatches prior to DNA replication and removal of the mis-inserted adenine by MutY glycosylases to initiate BER. The threat of OG and the importance of its repair is underscored by the association between inherited dysfunctional variants of the MutY human homolog MUTYH and colorectal cancer, known as MUTYH-associated polyposis (MAP). Our functional studies of the two founder MUTYH variants revealed that both have a compromised activity and reduced affinity for OG:A mismatches. Indeed, these studies underscored the challenge of recognition of OG:A mismatches that are only subtlely structurally different than T:A base-pairs. Since the original discovery of MAP, many MUTYH variants have been reported, with most considered "variants of uncertain significance." To reveal features associated with damage recognition and adenine excision by MutY and MUTYH, we have developed a multi-pronged chemical biology approach combining enzyme kinetics, X-ray crystallography, single-molecule visualization, and cellular repair assays. In this review, we highlight recent work in our laboratory where we defined MutY structure-activity relationship (SAR) studies using synthetic analogs of OG and A in cellular and in vitro assays. Our studies revealed the 2-amino group of OG as the key distinguishing feature of OG:A mismatches. Indeed, the unique position of the 2-amino group in the major groove of OG_{syn}:A_{anti} mismatches provides a means for its rapid detection among a large excess of highly abundant and structurally similar canonical base pairs. Furthermore, site-directed mutagenesis and structural analysis showed that a conserved C-terminal domain β-hairpin "FSH" loop is critical for OG recognition with the "His" serving as the lesion detector. Notably, MUTYH variants located within and near the FSH loop have been associated with different forms of cancer. Uncovering the role(s) of this loop in lesion recognition provided a detailed understanding of the search and repair process of MutY. Such insights are also useful to identify mutational hotspots and pathogenic variants, that may improve the ability of physicians to diagnose the likelihood of diseases onset and prognosis. The critical importance of the "FSH" loop in lesion detection suggests that it may serve as a unique locus for targeting of probes or inhibitors of MutY/MUTYH to provide new chemical biology tools, and avenues for therapeutic development.

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Introduction: The "OG" of DNA base lesions and BER "GO" to the rescue

The free radical theory of aging posits that aging is a result of the cumulative damage inflicted upon cells by reactive oxygen and nitrogen species (RONS).[1] RONS, arising from environmental sources, oxidative metabolism or inflammation, react with cellular macromolecules compromising structure and function.[2,3] DNA base and sugar modifications resulting from RONS, exacerbated by faulty repair mechanisms, result in a myriad of consequences, such as transcriptional arrest and replication errors, ultimately leading to genomic instability, carcinogenesis, aging and other disease phenotypes. [4,5,6]

Oxidized nucleobases are among the most common and well-studied DNA lesions, and circa 200,000 oxidatively damaged DNA bases are produced per cell per day.[7] Guanine is the most vulnerable nucleobase towards oxidation; indeed, the most prevalent and well-studied "OG" of oxidized base lesions is 8-oxo-7,8-dihydroguanine (OG).[8] The mutagenic potential of OG arises from its ability to base pair like the canonical base thymine (T), leading to adenine (A) misincorporation during replication forming an OG:A mispair; subsequent replication of the mispair seals the G:C to T:A transversion mutation (**Figure 1**).[4, 9] Increased mutagenesis due to accumulation of OG in the genome provided the basis for the free

radical theory of aging[10] and is correlated with a variety of diseases, including neurodegenerative disorders

and various types of cancer.[11,12,4] Most relevant to this review, the accumulation of OG in the tumor

suppressor gene *APC* due to inherited defects in OG repair has been linked with the formation of colorectal

polyps and a predisposition to colorectal cancer.[13]
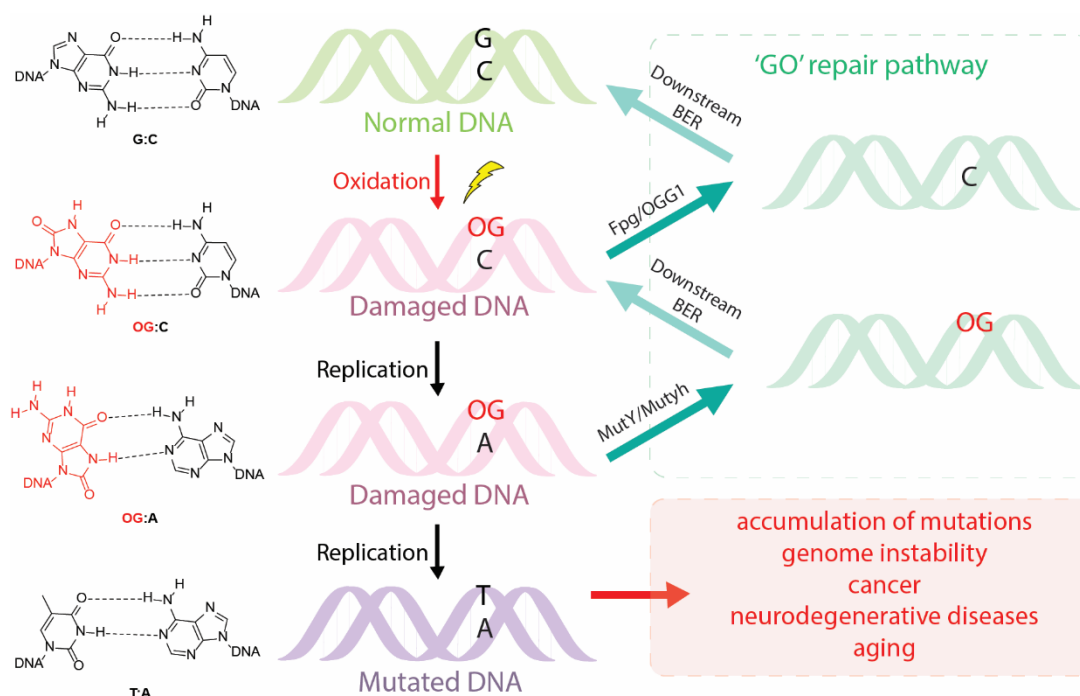

Figure 1 The presence of OG in DNA leads to the formation of G:C to T:A transversion mutations.
The GO repair pathway features two glycosylases, Fpg/OGG1 and MutY/MUTYH that prevent mutations associated

with OG by removing OG from OG:C base pairs or preventing propagation of the pro-mutagenic OG:A base pairs by

removal of the misplaced A, respectively.

The mutagenic consequences of OG accumulation in the genome makes it imperative for cells to

devote means to respond appropriately.[5] Lesions such as OG, that represent subtle chemical nucleobase

modifications, are typically removed by the base excision repair (BER) pathway where lesion-specific

glycosylases hydrolyze the *N*-glycosidic bond between the base and sugar, leading to the formation of an

apurinic/apyrimidinic (AP) site. [5,14] Downstream repair enzymes, such as AP endonucleases, polymerases

and ligases, excise the AP site, restore the proper nucleotide, and seal the backbone to complete the process of repair. The bacterial glycosylase MutM (also known as Fpg) and the human glycosylase hOOG1 are responsible for the removal of OG bases from OG:C base pairs (bps). However, pro-mutagenic OG:A mispairs are substrates of the MutY and MUTYH glycosylase in bacteria and humans, respectively. MutY enzymes are unusual among BER enzymes in catalyzing the removal of an undamaged but miscoding adenine base from OG:A rather than the damaged OG base itself; however, this process provides the crucial last stand against the mutation.[15, 5, 16] Bacterial genetic studies have shown that *mutY⁻ mutM⁻ E. coli* have extremely high mutation frequencies relative to single deletion *mutY⁻* or *mutM⁻ E. coli* strains indicating synergy in activity of the two enzymes toward the same lesion.[9,16] MutY together with MutM comprises what is termed the "GO" repair pathway to prevent mutations associated with OG.[16,17] The importance of preventing OG-associated mutations to maintain genomic integrity is also reflected in the high level of conservation of the GO-repair pathway across all domains of life.[18,19]

BER glycosylases took center stage when the correlation of inherited variants in MUTYH and colorectal polyposis and cancer was uncovered.[13] The recessive disorder is now referred to as MUTYH-associated polyposis, or MAP, and is characterized by accumulation of somatic G:C to T:A mutations in the tumor suppressor *APC,* leading to the polyposis phenotype and high penetrant colorectal cancer.[13,12] The David laboratory played a key role in the initial discovery of MAP by discerning the functional impact of the two founding variants, Y179C and G396D, using the bacterial enzyme as a model.[13,20] Notably, in vitro assays revealed that both bacterial variants exhibit defects in recognition and excision of OG:A mismatches.[20,21] Since the original landmark study, we have found that the mouse and human enzymes behave similarly.[22,23] Of note, we consistently observed that Y179C MAP variant exhibits greater dysfunction in *in vitro* assays than G396D, and this may be clinically significant.[12] A plethora of other MUTYH variants, with a multitude of missense mutations have since been associated with MAP and other cancers. Surprisingly, many MUTYH variants exhibit adenine glycosylase activity similar to the WT that presents a challenge for assessing the potential impact in people. Indeed, these findings prompted us to develop cellular assays to determine the impact in cells and reveal in molecular detail the intricacies of the

various steps in the overall process of repair mediated by MutY and MUTYH. We have summarized our contributions to discovery of MAP and much of our work on MutY and MUTYH variants in several reviews that we direct the interested reader to consult for additional details.[4,12,15]

Our aim in this review is to provide an overview of the research that our laboratory has performed to understand the exquisite specificity and efficiency with which MutY enzymes act on OG:A mispairs within DNA. Indeed, the rarity of OG:A base-pairs and their similarity to T:A base-pairs makes the task of MutY both onerous and remarkable. Fidelity of MutY-mediated repair requires verification of both OG and A to allow for contextually appropriate repair to avoid removing A from T:A bps and preventing genomic mayhem by random generation of abasic sites. Our laboratory, in concert with our collaborators, has used an array of chemical biology approaches leveraging nucleic acid chemistry, in vitro ensemble and single-molecule assays, structural studies and cellular assays to provide molecular insights into the many facets of this remarkable enzyme.

MutY is a retaining Glycosylase featuring a Covalent Intermediate

MutY enzymes catalyze removal of adenine by hydrolysis of the N-glycosidic linkage of 2'-deoxyadenosine with expected mechanistic similarities to acid-catalyzed depurination of 2'-deoxy-adeosine in nucleosides and DNA.[24] Kinetic isotope effect studies by McCann and Berti provided evidence that adenine excision by MutY follows an S_N1 reaction mechanism utilizing N7 protonation and general acid catalysis.[25] Structural studies, site-directed mutagenesis, and pH-dependent adenine glycosylase assays have revealed the structural domains and key amino acids involved in MutY adenine excision catalysis ([26,27]) (**Figure 2**). MutY is a member of the helix-hairpin-helix superfamily of BER glycosylases that "flips out" the target base into a catalytic pocket for excision.[19] A defining feature of the HhH BER superfamily is the presence of a critical catalytic Asp that is within a Gly-Pro rich region, referred to as a GPD motif. The Asp in MutY enzymes is required for catalysis, and pH dependence and site-directed

mutagenesis have shown that maximal activity requires a deprotonated Asp.[27] The Asp was initially proposed to be involved in activating the water nucleophile, as suggested for other BER glycosylases;[28,29] however, a structure of *Geobacillus stearothermophilus* (*Gs*) MutY bound to DNA containing a pyrrolidine transition state (TS) mimic (1N) paired with OG (referred to as TSAC, for transition state analog complex), inspired us to propose an alternative mechanism (**Figure 2**).   Indeed, the close positioning of the Asp to the N1' of 1N, that corresponds to C1' of the target A, and the presence of a potential nucleophilic water molecule on the opposite face of 1N in the position of the departed adenine (**Figure 2B**) suggested that the Asp (144 in Gs MutY) forms a covalent acetal DNA intermediate, thereby stabilizing the oxacarbenium ion while adenine departs as a neutral leaving group.[26] The inferences from the TSAC structure suggested that the stereochemistry at C1' would be retained, and this was confirmed by 2D-NMR stereochemical analysis of the acetal product formed via methanolysis in MutY reactions containing methanol in the buffer. Notably, in this mechanism, Glu 43 plays dual roles as the general acid in protonating the adenine base to enhance its departure and as the general base in deprotonating the water molecule to activate it as the nucleophile. The TSAC structure also suggested roles for Tyr 128 in electrostatic stabilization of the oxacarbenium ion TS and for Glu 43 in positioning the water molecule for hydrolysis of the covalent acetal intermediate.[26]

Recent work by our laboratory involving combining mutation of an Asn residue that H-bonds to the catalytic Asp, along with an alternative substrate, purine (P) paired with OG, and crystallization under different conditions lead to a suite of new Gs MutY complex structures with the substrate and three enzyme-generated products.[30] In all three product complex structures, only the *beta*-anomer was observed, consistent with retention of configuration, and aligning with the mechanism we had previously proposed. In addition, two recent computational studies have provided additional support for this mechanism that is unique for MutY relative to other glycosylases.[31, 32]
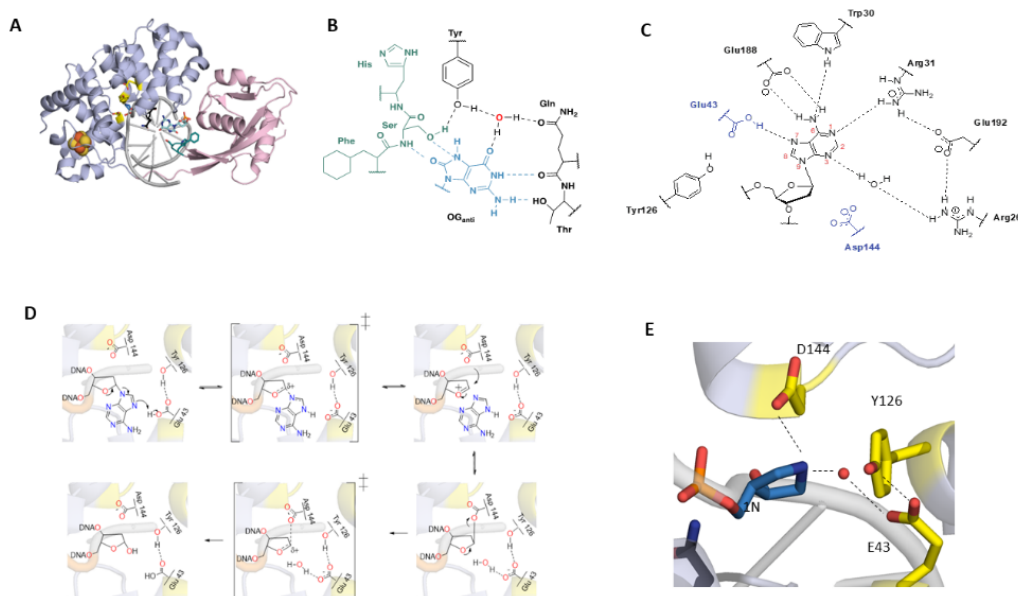
Figure 2 Crystal structures of *Gs* MutY reveal details of the catalytic mechanism. A Overall structure of *Gs* MutY bound to DNA containing transition state analog, 1N, across OG (PDB ID 6U7T) **B** Key residues from C- and N-terminal domain form a network of interactions around OG$_{anti}$ **C** Contacts observed in FLRC to the A base flipped into the active site with the N-terminal domain **D** Proposed mechanism for MutY mediated adenine excision. Protonation of the A at N7 enables it to leave as a neutral molecule. The oxacarbenium ion hence formed is stabilized by the catalytic aspartate following which an active site water molecule leads to the formation of the abasic site product. **E** Extruded 1N in the active site and active site residues (PDB ID 6U7T)

STRUCTURE ACTIVITY RELATIONSHIPS (SAR) FOR OG:A REPAIR BY MUTY

Inspired by the medicinal chemistry approach of defining structure activity relationships (SAR) of small molecules and natural products to glean insight into their biological targets, we have used analogs of OG and A to develop SAR for MutY.[33,34,35,36] Structures analyzed in the SAR studies included the structure of Gs MutY bound to a noncleavable A analog, 2'-deoxy-2'-fluoroadenosine opposite OG, which is referred to as the "Fluorine lesion recognition complex" or FLRC. In the FLRC, the "A" is extruded from the helix and placed in the catalytic pocket within the N-terminal domain, while the "OG" base has been rearranged to its *anti* conformer remaining stacked within the DNA helix with contacts from both C- and N-terminal domains (**Figure 2**). Since initial lesion detection and interrogation by MutY occurs when the target bp is

within the DNA helix, we also considered the structure of an OG_{syn}:A_{anti} bp within duplex DNA in our SAR studies (**Figure 1**). In terms of activity (**Figure 3**), we analyzed the glycosylase activity of MutY on the modified substrates using a minimal kinetic scheme to define core parameters of DNA duplex affinity (K_d), base excision catalysis (k_2) and DNA product release (k_3). [37] The kinetic parameters k_2 and k_3 were measured using gel-based glycosylase assays that monitor strand scission at the A or A analog nucleotide, and were performed under conditions of single or multiple turnover conditions to isolate the relevant rate constants. To simplify binding affinity measurements we used catalytically inactive E37S MutY that binds with high affinity to substrate OG:A bp-containing duplexes, but is unable to mediate base excision.[38] To evaluate the MutY activity in a cellular context, we developed a repair assay that uses a plasmid carrying a site-specific OG:A, OG:X, or Y:A mispair strategically positioned within a BMT1 restriction site such that repair to G:C will restore the restriction site.[39] After transformation of the lesion-containing plasmid into *muty*[+] or *muty*[-] *E. coli* cells, amplification and extraction, restriction digestion analysis and DNA sequencing was performed to determine the distribution of bps at the lesion site (**Figure 3**). In these assays, OG:A lesion bps within the plasmid DNA are fully repaired in the presence of MutY to the correct G:C bps (> 95%), while in the absence of MutY a mixture of G:C and T:A bps are observed at the location of the lesion site (35% G:C, 65% T:A) consistent with equal replication of both bp partners and the expected levels of correct versus mutagenic replication opposite OG. The extent of MutY-mediated repair of the OG or A analogs can be delineated by comparing the differences in *muty*+ versus *muty*- cells. Using this multi-pronged approach with a series of OG and A analogs (**Figure 4**), we revealed exquisite detail on the features important for repair of the OG:A bp by MutY.[35,36]

A) **Minimal Kinetics Scheme**

$$\text{MutY} + \text{DNA}_S \underset{K_D}{\rightleftharpoons} \text{MutY.DNA}_S \xrightarrow{k_2} \text{MutY.DNA}_P \xrightarrow{k_3} \text{MutY} + \text{DNA}_P$$

B) **Binding Assay**

25° C

Native PAGE [MutY]

Bound DNA

Free DNA

C) **Glycosylase Assay**

NaOH 90° C

Dye 90° C

Denaturing PAGE
Time

Substrate

Product

D) **Cellular Repair Assay**

3,056 bp

*Bmt*I

transform

repair

*mut*Y+

no repair

*mut*Y-

*Bmt*I

*Bmt*I

*Bmt*I

*Bmt*I digest

bp

1,748
1,308
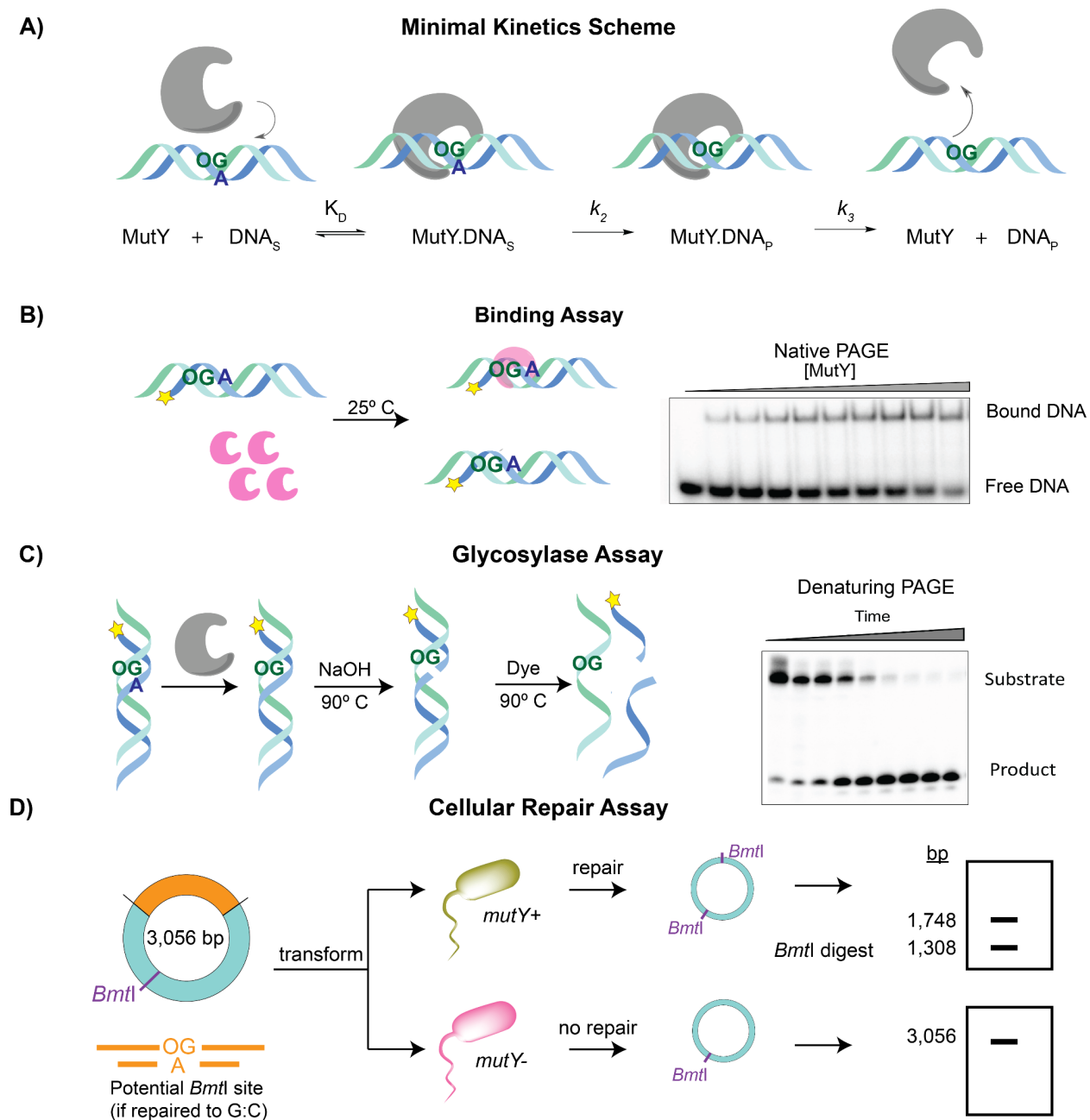
3,056

OG
A

Potential *Bmt*I site
(if repaired to G:C)

Figure 3 Schematic description of assays used to evaluate different stages of repair A Minimal kinetics scheme depicting the major steps in the enzymatic activity of MutY in terms of the binding constant, K_D, rate of adenine excision, k_2, and rate of abasic site product release, k_3. **B** Electrophoretic mobility shift assays, using either a catalytically inactive enzyme or a non-hydrolysable substrate, are employed to evaluate the binding affinity of the enzyme for substrate analogs. **C** Glycosylase assay used to evaluate the kinetic parameters k_2 and k_3. **D** An *E. coli* based cellular repair assay is used to evaluate the overall extent of repair in terms of conversion of an OG:A mispair to G:C.
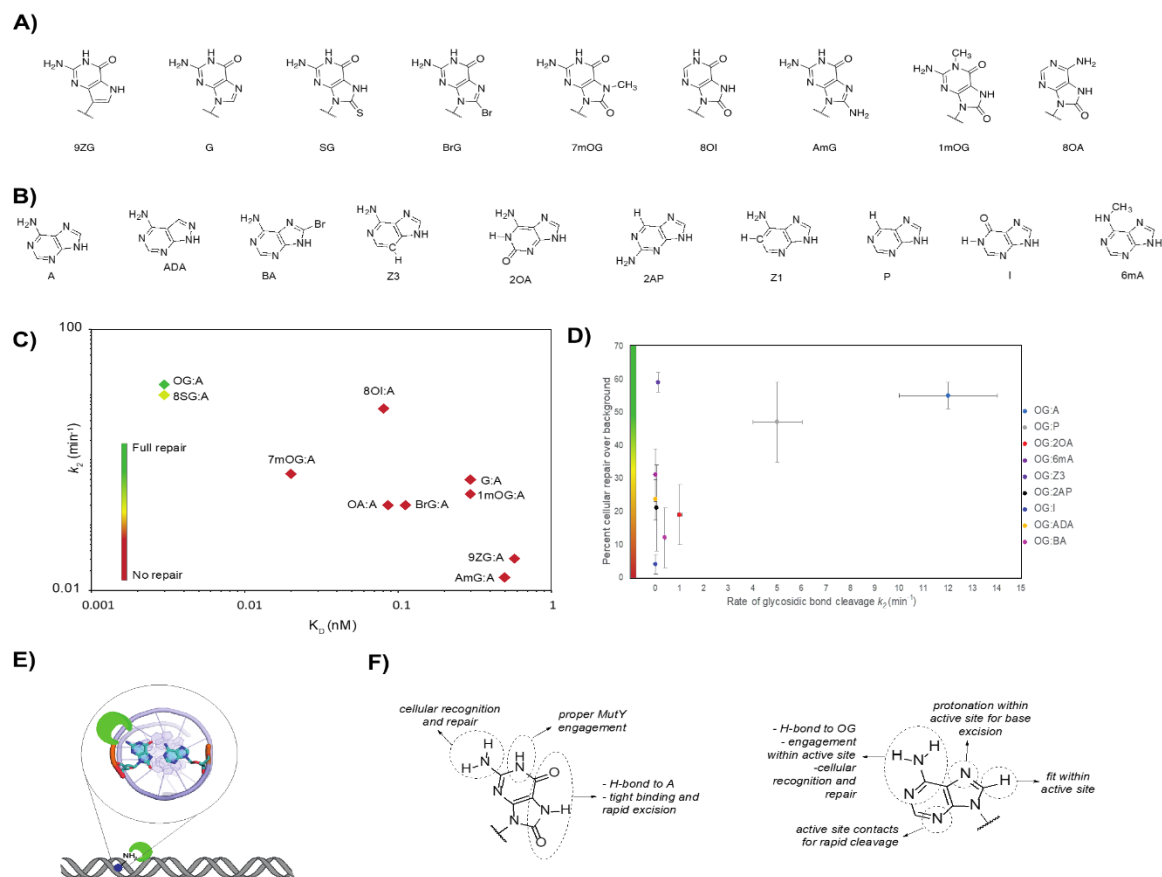
Figure 4 Structure activity relationships (SAR) studies reveal key features needed for recognition and repair by MutY. **A** Chemical structures of OG analogs. **B** Chemical structures of adenine analogs. **C** Relationship between the rate of A cleavage, binding constants of the catalytically inactive E37S MutY variant to duplexes containing X:A mispairs, where X is the OG analog. **D** Relationship between rate of excision of adenine analogs and overall cellular repair in *E. coli*. The adenine analogs exhibited tight binding ($K_D < 5$ pM) when paired opposite OG. **E** The unique H-bonding ability of A to OG positions the 2-amino group in the major groove of DNA to enable recognition by MutY **F** Summary of the roles of structural features of OG and A as determined by SAR analysis.

The selected OG and A analogs systematically modified different structural aspects of the target mispair and revealed details on specific aspects of the repair process mediated by MutY. Indeed, these studies highlighted the importance of *OG detection* in the search and rescue mission of MutY. Alterations to the OG structure decreased both binding affinity to MutY, as well as the catalytic rate of A excision, indicating the high degree with which the structure of OG is required for proper engagement in the OG binding site. Indeed, OG analogs retaining the 8-oxo (or chemically analogous 8-thio) structural feature exhibited the highest affinity for MutY, indicating the importance of the thymine-like Hoogsteen face of OG for high affinity to MutY. In contrast adenine analogs retained high affinity for MutY and were excised

to near completion as long as they were paired across OG,[35] suggesting sequential recognition of the base-pairing partners, with OG structure guiding initial recognition.

The high sensitivity of the adenine excision rate constants k_2 to OG structural modifications was somewhat surprising due to the distal location of the OG binding pocket from the active site. The long-range impacts suggest that OG:A lesion engagement elicits a conformational change that "locks" MutY into a catalytically competent state that is only enabled by the chemical structure of OG.[36] The post-recognition FLRC structures of MutY shows that upon localizing to the mispair, MutY rotates OG from its *syn* conformation when in the duple to an *anti* conformation, while also extruding the A nucleotide out of the helix to place the adenine base into an adenine binding pocket. Once lodged in the adenine binding site, extensive H-bonding contacts to every heteroatom on A are made to align the A nucleotide for contacts with the catalytic Glu and Asp residues (**Figure 5C**).[29] Correlation of experimental acid labilities and gas phase acidities with *in vitro* excision rates of A analogues revealed the H-bonding patterns also modulate the acidity of the A to affect its rapid protonation and release. These analog studies demonstrate that *only adenine* was rapidly oriented and cleaved within the active site of MutY.[35]

The effect of OG and A structural modifications on MutY-mediated repair in *E. coli* revealed how differences observed in vitro translate to a cellular context. These results drew even more attention to the high reliance on recognition of unique features of "OG" by MutY for facile repair. Despite exhibiting a range of reduced activity in vitro, all of the OG analogs tested, except 8-thioG that is the most similar to OG, exhibited minimal repair in the bacterial cell assay. Most surprising to us was the observation of minimal repair of OI:A bps, since these were found to be good substrates in vitro. There are contacts made to the 2-amino group of OG in the FLRC, that might account for some reduction in the lesion affinity and glycosylase activity; however, it wasn't immediately obvious why the loss of the 2-amino would be so much more dramatic in cells. Inspection of the structure of the OG$_{syn}$:A$_{anti}$ bp within the DNA helix provided the potential rationale: the 2-amino group of OG is uniquely located in the major groove of DNA in OG$_{syn}$:A$_{anti}$ bps. Indeed, in G:C and OG:C bps, the 2-amino group is located in the minor groove. In addition, the major group methyl group of T in T:A bps is distinctly different than a 2-amino group. In the

case of the A analogs, MutY mediated repair was independent of *in vitro* rate of A excision; indeed, some analogs that were very poorly processed in vitro (e.g. 3-deazaA:OG, 200-fold slower) were repaired almost as well as OG:A, while some that were decent substrates in vitro (G:A), were barely repaired in the cellular context. The only common feature of A analog bps that correlated with high repair was the ability to bp with the syn conformer of OG! In this way, the "A" or "A analog" effectively positions the 2-amino group to project from the DNA major groove. These results illustrate the indirect nature of detection of misplaced "A" opposite OG by relying on the unique base pairing structure of A with OG. The disparity between processing modified substrates *in vitro* and in cells demonstrates that in the presence of vast tracts of undamaged DNA within the genome, lesion detection and recognition rather than rate of excision is the limiting factor for repair.[35]

IDENTIFICATION OF OG SENSING RESIDUES IN MUTY

Looking for signs: The CTD of MutY recognizes OG

MutY enzymes are distinct from other BER superfamily glycosylases in harboring a MutT-like C-terminal domain (CTD) that recognizes OG.[33,40,41] When the CTD is removed, *Eschericia coli* (*Ec*) MutY N-terminal domain (MutY CΔ225) was able to process both OG:A and G:A bps but with a diminished preference for the cognate OG:A lesion. Though *in vitro* catalytic activity was retained in the absence of the CTD, *E. coli* expressing MutY CΔ225 exhibited a significantly higher mutation frequency (500-fold increase)[33] and were unable to repair OG:A in the bacterial lesion reporter assays;[27] both studies pointing to the key role of the CTD and OG recognition in repair of OG:A mismatches in a cellular context. Since the base excised by MutY is the undamaged A paired opposite OG, rather than the damaged base itself, initial recognition of the lesion is necessary to avoid indiscriminate excision of other undamaged DNA bases. The two domains of MutY enable complete engagement and interrogation of both DNA strands (**Figure 2A**), in contrast to other HhH glycosylases that solely contact the lesion-containing strand.[42] The dual domain structure enhances substrate specificity as observed by the fact that MutY can recognize two

structurally related DNA lesions, OG:A and G:A. However, subtle differences in structure have a pronounced effect on catalysis - MutY processes G:A almost 30-fold less efficiently than OG:A *in vitro*, and negligibly repairs G:A in a cellular context.[36,39] From these findings, we suggest *in vitro* excision of A from G:A mismatches by MutY is a precarious evolutionary artefact rather than a biologically relevant activity, especially as this mismatch is primarily repaired by mismatch repair DNA repair pathway.[43]

Structural studies reveal the importance of a "FSH" loop in damage detection

To reveal insight into motifs in MutY responsible for OG specific recognition, we swapped OG for G, and determined a structure of Gs MutY bound to the pyrrolidine transition state analog, 1N, opposite G (TSAC-G:1N), in collaborative studies with the Horvath laboratory (University of Utah).[44] The structural overlay of the TSAC-G:1N structure with corresponding OG:1N structure (TSAC-OG:1N PDB ID 6U7T) showed remarkably similar structures overall with the only changes localized at Ser 308 within the CTD. The perturbed Ser308 normally H-bonds to the N7-H of OG but rotates away and disengages from the N7 lone pair of G (**Figure 5B**). The Ser308 rotamers in both structures retain H-bonding to Tyr88 (Tyr179 in human MUTYH, founder MAP variant) and preserve the connection of N7-H and O8 of OG with Tyr88 *Gs* MutY. Tyr88 also forms a hydrogen bond with a water molecule that also interacts with Gln48 and O4 of OG (**Figure 5C**). The Watson-Crick face of OG forms hydrogen bonds with two other N-terminal domain residues, Gln48 and Thr49 that intercalate into the void left by the extruded adenine (**Figure 5C**). Conspicuously, Ser308 resides at the tip of a β-hairpin loop that penetrates the DNA helix and positions itself close to OG (**Figure 5B**). The network of interactions mediated by Ser308, and the presence of a

similar loop in the d(OG)TPase, MutT, implicated the FSH loop as an essential OG recognition motif (**Figure 5**).

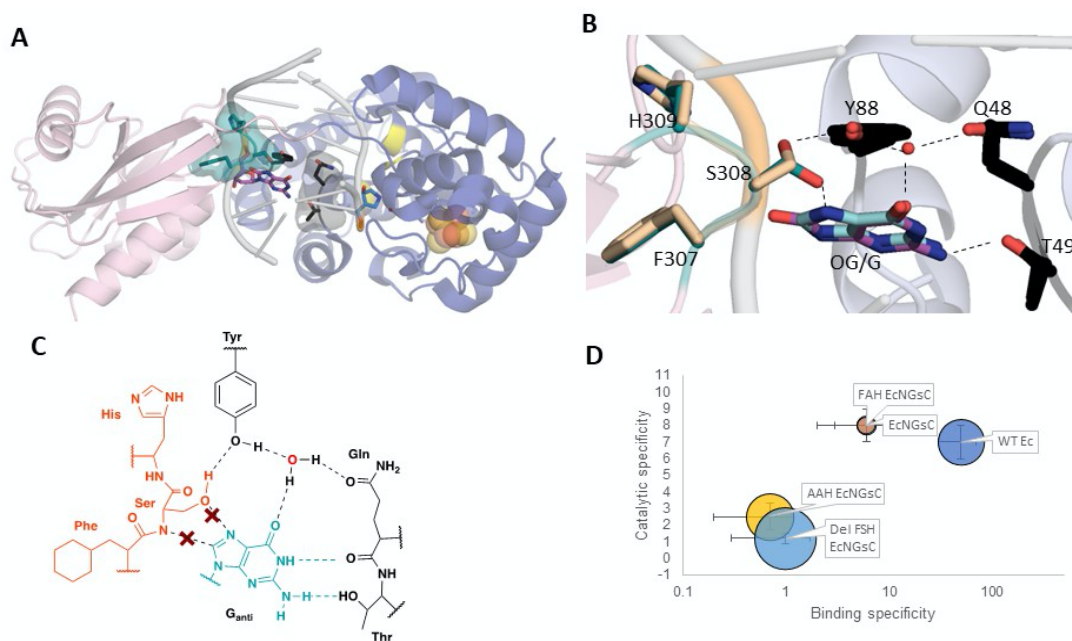**Figure 5 Recognition of OG:A by MutY is dependent on a conserved C-terminal loop. A.** FSH loop invades into the DNA helix searching for OG (green, PDB ID: 6U7T) **B.** Overlay of crystal structures of *Gs* MutY bound to the transition state analog paired opposite OG (magenta; PDB ID: 6U7T) and G (cyan; PDB ID 6Q0C) showing the invasion of the DNA helix by the FSH loop. The FSH loop serine (S308 in *Gs* MutY) is proposed to aid discrimination between OG and G through the formation of an OG specific hydrogen bond. **C.** Key residues from C- and N-terminal domain form a network of interactions around OG$_{anti}$ while G$_{anti}$ lacks the interactions (denoted with red X) with serine residue that has rotated 120° and maintains interactions with tyrosine **D**. Graph showing binding and catalytic specificity of FSH loop mutants. The mutation suppression frequency is represented by the size of the circles corresponding to each variant. Smaller circles indicate the MutY variant is competent in suppressing G:C → T:A mutations in cells.[44]

The role of the FSH loop in ensuring OG:A repair in a cellular context was further demonstrated using a rifampicin resistance assay[45,46,47] to measure mutation suppression by MutY variants in which the amino-acid side chains were replaced or the loop was deleted entirely (**Figure 5**). The toxicity of full-length *Gs* MutY expression in the reporter *E.coli* strain, necessitated using a chimera containing the N-terminal domain of *E. coli* MutY and C-terminal domain of *Gs* MutY (*Ec*N*Gs*C MutY). We observed single amino acid replacements in the FSH loop minimally impacted mutation frequency, whereas double amino acid substitutions or loop deletion resulted in significant increases in mutation frequencies, showing the residues cumulatively play a role in OG:A repair (**Figure 5**).[44]

In vitro binding and kinetics experiments additionally demonstrate the importance of the FSH loop in discrimination of OG from undamaged G. For this discussion, we define "binding specificity" as K_D of MutY for G over OG, and "catalytic specificity" as k_2 for OG over G. Single substitution of Ser308 to an alanine (FAH *Ec*N*Gs*C) decreased affinity for both OG:FA and G:FA duplexes while maintaining binding specificity. Double replacement AAH variant and loop deletion (Del FSH) impacted binding more drastically indicating both residues are necessary for binding and catalytic specificity for OG (**Figure 5**).[44]

Seeing is Believing: Single-molecule studies reveal features MutY uses the FSH loop His to detect OG:A bps via the OG 2-amino group

Accurate and effective selection of damaged bases by glycosylases within vast excess of undamaged DNA is a statistically daunting feat that for decades has fascinated the DNA repair community.[48] Many BER glycosylases are able to identify subtle chemical and/or structural modifications that are minimally disruptive or destabilizing to the DNA helix, thus providing no obvious sign-post to telegraph their location. It has been postulated that glycosylases utilize facilitated diffusion to slide along the DNA backbone to detect minor alterations in DNA structure which is achieved through thermal energy alone, rather than ATP hydrolysis as in the case of DNA helicases, MutS and other DNA repair enzymes.[49,50]

Direct visualization of glycosylase motion by single molecule fluorescence microscopy (SMFM) has provided invaluable insight into glycosylase motion.[51,52,53,54] By stretching λ-DNA across silica beads to create DNA tightropes, trajectories of single Q-dot labeled glycosylases have been visualized in real time.[52,55,56] Using this approach, the glycosylase hOGG1 was shown to repeatedly interrogate a section of DNA through repeated one-dimensional scanning.[57,56] Despite the redundancy in this search mechanism, the probability of OG capture is enhanced by increasing the number of encounters between glycosylase and lesion.[57] Subsequent studies with *Ec* glycosylases Fpg, Nei, and Nth showed similar diffusive behavior wherein each glycosylase repeatedly scanned over a single area of DNA.[58] Studies using DNA with randomly introduced abasic sites showed that glycosylases "pause" more frequently due to binding the damaged sites.

Single molecule fluorescence microscopy search assays were used to directly observe the real-time search behavior of WT Ec MutY on OG:A and OI:A bps to test the importance of the 2-amino group in the lesion recognition process (**Figure 6**). [59] These studies directly visualized Q-dot labeled MutY searching DNA tightropes containing OG:A or OG:I bps positioned every 2726 bps, and identifiable via their relative positioning to a fiducial Cy5 label. SM trajectories showed long paused events for MutY with OG:A bps (>300s) while noticeably absent were such long pauses for WT MutY on OI:A sites. Time-weighted sliding window diffusion analysis revealed that MutY scans rapidly on undamaged DNA tightropes at rates consistent with random diffusion ($D_{max} \sim 0.01$ um^2/s) while the presence of OG:A sites leads to a significant decrease in diffusion rate consistent with pausing ($D_{max} < 0.001$ um^2/s). In the presence of OI:A bps, MutY shows primarily fast diffusion, indicating no recognition of the damaged base analog. This work demonstrates that the 2-amino group of OG is essential for detection of the OG:A bp, and its absence leads to an inability to find OI:A bps and mediate their repair. [59]

The sensitivity of MutY repair to the absence of the 2-amino group in OI:A substrates, and the conspicuous positioning of the FSH loop near OG, suggested to us that a residue within the FSH loops serves as the "sensor" of interhelical OG:A bps.[59] Modeling using several *Gs* MutY structures suggested that His 309 (*Gs* MutY) would be appropriately positioned to detect the 2-amino group of OG$_{syn}$. Indeed, the in vitro and cellular repair of a variant at the corresponding position in *Ec* MutY (H296A) acting on OG:A substrates mirrored the results of WT acting on OI:A bps. Specifically, H296A acting on OG:A, and WT acting on OI:A both showed only 2-fold reduced adenine excision (k_2). In terms of binding affinity (K_d), the affinity was more dramatically reduced for H296A with OG:A than WT with OI:A (150-fold vs ~12-fold respectively). Cellular repair with H296A was significantly less than WT with OG:A bps, but slightly above that observed with WT on OI:A bps. These results suggest that in a cellular context where there is a larger amount of undamaged DNA, H296A MutY is severely compromised in detecting OG:A bps.

Single molecule visualization of the behavior of H296A MutY on OG:A containing DNA tightropes was similar to that observed for WT MutY on *undamaged* DNA, indicating without the histidine,

MutY is "blinded" to the lesion (**Figure 6B-D**). [59]   A small population of H296A MutY encountering OG:A

lesions was observed, however, persistent H296A pausing at damaged sites was not observed. Only WT

MutY on OG:A bps showed significantly longer binding lifetimes, than the combination of H296A/OG:A

or WT/OI:A (**Figure 6C,D**). These results showed the histidine residue was indispensable in initial OG:A

lesion detection. Collectively, the evidence from structural studies, biochemical characterization, cellular

assays and single molecule visualization demonstrate the FSH loop is important for detecting and binding

to OG:A. These results demonstrated that both the 2-amino group of OG, and His 296 of MutY are required

for detection of OG:A bps in the context of large tracts of undamaged DNA, as would be present in cells,

and is borne out by the lack of repair in cells when either feature is absent.
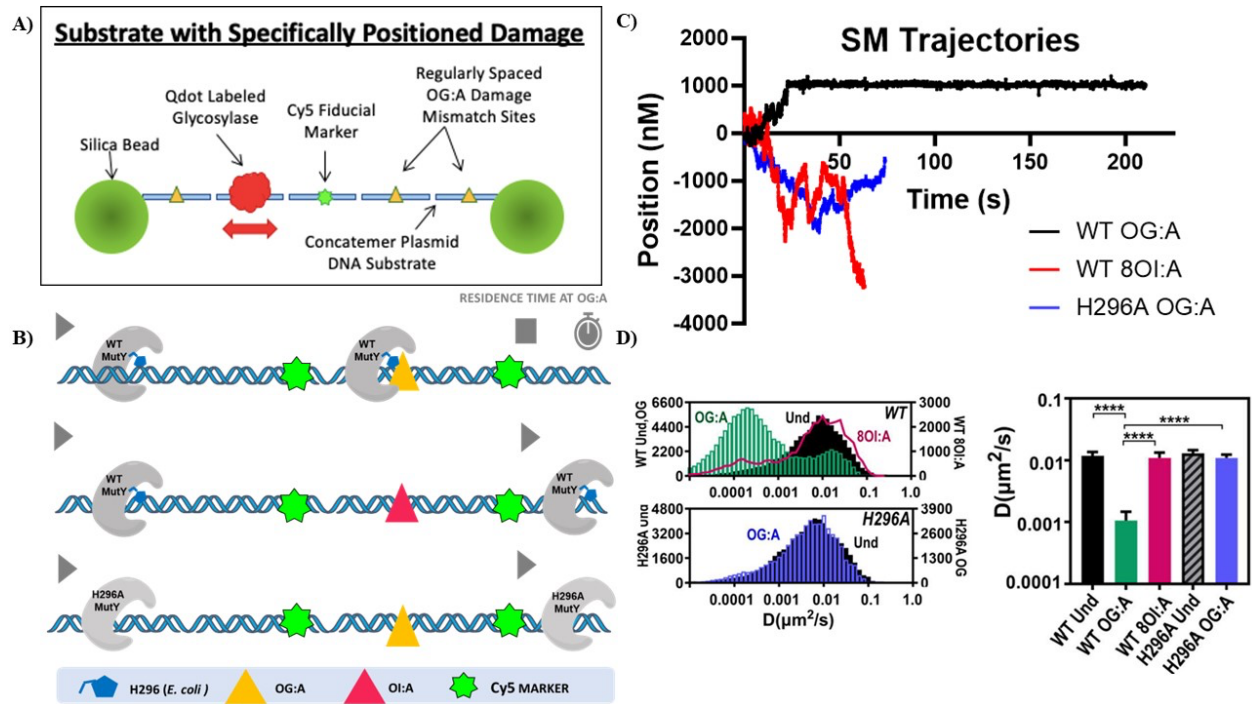

Figure 6 Single molecule studies using *E. coli* MutY revealed the role of the C-terminal domain histidine, H296, in OG:A detection. [59] **A.** Overview of Single molecule based assay utilizing Q-Dot conjugated glycosylase molecules to monitor in real time diffusion on lesion or non-lesion containing stretched DNA. Lesions are equally spaced between CY5 markers to correlate periods of "pausing" with sensing of lesions. **B** Cartoon depiction highlighting observed outcomes when: WT *MutY* is monitored in search of OGA (Top) OI:A (middle) Histidine 296 is searching for OG:A (bottom). **C.** Representative displacement trajectories of WT *MutY* and H296A variant on OG/OI:A lesion containing concatemerized substrate DNA. **D.** Histogram showing counts for individual glycosylase molecules and computed diffusion rates.

Implications for MUTYH, MAP and other human diseases

We developed a multipronged approach towards uncovering features of lesion recognition and base excision aspects of MutY. Our work has shown successful lesion recognition results from cooperation between both domains, and is dependent upon the precise base pairing pattern of OG and A. We also demonstrate the significance of a conserved CTD β-hairpin loop, bearing the residues Phe-Ser-His (FSH loop), in damage detection and OG vs G differentiation. Our SAR studies indicate that initial lesion detection is highly dependent on the OG base structure, OG and A binding likely occur sequentially and proper engagement of the OG base in the CTD exerts long range effects on A orientation within the active site in the catalytic NTD. In addition, misplaced As are detected indirectly by their ability to hydrogen-bond and form a stable bp with OG in its *syn* conformer. Once the A has been extruded, H-bonds to adenine within the MutY active site mediate proper alignment for excision. Taken together, we provide a molecular view of the damage location and excision process by MutY. In addition, this work has shown how MutY variants that are deficient in any of the steps preceding catalysis, such as lesion detection (H296A) and differential lesion binding (AAH MutY) compromise the ability of MutY to successfully repair OG:A in cellular contexts.

Our SAR results with MutY provide an important backdrop for evaluating and predicting functional and clinical consequences of MUTYH variants. Indeed, we have recently found in SAR studies of OG with MUTYH that efficient MUTYH-mediated cellular repair is also critically dependent upon the 2-amino of OG (unpublished results, *submitted for publication*). Taken all together our work is highly suggestive that MUTYH variants that exhibit compromised OG lesion detection and affinity will be particularly disabled in their ability to mediate repair inside cells. Our work also provides a cautionary tale that relying on a single type of assays to reveal functional properties of MUTYH variants would be exceedingly misleading and therefore underscores the need for multiple functional assays and structural studies. Among the >800 catalogued variants of MUTYH reported in clinical databases, many (~20%) result in functional defects and are associated with cancers, including breast, ovarian, gastrointestinal and gliomas, while most (~70%)

are variants of uncertain significance (VUS).[60, 12] These extensive and potentially elusive variants predicate the need for detailed functional characterization.

Despite its role as a tumor-suppressor, MUTYH is among a host of DNA repair proteins, most notably Poly(ADP-ribose) polymerase (PARP), whose inhibition presents a promising chemotherapeutic modality.[61] MUTYH inhibition offers a broad range of therapeutic potential, as its activity is implicated in various disease phenotypes.[12] For example, results from Gao *et al.* indicate that MUTYH expression is upregulated in SW780 bladder cancer cells, and shRNA knockdown of MUTYH inhibited proliferation, migration, and induced apoptosis in the cells.[62] Additionally, in murine models of ulcerative colitis, inflammation was reduced in *Mutyh*^{-/-} mice compared to wildtype.[63] Moreover, MUTYH is implicated in Alzheimer's Disease (AD), a neurodegenerative disease characterized by a high degree of oxidative stress.[64,65] In recent work, Mizuno et al. determined that MUTYH deficiency (achieved in a protease-dependent manner) reduced microgliosis and ameliorated memory impairment associated with AD by restoring hippocampal neurogenesis in mice.[66] Thus, inherited defects in MUTYH resulting in increased cancer predisposition, in addition to growing associations between MUTYH activity and various disease states, suggest potential utility of MUTYH activators and inhibitors, respectively. Despite the growing interest in discovering small-molecule modulators of DNA repair enzymes,[67,68] there are no such reported molecules targeting MUTYH. As such, an understanding of the chemical basis of MUTYH mediated repair can guide the design of DNA damage probes and inhibitors and can help relate clinically observed functional patterns to defects in the chemistry of the enzyme.

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Biographies

Sheila S. David received her B.A. in Chemistry from St. Olaf College, and Ph.D. from the University of Minnesota under the supervision of Professor Lawrence Que, Jr. She then pursued postdoctoral studies at Caltech with Professor Jacqueline K. Barton and began her academic career as an Assistant Professor at the

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Mohammad Hashemian obtained his B.S. in Biological Sciences from the University of California, Irvine in 2019. He is currently pursuing his Ph.D. in chemistry and chemical biology in the David lab at the University of California, Davis pertaining to structural and functional studies of MUTYH metal cofactors, how their roles deviate within cancer-associated variants, and how MUTYH native properties may be leveraged to guide ligand discovery.

Acknowledgments

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Conspectus Figure

single molecule fluorescence microscopy

structural studies

In vitro enzymology   cellular assays

OG:A