A role for N6-methyladenine in DNA damage repair:

Prepared as an opinion for TiBS

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Abstract

The leading cause of mutation due to oxidative damage is 8-oxo-2'-deoxyguanosine (**8-oxoG**, see Glossary) mispairing with adenine (Ade), which can occur in two ways. First, guanine of a G:C DNA base pair can be oxidized. If not repaired in time, DNA polymerases can mispair Ade with 8-oxoG in the template. This 8-oxoG:A can be repaired by enzymes that remove Ade opposite to template 8-oxoG, or 8-oxoG opposite to Cyt. Second, free 8-oxo-dGTP can be misincorporated by DNA polymerases into DNA opposite template Ade. However, there is no known repair activity that removes 8-oxoG opposite to template Ade. We suggest that a major role of N6-methyladenine in mammalian DNA is minimizing incorporation of 8-oxoG opposite to Ade by DNA polymerases following adduct formation.

Keywords

8-oxoguanine; DNA adenine methylation; single-stranded DNA; MettL3-MettL14; YTHDC1

Generation of ssDNA segment during repair

Genomic DNA is constantly subject to damage, induced by incidents from UV irradiation and oxidative stress to environmental mutagens and cancer chemotherapeutic drugs [1]. **Nucleotide excision repair** (NER, see Glossary) is the main pathway used by mammals to remove bulky DNA lesions [2]. Mutations in NER proteins are associated with (at least) three inherited human diseases: Xeroderma pigmentosum with its tremendously increased risk of skin cancer, Cockayne syndrome with its developmental and neurological abnormalities, and Trichothiodystrophy, with its brittle hair, hematological disorders, and intellectual impairment [3, 4]. The NER pathway involves a set of coordinated enzyme activities that recognize a wide range of DNA substrates, including **UV-radiation** induced

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photoproducts (cyclopyrimidine dimers or 6,4-photoproducts). NER proteins unwind the DNA and excise a segment (~25–30 nucleotides) of the damaged strand, spanning the site of damage.

After removal of a stretch of single-strand (ss)DNA containing the lesion, **repair-associated DNA synthesis** requires DNA replication proteins for gap-filling synthesis by DNA polymerase(s), and ligation. DNA polymerase beta (β) is required for base excision repair [5], but other polymerases including Y family members delta (δ), epsilon (ε), and kappa (κ) are important for NER repair synthesis in human cells [6]. Significantly, some DNA polymerases have a higher tendency to misincorporate 8-oxo-dGTP into DNA opposite template adenosines, using **Hoogsteen pairing** [7–10] (Figure 1; compare panels A and C). This 8-oxoG:A pairing requires the exocyclic N6 amino group of adenosine, which has implications we will discuss below. First, we will summarize what is known about sources of 8-oxoG.

Origins and repair of 8-oxoG in DNA

Free 8-oxo-dGTP in the deoxynucleotide triphosphate pool is believed to result from (age-related) oxidative stress [11–14]. There are cellular quality control activities such as MTH1 8-oxo-dGTPase (a MutT ortholog also called oxidized purine nucleoside triphosphatase). Mutant studies reveal that MTH1 reduces 8-oxoG nucleotide pool levels by about a third – from a range of 3–6% down to 2–4% of all guanine nucleotides [15] – but the remainder still poses a significant risk for incorporation into DNA. Surprisingly, the efficiency of β polymerase 8-oxo-dGTP incorporation opposite a template Ade is higher, by a factor of 40, than that opposite the normal (for Gua) Watson-Crick template Cyt [10, 16]. This 8-oxoG:A misincorporation, if it cannot be prevented or repaired appropriately, results in a transversion mutation from T:A to G:C (or T→G in which the mutation is represented by the pyrimidine of the base pair) (Figure 2A). Earlier studies revealed that MutT-deficient *Escherichia coli* show 1000-fold higher frequency of T→G transversion than wild type cells [17]; mutations arising in MutT-deficient cells are strictly T→G transversions [18]. Mouse Mth1 protein substantially reduces T→G mutations when expressed in MutT-deficient bacterial cells [19]. Mice lacking Mth1 activity were shown to be hypersusceptible to lung, liver and stomach cancers [20]. In humans, age-associated T→G somatic mutations are more abundant in liver than in colon and small intestine [21], and are significantly elevated, for example, in esophageal adenocarcinoma and B-cell non-Hodgkin lymphoma [22].

This opinion piece is focused on a means of preventing 8-oxoG incorporation during repair synthesis but, for completeness, we note that oxidation can occur directly to a G:C base pair already in the DNA, resulting in an 8-oxoG:C pair (Figure 2B, top). This is particularly likely to occur when the paired Cyt is methylated (5-methylcytosine), which may have epigenetic consequences [23]. 8-oxoG is the main product of oxidative damage to both DNA and nucleotide pools (reviewed in [24]). In fact, there is evidence that 8-oxoG presence in DNA is used as a gene-regulatory signal of oxidative damage [25].

Base excision repair (BER) enzymes such as the DNA glycosylase OGG1 from eukaryotes or formamidopyrimidine-DNA-glycosylase (Fpg) from *Escherichia coli* remove 8-oxoG

from DNA when it is paired with cytosine (8-oxoG:C) [26] (Figure 2B). However, if not repaired in time, the replication DNA polymerases can misread 8-oxoG in the template and incorporate Ade instead of Cyt [27], and a second round of replication would generate a G:C to T:A transversion (C→A mutation) (Figure 2B). Importantly, after removing the misincorporated Ade by the MutY glycosylase homolog (MYH), DNA polymerase λ - together with proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A) - correctly incorporates dCTP (instead of dATP) opposite 8-oxoG, leading to the formation of C:8-oxoG (Figure 2B) [28] (reviewed in [29] and references therein).

Misincorporation of 8-oxo-dGTP (Figure 2A), rather than direct oxidation of an existing G:C base pair, also yields an 8-oxoG:A mismatch, but the consequences of 8-oxoG:A mismatches are not equal in the two situations. OGG1 is unable to remove 8-oxoG from 8-oxoG:A pairs [30], but they are subject to repair by MYH, which removes the Ade, allowing its eventual replacement with Cyt (Figure 2A). Similarly, in *Saccharomyces cerevisiae*, the MSH2-MSH6 mismatch repair heterodimer removes Ade paired with 8-oxoG [31]. This is a double-edged sword, however: MYH or MSH2-MSH6 do correctly repair 8-oxoG:A resulting from misincorporation of Ade across from 8-oxoG (Figure 2B, middle right); but in the case of misincorporation of 8-oxoG across from Ade this "locks in" a T→G transversion (Figure 2A). Mismatch repair (MMR) systems [32, 33] may also provide an independent supplementary layer of protection by excising 8-oxoG from DNA following incorporation from the dNTP pool [30]; though the extent to which this occurs is not yet clear. The steady-state level of DNA 8-oxoG is significantly elevated in MMR-deficient mouse [34] and human cells [30], and the oxidized purine dNTP pool contributes significantly to the genetic instability of MMR-defective cells [35]. To our knowledge, there is no proven activity that removes 8-oxoG from 8-oxoG:A base pairs. Clearly, the mispairing of 8-oxoG with Ade poses an unacceptable mutation risk, despite the OGG1, MYH, and MMR-associated repair capabilities.

DNA adenine methylation may reduce misincorporation of 8-oxoG

It would be counterproductive to start repairing one type of DNA damage, and end up with another (due to 8-oxoG:A mispairing). A means of preventing 8-oxoG incorporation in the first place would presumably have strong selective value. In fact, there exists a mechanism by which 8-oxoG misincorporation across from Ade can be greatly reduced. Specifically, methylation of the template adenosine at the amino group - required for Hoogsteen pairing - generates **N6-methyladenine** (N6mA). N6mA:8-oxoG is much less stable than A:8-oxoG, resulting in less efficient misincorporation of 8-oxo-dGTP opposite N6mA by polymerases such as human pol β [36]. Examined polymerases exhibit a discrimination factor between 7–19 fold, meaning that there is on average about an order of magnitude lower incorporation of 8-oxo-dGTP opposite N6mA than across from unmethylated Ade [36]. We propose that a major role of N6mA in mammalian DNA is minimizing incorporation of 8-oxoG opposite to A by DNA polymerases.

DNA methylation, including generation of N6mA, is common and has long been known to occur in bacteria and archaea [37], where it plays roles in various cellular functions including adenine methylation-directed mismatch repair in *E. coli* [38]. In contrast, N6mA

was detected in mammalian DNA only in 2016 [39], and its further study has grown in parallel with development and application of ultrasensitive mass spectrometry [40–44]. There are three currently-debated issues regarding N6mA in mammalian DNA: (A) whether low-level N6mA can be accurately detected [45–48]; (B) how N6mA in DNA is generated [42, 43, 49–53]; and (C) what the potential functions of N6mA in DNA might be. Here we suggest one functional, highly significant role for N6mA in DNA.

The following considerations prompt us to suggest the potential involvement of DNA adenine methylation in DNA repair. First, the initial observation in embryonic stem cells identified N6mA enrichment in H2A.X deposition regions [39], where H2A.X is a histone variant typically associated with DNA double-strand breaks (DSBs). N6mA DNA modification is also elevated in glioblastoma (a disease partially associated with the cumulative effects of high-dose exposure to ionizing radiation, or to chemical carcinogens), but not in normal adult tissues or mammalian cells [42]. Second, upon ultraviolet irradiation of human sarcoma U2OS cells pretreated with bromodeoxyuridine, the MettL3–14 methyltransferase complex – which generates N6mA in RNA or DNA – is recruited within 2 min to the damaged sites, and MettL3 catalytic activity is required for the DNA repair [54]. Further, DNA polymerase κ requires the catalytic activity of MettL3 for its immediate localization to sites of DNA damage [54]. Third, again in human sarcoma U2OS cells, irradiation with X-rays or treatment with chemicals that induce DSBs leads to colocalization of MettL3 and γH2A.X foci [55]. In addition, YTHDC1 – which binds to N6mA in RNA or DNA – is recruited to DSBs [55]. Fourth, MettL3–14 and YTHDC1 have been characterized extensively as, respectively, generating and binding N6mA in RNA ([56, 57] and references therein). The data on repair of UV-induced and DSB DNA damage suggested a plausible, if complex, RNA methylation-mediated mechanism [54, 55].

Here we offer a simpler model of DNA adenine methylation in preventing or reducing DNA polymerase incorporation of 8-oxoG opposite to A, based on MettL3–14 and YTHDC1 having the respective abilities to methylate and bind N6mA in ssDNA [58, 59] – an intermediate substrate, after excision and before the gap-filling synthesis (Figure 2C). We note that, while Occam's Razor and evolutionary constraints tend to favor simplicity, the RNA-associated and DNA-only mechanisms are not mutually exclusive, and further experiments are needed to clearly distinguish between them.

MettL3–14 methyltransferase complex and YTHDC1 are active on ssDNA, including unpaired DNA

MettL3 and MettL14 are members of a family of class beta methyltransferases [60]. The beta methyltransferases act on the amino groups of adenine or cytosine in DNA or RNA, and have their conserved motifs in a particular order in their amino acid sequences [61]. The beta methyltransferases also have a unique requirement for being multimeric, forming either homo- or hetero-dimers (such as MettL3-MettL14). MettL3–14 has been studied widely for its role in generating N6mA in RNA [56, 57], at the degenerate consensus sequence RR**A**CH (R=purine and H=not G) [62]. Prompted by the observations noted above, we investigated whether the MettL3–14 heterodimer, known for RNA methylation, also possesses methyl

transfer activity onto DNA adenines *in vitro*. In fact, on synthetic substrate oligonucleotides, MettL3–14 shows >10-fold greater catalytic efficiency for methylation of ssDNA than for ssRNA under the same conditions [58]. In addition, MettL3–14 is active on unpaired DNA, but is inactive on double-stranded DNA [58], suggesting a means of targeting methylation to damaged regions of DNA.

For the same reason, we investigated the YTH domain of mammalian YTHDC1 as a N6mA DNA-binding domain *in vitro*. YTH domain-containing proteins have been studied extensively for their ability to bind N6mA-containing RNA in mammalian cells [63–65]. There are five YTH domain-containing proteins in humans (DC1, DC2, DF1, DF2, and DF3), and among them only YTHDC1 is known to localize in the nucleus [66]. We showed that the YTH domain of YTHDC1 binds to ssDNA containing N6mA. Under the same conditions, this YTH binding affinity for N6mA in a DNA context is stronger by a factor of 5 than such binding in an RNA context [59]. For comparison, YTHDF1 is predominantly cytoplasmic, while YTHDF2 migrates to mitotic chromatin in human induced pluripotent stem cells [67], and the YTH domains of these two proteins exhibited the opposite effect from that of YTHDC1, with ~1.5–2X stronger binding to N6mA in ssRNA than in the corresponding DNA. Importantly, no measurable binding was observed to double-stranded (ds)DNA, or to RNA/DNA hybrid oligos containing N6mA on one strand.

A biochemically-derived model for a DNA N6mA role in nucleotide excision repair

Here, we build on the established NER pathway, recruitment of MettL3–14 and YTHDC1 to the UV-induced or DSB damaged site [54, 55], and our *in vitro* characterization of MettL3–14 and YTHDC1 activity on ssDNA – an intermediate substrate between excision and the gap-filling synthesis. We hypothesize that damage-induced generation of N6mA in DNA reduces misincorporation of 8-oxoG opposite to Ade (Figure 2C). UV damage results in bulky DNA adducts – these adducts are mostly cyclothymine dimers and 6,4-photoproducts opposite to the AA dinucleotides. Recognition of this damage by XPC - one of the XP protein factors named from their association (when defective) with Xeroderma pigmentosa - leads to subsequent excision and removal of a segment of ssDNA that contains the lesion, resulting in single-stranded regions in the genome (Figure 2C). Recruitment of MettL3–14 to the damaged sites yields N6mA in the transiently-generated ssDNA, followed by the recruitment of YTHDC1 to N6mA-containing ssDNA. The YTHDC1 could protect against, for example, demethylation by Alkbh1 which also acts on damaged or unpaired DNA [68]. Finally, DNA polymerases use the undamaged, adenine-methylated ssDNA to synthesize a short complementary sequence with little or no 8-oxoG opposite to N6mA. Thus, in this model, the N6mA is not just a signal to recruit proteins, but is an intrinsically-protective modification to potential mutation hotspots.

We note that our simpler DNA-only model is not readily applicable to all steps along the DSB repair pathways, which are complex ([69] and references therein). Choice among the available DSB repair pathways (non-homologous end joining and homologous recombination) is affected by the initiating DNA lesion itself (whether the DNA ends

contain single-stranded tails). Because our DNA-N6mA model is based on transiently ssDNA substrates for MettL3–14 and YTHDC1, the emergence of ssDNA regions (as well as non-B DNA structures of D-loops or resolution of the four-way Holliday junction) during repair – traditionally considered the error-prone step of single-strand annealing [70] – might be where N6mA methylation could have its greatest impact.

It is not yet clear which proteins control mammalian DNA N6mA or its effects

Ever since the initial report of N6mA existence in the DNA of mouse embryonic stem cells [39], the identification of potential adenine DNA methyltransferase(s) has been an unsettled issue. Two recent developments might point to where and when the enzyme or enzyme complex might function. First, the same laboratory that made the initial claim has reported that N6mA in DNA increases during the development of mouse trophoblast stem cells (which eventually give rise to the placenta), specifically at regions of stress-induced DNA double helix destabilization (SIDD) [44]. Single-stranded DNA that persists in that state is one of the possible SIDD aftereffects [71]. Second, the characterized candidates for DNA N6mA writer (MettL3–14), reader (YTHDC1), and eraser (ALKBH1), all prefer locally unpaired DNA substrates *in vitro* [58, 59, 68]. Of these three proteins, the activities of YTHDC1 and ALKBH1 are independent of sequence context aside from the methylated adenine itself. In contrast, MettL3–14 is an adenine methylase complex long studied for its activity on RNA, where it targets the sequence RR**A**CH, with particular specificity for the ApC motif. Interestingly, many nucleic acid-modifying enzymes are able to modify both DNA and RNA, with examples including human MettL4 [51, 72] - another candidate adenine methylase. As noted above, MettL3–14 and YTHDC1 have the respective abilities to methylate and bind N6mA in ssDNA [58, 59]

We suggest that a proper biochemical investigation of proteins that copurify with DNA adenine methylase activity will be needed to properly identify all necessary components of the catalytically active methylase complex. A functional homolog DNA methyltransferase does exist in *Escherichia coli* (M.EcoGII), that meets the requirement of sequence independence aside from the target adenine, ability to act on both nucleic acid types (DNA and RNA), and impartiality regarding strandedness (single or double) [60]. Such a gene might only be expressed at specific time of development, and might be induced only under stress.

Concluding remarks

Our model predicts that the DNA N6mA level will be elevated in cells immediately after repair of DNA damage, since there is no immediate need to remove it from the template strand following repair synthesis. We do note that mammalian Alkbh1 and Alkbh4 have been implicated in DNA N6mA demethylation [39, 42, 43] so, in theory, N6mA accumulation could result from either targeted methylation or targeted depression of demethylating activity. In either case, as damage could occur anywhere in the genome, the appearance of N6mA would appear to be random. The role of N6mA we propose is not to attract repair enzymes, but to prevent 8-oxoG incorporation during repair, so there is in

theory no need to remove the N6mA following repair. In the absence of MettL3–14 and/or YTHDC1, increased incorporation of 8-oxoG should be observed in the genome. We note that the recent study on damage-induced recruitment of MettL3–14 focused on its RNA methylation activity [54, 55]. A genome-wide study on the DNA N6mA induced by UV irradiation, particularly in skin cancer-prone cells, should be carefully examined with considerations of potential experimental caveats [48].

Because the methyltransferase complex MettL3–14 is recruited to damaged sites within 2 min of ultraviolet irradiation, it is possible that methylation occurs before excision. If so, the enzymatic activity of MettL3–14 should be tested on dsDNA containing a photoproduct or other lesion. We note that MettL3–14 is active on dsDNA-containing unpaired, mismatched bases centered on the target adenine, but is inactive on the fully-paired duplex [58]. The effects of template strand N6mA on the activities of NER enzymes should also be examined.

How repair-associated polymerases (δ, ε, and κ) discriminate between damaged and undamaged nucleotides is not well understood. The replicative fidelities of the Y-family enzymes (such as η and κ) are relatively low [73] – they induce mutations at a frequency of $\sim 10^{-3}$ / generation or higher when acting on undamaged templates [74, 75], and this may be one reason for which polymerase κ is kept outside the nucleus under nonstress conditions [76]. Aside from cases in which an elevated mutation rate may yield beneficial adaptations [77–79], it seems counterproductive for cells to use an **error-prone polymerase** to carry out repair synthesis even for a short patch size of 30 nucleotides, which would result in about one error every 30 repair patches. Perhaps an error prevention mechanism such as proposed here serves to adjustably reduce the mutational price paid for the post-polymerase repair. In light of our model, it is critical to determine the effects of DNA damage on the rate of incorporation of 8-oxoG opposite to A *vs.* N6mA.

Finally, the effects of N6mA on the post-polymerase repair enzymes (such as MYH and MMS in Figure 2A) acting on mispaired DNA substrates (8-oxoG:N6mA *vs.* 8-oxoG:A) needs to be examined. Perhaps the adenine methylation provides a sign for the repair enzymes that, when 8-oxoG is nevertheless incorporated opposite Ade, the adenine should not be cleaved from the template (presumptively correct) strand – a strategy used in *E. coli* for strand discrimination during mismatch repair [80]. We have raised some outstanding questions (see Outstanding Questions), but much remains to be learned about the **writers**, **erasers**, **and readers of N6mA** in mammalian DNA, as well as its roles that may transcend control of gene expression.

Acknowledgements

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Outstanding Questions Box:

- Are other RNA adenine methyltransferases active on DNA?

- Does RNaseH-resistant DNA N6mA increase following DNA damage that results in ssDNA?

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- Are the ssDNA regions identified by permanganate enriched for N6mA?

- Do MettL3–14 and/or YTHDC1 mutants show increased 8-oxoG levels in DNA or elevated T →G transversions (particularly in an MTH1 background following oxidative stress)?

- What are the step-by-step mechanisms in recruitment of MettL3–14 and YTHDC1 to the damaged sites?

- Are there direct interactions between MettL3–14 (and YTHDC1) and known DNA repair factors?

Highlights

- DNA polymerases can misincorporate 8-oxoguanine into DNA opposite template adenine.

- **DNA damage induced by ultraviolet and double-strand breaks** recruit the adenine methyltransferase MettL3-MettL14, and N6-methyladenine reader YTHDC1, to the damaged sites.

- **MettL3-MettL14 and YTHDC1 are** active on single-strand DNA, as well as RNA.

- DNA polymerases exhibit reduced misincorporation of 8-oxoguanine opposite N6-methyladenine, compared to opposite unmethylated adenine.

- DNA adenine methylation may thus provide a significant error prevention mechanism during repair.
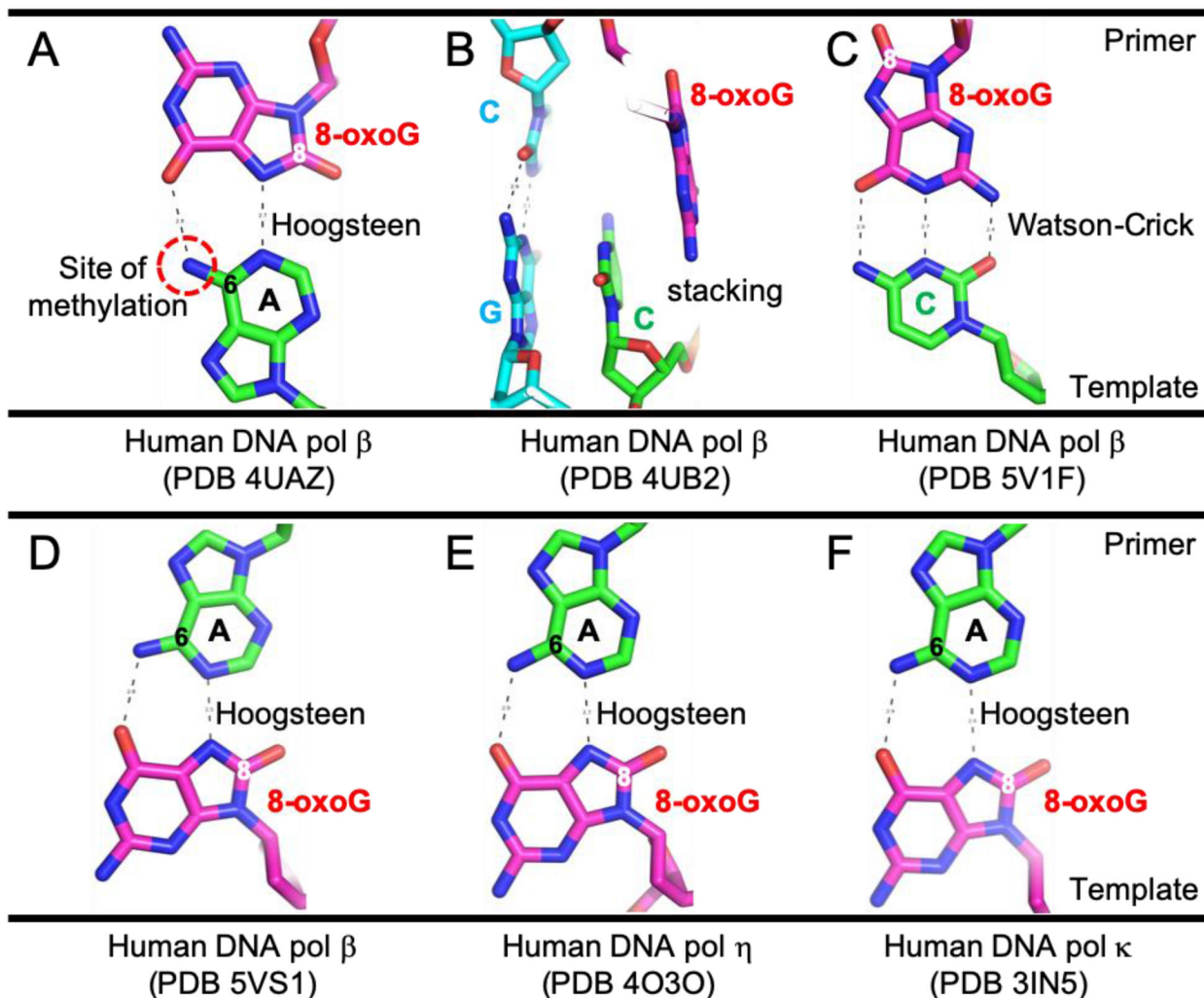
Figure 1. Examples of 8-oxoG:A mispairs by DNA polymerases.
(**A**) 8-oxoG incorporation opposite to template Ade. (**B-C**) For comparison, 8-oxoG incorporation opposite to Cyt involves steps of stacking (panel B) and Watson-Crick pairing (panel C). 8-oxoG in the *syn*-conformation pairs with Ade (panel A), whereas 8-oxoG in the *anti*-conformation pairs with Cyt (panel C). The two conformations are rotated ~180° relative to each other, along the glycosidic bond that joins base and sugar. (**D-F**) Ade incorporation opposite to template 8-oxoG in the context of three different DNA polymerases.
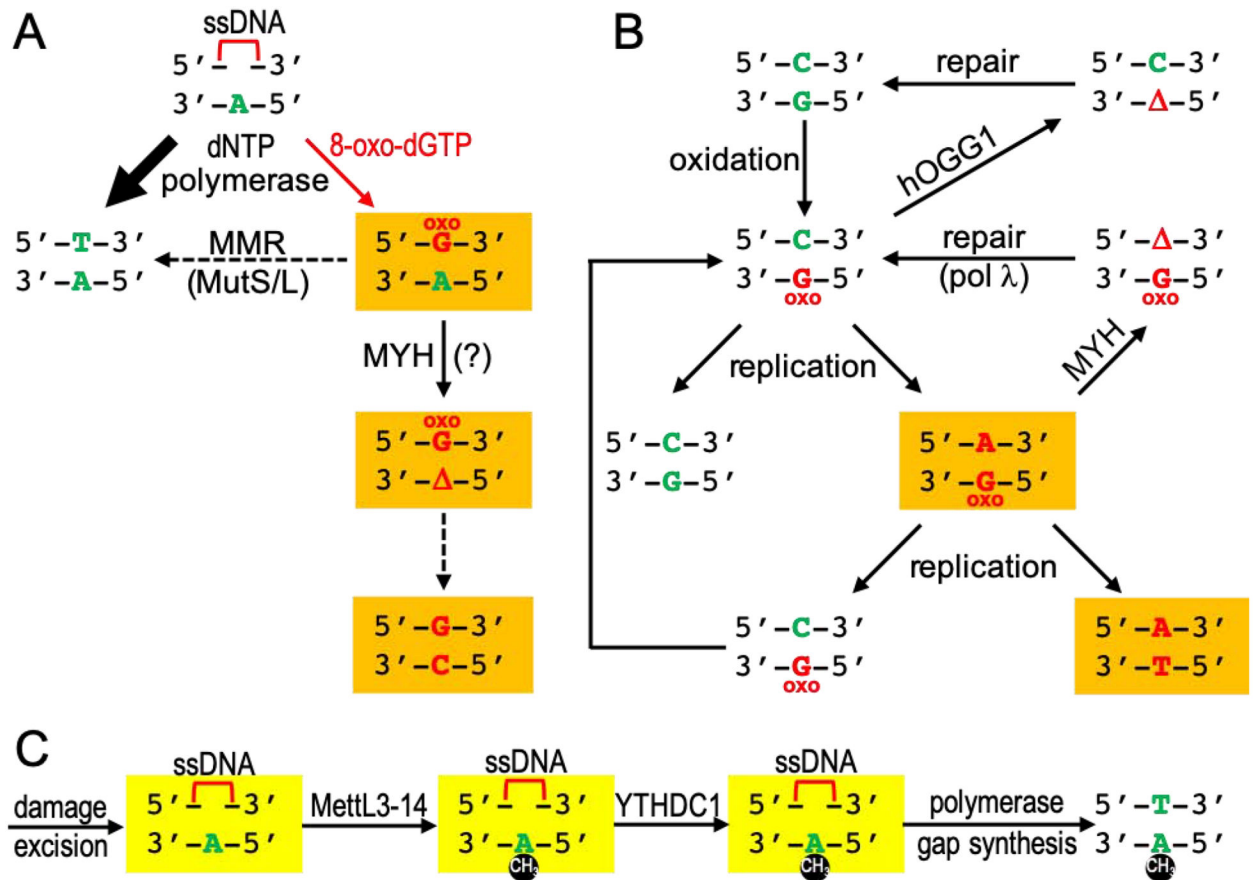
Figure 2. A biochemically-derived model for a DNA N6mA role in nucleotide excision repair.
(**A**) The red bracket represents an ssDNA gap (~25–30 nt) after nucleotide excision (top).
DNA synthesis would be largely accurate because T is preferentially inserted opposite A
(left bold arrow). However, reactive oxygen species can lead to the formation of 8-oxo-
dGTP in the dNTP pool, and in the event that 8-oxo-dGTP is incorporated into DNA during
synthesis, it may be either correctly base-paired opposite C (see Figure 1C) or mispaired
opposite Ade (right red arrow; see Figure 1A). In the latter event, MMR most likely excises
incorporated 8-oxoG from newly synthesized daughter DNA (dashed line) or base excision
of the (correct) Ade by the MYH DNA glycosylase can result in a T→G transversion
mutation (orange boxes). (**B**) Oxidation can occur directly to a G:C base pair already in the
DNA, resulting in an 8-oxoG:C pair. The adduct can be excised from DNA by hOGG1
glycosylase, and subsequently repaired via base excision repair (BER) to restore the original
G:C base pair (top right). If the 8-oxoG adduct is not removed prior to DNA replication,
DNA synthesis may retain the 8-oxoG and form an 8-oxoG:A mispair (middle right, orange
box; see also Figure 1D–F). The misincorporated Ade can be removed by the MYH DNA
glycosylase and replaced by C in the single-nucleotide gap via polymerase λ, yielding a
further chance for removal of the 8-oxoG lesion by hOGG1. If not repaired in time, a second
round of replication can yield a C→A transversion mutation (orange box, bottom right). (**C**)
A model of N6mA reducing misincorporation of 8-oxoG opposite to Ade. Between the

nucleotide excision and DNA synthesis, a transient ssDNA gap (~25–30 nt; the red bracket) can become methylated by MettL3–14 and protected by YTHDC1.