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Delineating the Roles of Genotoxic Stressors in Adaptive Mutations and DNA Repair Inactivation

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B.S., The University of North Carolina at Chapel Hill, 2008

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Abstract

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By Jordan Frederick Morreall

DNA damage threatens genomic integrity by inducing mutations. Although mutations can arise prior to selection, they can also arise under selective non-growth conditions. During transcription, RNA polymerase can bypass DNA damage and generate mutant transcripts, called transcriptional mutagenesis (TM). TM can encode a mutant protein allowing a cell to switch to a pro-growth state, causing DNA replication that bypasses the original DNA lesion and encodes analogous mutations in a process called retromutagenesis. One major goal of this work is to determine how retromutagenesis can contribute to adaptive mutations, which allow cells to escape selection. To study retromutagenesis, we constructed *Escherichia coli* strains containing a premature stop codon in *lacZ* preventing growth on lactose-selective media. Nitrous-acid mutagenesis then gave rise to different revertant mutations indicative of damage to the transcribed or non-transcribed strand of the stop codon. After mutagenized cells were incubated in rich broth before growth on selective media, revertant colonies contained similar numbers of mutations on both strands, indicating similar mutagenic sensitivity of the two strands. However, revertant colonies arising after immediate selection contained mutations almost exclusively on the transcribed strand, implicating retromutagenesis. Other studies in this work examined loss in repair activity of mammalian cells under oxidative stress. A major context of physiological oxidative stress is inflammation, which can be mediated by the cytokine tumor necrosis factor alpha (TNF-α), implicated in every stage of cancer. TNF-α induces oxidative DNA lesions such as 8-oxoguanine, excised by 8-oxoguanine glycosylase 1 (OGG1). One common *Ogg1* allelic variant is S326C-*Ogg1*, which is associated with various forms of cancer and is known to be inhibited by oxidative stress. However, the impact of inflammatory cytokines on OGG1 variant repair activity remains poorly understood. We determined that S326C-OGG1 activity is impaired after exposure to H_2O_2. Also, we found that TNF-α induces oxidative stress that causes DNA damage and inactivates S326C-OGG1 *in vitro*, as well as in a cellular DNA repair assay. These experiments help explain the increased risk of cancer among S326C-*Ogg1* individuals. Ultimately, examining the roles of retromutagenesis and inflammation in response to genotoxic insults could contribute to a better understanding of human pathologies.

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CHAPTER 1

GENERAL INTRODUCTION

1. DNA Damage

DNA is the molecule of heredity for both prokaryotes and eukaryotes. As such, its integrity is vital to the propagation of healthy offspring, both at an organismal and cellular level (1). However, cellular DNA is under constant attack from a variety of endogenous and exogenous sources (2). There is are a multiplicity of forms of DNA damage, each of which gives rise to a different spectrum of mutations based on the base-miscoding properties of the damaged nucleotide. Exogenous sources of DNA damage include UV light, ionizing radiation, free oxygen radicals, alkylating compounds, polycyclic aromatic hydrocarbons, biphenyls, and heterocyclic amines (3). DNA damage is strongly associated with the accumulation of mutations, as well as the development of cancer in higher organisms (4). Mutations often cause dysregulation in the cellular pathways necessary to the maintenance of genomic integrity, such as a DNA damage repair pathway (5). Alternatively, mutations can arise from DNA-damaging species within the cell (6). There are many forms of DNA damage, including oxidation, deamination, methylation, and depurination. Endogenous sources of oxidation cause the greatest amount of mutagenic DNA damage, largely arising from the electron transport chain within the mitochondrial membrane (7).

1.1. Oxidative DNA Damage

One of the most common forms of DNA damage occurs through oxidation (7). Oxidation of DNA bases occurs when a cell is under oxidative stress (8). Oxidative

stress is the cellular condition in which more oxidizing species exist than can be neutralized by cellular antioxidants, which can cause damage to proteins, lipids, and nucleic acids (9). Oxidizing species arise from many sources, both endogenous and exogenous. Mitochondria produce oxidizing species, a natural byproduct of the electron transport chain (10). In metabolically normal cells, such endogenous oxidizing species do not give rise to oxidative stress (11).

Reactive oxygen species (ROS) encompass a variety of molecules. Such molecules include hydrogen peroxide (H_2O_2), superoxide ($^{\cdot}O_2^-$), and the hydroxyl radical ($^{\cdot}OH$) (12). ROS are thought to damage DNA via conversion into the hydroxyl radical through the Fenton reaction (13). Oxidative attack occurs at a variety of molecular sites within the nucleotide (**Figure 1**) (14). Different ROS molecules have different affinities for reaction with each nucleotide, and each ROS is targeted with different affinity by cellular antioxidant systems (15). Therefore, each nucleotide exhibits a different response to oxidative stressors, and the complexity of such responses may underlie the heterogeneity among responses to oxidative stressors (16).

ROS cause oxidative damage to nucleotides. Among the nucleotides, guanine is one of the most vulnerable to oxidation. One of the most common forms in which oxidatively-damaged guanine is found is 8-oxoguanine (8-oxodG) (17). 8-oxodG is mutagenic both at the level of transcription and replication because 8-oxodG undergoes base mispairing with adenine at a higher frequency than does undamaged guanine (18).

One molecule that causes DNA oxidation is potassium bromate ($KBrO_3$). In particular, treatment of cells with $KBrO_3$ causes a dramatic increase in 8-oxodG. However, it does not contribute to oxidation of amino acids (19), and in these studies,

serves as a control by which to discriminate between the effects of guanine oxidation and cysteine oxidation.

1.2. Deamination of DNA

Another form of ubiquitous DNA damage is deamination. Nucleotides undergo spontaneous deamination at a relatively slow rate, but deaminated bases can undergo mutagenic base mispairing (20). The rate of nucleotide deamination increases in the absence of DNA deamination repair enzymes, such as endonuclease V (Endo V) (21) in *E. coli* (22). Likewise, DNA deamination is substantially increased in the presence of an exogenous deaminating agent, such as sodium nitrite ($NaNO_2$) (23). $NaNO_2$ primarily reacts with adenine to give rise to hypoxanthine (H_x), which gives rise to AT>GC mutations due to the altered base pairing specificity of H_x during replication (24).

1.3. Ultraviolet Radiation Damage to DNA

One of the most potent ubiquitous environmental mutagens, ultraviolet (UV) radiation reaches Earth in light emitted by the sun. UV irradiation causes increased levels of cellular ROS, partly via the dissociation of H_2O molecules, which directly gives rise to a variety of oxygen radicals (25). Chronic exposure to UV causes direct damage to DNA, including cyclobutane pyrimidine dimers, 6-4 photoproducts (26), and 8-oxodG (26), and is highly correlated with the development of skin cancer. Likewise, ionizing radiation, largely arising from cosmic radiation, causes DNA damage through the dissociation of H_2O into ROS that cause DNA damage (27).

1.4. Biological Consequences of DNA Damage

1.4.1. Adaptive Mutagenesis

Historically, mutagenesis has primarily been considered as a consequence of replication. As such, studies of mutagenesis have typically employed continuously dividing cells under non-selective conditions (28). However, bacteria in selective conditions undergo a different set of mutations than do bacteria in non-selective conditions (29). Such selective conditions are expected to be a more biologically relevant model for mutagenesis because bacterial cells in an environmental or physiological context do not undergo continuous cell division (30). However, bacteria undergo mutagenesis even in the absence of continuous cell division. Mutations arising in stationary-phase cells have even been demonstrated to contribute to antibiotic resistance (31). Some relatively recent studies have focused on mutagenesis occurring in non-dividing cells that allow them to overcome growth arrest, called adaptive mutagenesis (32). Adaptive mutagenesis has been explained by a variety of mechanisms (33), including homologous recombination (34), amplification (35), and recombination (36) (**Table 1**).

Most of the characterized mechanisms of adaptive mutagenesis describe ways by which DNA is modified (**Figure 2**) (37-39). However, adaptive mutagenesis could occur through the expression of cryptic metabolic genes (40), or because of DNA lesions causing altered base specificity in transcribed genes (41). The latter transcription-dependent model of adaptive mutagenesis has been buttressed by many studies illustrating phenotypic reversion occurring in stationary-phase cells, often many days

after the imposition of selection (42), thought to demonstrate the absence of revertant mutations prior to selection. Many experiments have illustrated that such reversion is dependent on unrepaired DNA damage (43, 44). Collectively, the studies described raised the possibility that adaptive mutagenesis may occur through a transcription-dependent mechanism.

1.4.2. Transcriptional Mutagenesis

Damaged DNA bases give rise to the same mutations in both DNA and RNA, specific to their base-miscoding properties (46). However, such mutations arise only when the relevant polymerase is capable of bypassing the causative DNA lesion (47-50). The process by which RNA polymerase bypasses DNA lesions and generates mutant transcripts is called transcriptional mutagenesis (TM) (**Figure 3**) (51). This process has been characterized *in vitro* at sites of various DNA damage, including abasic sites (52), dihydrouracil (51, 53), uracil, O^6-methylguanine, and 8-oxoguanine (54, 55). *In vivo*, a slightly different set of DNA lesions have been shown to mediate transcriptional mutagenesis, including 8-oxoguanine (18), 6-thioguanine (56), uracil (57), and O^6-methylguanine (58). The DNA lesions giving rise to TM have each been shown to have different levels of mutagenicity (59).

Transcriptional mutagenesis only occurs when RNA polymerase encounters a mutagenic DNA lesion that can be bypassed by the polymerase (60). The bypass of DNA lesions can occur even after prolonged stalling of the RNA polymerase (61). DNA lesions are bypassed with different levels of efficiency depending on the type of RNA polymerase involved, i.e., the organism in which they are found (62, 63). Interestingly,

some DNA lesions may undergo a change in polymerase blocking properties after interaction with repair enzymes (64).

The consequences of TM have yet to be fully appreciated, as TM has been shown to contribute to only a handful of phenotypes. Previous studies using reporter systems have illustrated that TM can give rise to the expression of luciferase in *E. coli* (65, 66) and in mammalian cells (67), and, using a different reporter system, p-ERK in mammalian cells (18). Since TM gives rise to mutant proteins that could allow a cell to undergo replication before repair of the causative DNA damage, TM may promote DNA mutations via DNA polymerase bypass of the same DNA damage that caused TM, a phenomenon known as retromutagenesis (**Fig. 4**) (68). Since it arises from TM, which occurs from damage to the transcribed strand, retromutagenesis should give rise to mutations on the transcribed strand. It is critical to note that RNA and DNA polymerase have been found to give rise to analogous mutations when bypassing DNA damage (53). Presumably through retromutagenesis, there is substantial evidence that TM may contribute to biological endpoints as diverse as tumorigenesis (69, 70) and antibiotic resistance (71). Retromutagenesis is thought to be an important mechanism by which cells are capable of "testing" mutations in a transient pool of relatively short-lived proteins before such mutations are established in the DNA of a dividing cell population, and thus may have considerable evolutionary importance (72).

1.4.3. Antibiotic Resistance

In bacteria, one of the major pathological consequences of mutagenesis is the acquisition of antibiotic resistance. Some antibiotics have cytotoxic effects, although most antibiotics are cytostatic, meaning that cells under their treatment would be subject to growth arrest (73). Antibiotic resistance occurs whenever a bacterium is able to overcome the cytostatic or cytotoxic effects of a given antibiotic concentration.

Antibiotic resistance arises from any genetic mechanism allowing a cell to lessen its exposure or vulnerability to a given antibiotic. These mechanisms include sequestering antibiotic targets, enzymatically degrading the antibiotic, over-expression of target proteins, or the alteration of antibiotic targets (74). There are many genetic mechanisms by which antibiotic resistance can originate, including conjugation, transduction, transformation, recombination, and point mutations (75-79). Point mutations, such as those caused by TM, contribute to resistance to a subset of important antibiotics, predominantly the fluoroquinolones (80). Such point mutations alter the target of fluoroquinolone activity (81, 82). Individual mutations have been shown to iteratively increase the level of antibiotic resistance within a population, and thus each may provide a selective advantage that allows them to be established within a dividing population of cells (83). The point mutations generated by TM may play a role in the development of antibiotic resistance. Interestingly, mutations that promote antibiotic resistance can themselves increase translational infidelity, increasing the likelihood of mutant proteins arising (84).

In wild-type cells, an individual mutagenic lesion would not be expected to exist for a sufficient duration to give rise to many mutant transcripts. However, in cells containing a hypomorphic allele of a repair enzyme, or a repair enzyme whose activity is compromised, such lesions may persist long enough to drive transcriptional and/or replicative mutagenesis. This is especially possible when a cell undergoes chronic oxidative stress, as is seen in infected microenvironments and in the gut (85, 86). Indeed, oxidative stress has been demonstrated to contribute to antibiotic resistance (87), possibly via TM. TM may be particularly important in generating antibiotic resistance-associated mutations because bacteria under non-growth conditions have been shown to give rise to antibiotic resistance (88).

1.4.4. Inflammatory Microenvironment

Some of the most genotoxic physiological microenvironments arise during inflammation. Inflammation is a physiological response to a variety of stimuli, including necrotic cell death. Inflammatory signaling causes the recruitment of cells that promote angiogenesis, invasion, and proliferation. This pro-growth signaling poses a hazard to viable cells in an inflammatory microenvironment, as chronic inflammation has been shown to be associated with tumorigenesis (89-91). Another stimulus that provokes inflammation is bacterial infection. For example, infection with *Streptococcus pneumoniae* has been observed to promote inflammation through the recruitment of neutrophils that cause oxidative stress (92). Therefore, cells in either a tumor or infected microenvironment can be expected to undergo substantial oxidative stress (93).

Oxidative stress causes damage to a variety of molecular targets, partly determined by the source of the oxidative stress itself (94).

ROS cause oxidative damage to amino acids as well as nucleic acids. The amino acid most sensitive to oxidation is cysteine, whose thiol group is easily oxidized. Cysteine thiol oxidation can cause a multiplicity of biological effects, depending on the protein in which the cysteine is present and the particular oxidation state of the thiol group (95). Such oxidation states include the sulfenic acid (SOH), sulfinic acid (SO_2H), sulfonic acid (SO_3H), nitrosothiols (SNO), and disulfide bonds (PrSSPr) (16). Some of these oxidation states are reversible, such as sulfenic acid (96). The variety of often-reversible cysteine modifications provides a modality for protein regulation or pathogenesis, depending on its context (97). Oxidized cysteines can undergo disulfide bond formation, which creates secondary interactions that can alter the mobility and function of a protein (98-100).

1.4.5. Tumorigenesis

DNA damage can give rise to mutagenic DNA bases, which substantially increase the rate of mutagenesis. Such mutagenesis can cause the dysregulation of any cellular pathway, and in particular, a number of pathways necessary to prevent tumorigenesis (101). In fact, the genomic instability that arises from chronic mutagenesis is an important characteristic of cancer. Cancer is associated with exposure to a variety of mutagens, including ionizing radiation, UV irradiation, and polycyclic aromatic

hydrocarbons (PAHs). One common property of all these mutagens is the ability to induce oxidative stress (102).

Tumorigenesis is associated with the oxidative stress, partly due to the mutagenesis arising from the oxidation of nucleotides (103). In tumor cells, up to 200 modified nucleosides have been measured per 10^5 nucleosides (104). Such high levels of DNA damage present a challenge to the repair capacity of a wild-type cell, and a cell with a dysfunctional repair phenotype will incur an even more elevated frequency of mutations as a result (**Figure 5**). Higher mutation frequencies increase the likelihood of tumorigenesis (105).

The oxidative stress associated with tumorigenesis is particularly pernicious during inflammation. Inflammation is often found within tumors, and involves the signaling of networks of cytokines that promote the recruitment of cells such as neutrophils that cause an increased level of oxidative stress. This oxidative stress, in turn, promotes the pathways such as JNK that mediate increased levels of ROS in a positive feedback loop. Such feedback loops help explain why tumor microenvironments are often subject to chronic oxidative stress (106).

2. DNA Damage Repair Systems

DNA damage can take several different forms. Some sources of damage, such as UV irradiation and chemotherapeutic agents, can cause crosslinks to form between DNA bases. Other sources of damage can damage DNA bases in such a way that gives rise to bulky, strand-distorting DNA adducts, all typically repaired by the nucleotide excision

repair (NER) pathway. NER occurs through two sub-pathways: global genomic repair (GGR) and transcription-coupled repair (TCR) (107). TCR occurs when RNA polymerase arrests at a lesion in the transcribed strand, allowing the polymerase to bind to proteins that target RNA polymerase in elongation mode. However the oxidative and deaminative DNA lesions in these studies are non-bulky, decreasing the likelihood of the arrest required for TCR, and are primarily repaired by the base excision repair (BER) pathway. There are several other mechanisms by which DNA can be repaired, including direct repair, double-strand break repair, and crosslink repair (108). In addition, one enzyme important in the studies herein, Endo V, initiates an alternative excision repair pathway in *E. coli* (109). Among the DNA damage repair systems mentioned, the systems most relevant to the studies described herein will be reviewed below.

2.1. Base Excision Repair (BER)

The base excision repair (BER) pathway repairs primarily non-helix distorting DNA damage. Such damage encompasses oxidation, alkylation, hydrolysis, and single-strand breaks (110, 111). BER is carried out by a number of cellular enzymes that first recognize a specific DNA lesion, remove it, and then replace it with an undamaged nucleotide. BER begins when a DNA lesion is recognized by a glycosylase specific to the DNA lesion (112). Such recognition is thought to occur through a combination of glycosylase sliding along DNA strands and transfer among DNA strands, a process known as facilitated diffusion (113). The glycosylase cleaves the N-glycosidic bond connecting the DNA lesion to the DNA backbone, releasing the DNA lesion and leaving an apurinic/apyrimidinic (AP) site. The AP site is then cleaved by AP endonucleases or

by the lyase activity of a bifunctional glycosylase enzyme. The resulting 3' and 5' ends of the DNA backbone are then processed to convert them into a 3' hydroxyl and 5' phosphate group. This processing allows DNA polymerase to synthesize the appropriate DNA nucleotides to replace the DNA lesion. Finally, DNA ligase seals the nicks in the DNA backbone to complete BER (**Figure 6**) (114).

One of the most common forms of oxidative DNA damage is 8-oxo-7,8-dihydroguanine (8-oxodG). 8-oxodG undergoes base mispairing with adenine (**Figure 7**), which, if present during replication, can lead to GC>TA mutations (115). This DNA lesion arises in non-random patterns across the human genome, which suggests that there may be "hot spots" for such oxidative, mutagenic DNA damage across the genome (116). Likewise, the repair of 8-oxodG is influenced by the sequence context in which it is found (117).

The enzyme primarily responsible for the excision of 8-oxodG in humans is the human 8-oxoguanine glycosylase 1 enzyme (*hOgg1*) (118, 119). *Ogg1* is located on the petite arm of human chromosome 3, and encodes two different isoforms, isoform 1a, localized primarily to the nucleus, and isoform 2a, localized primarily to the mitochondrion (120, 121). For the purposes of the studies described herein, the nuclear isoform 1a will be the focus. OGG1 isoform 1a is a roughly 39 kDa enzyme containing 345 amino acids. The structure of most residues of OGG1 has been resolved (residues 1 – 325) (122). The residues necessary for DNA binding include residue 319, but the enzymatic roles of the more C-terminal residues have not been identified. OGG1 cleaves the N-glycosidic bond connecting 8-oxodG to the DNA backbone. However, OGG1 targets other DNA lesions, including fapyG and Me-fapyG (123). OGG1 is a

bifunctional glycosylase, meaning that it also has AP lyase activity, which allows OGG1 to cleave the DNA backbone 3' to AP sites. This AP lyase activity gives OGG1 an even more substantial role in carrying out 8-oxodG repair, since it is capable of participating in multiple steps of the BER pathway (124). OGG1 has a demonstrable importance in preventing mutagenesis and tumorigenesis because OGG1 deficiency has been found to be associated with a variety of cancers (125), including colorectal adenocarcinoma (126).

The wild-type OGG1 enzyme catalyzes the efficient excision of 8-oxodG from DNA (127, 128). However, there are several variants of this enzyme that undergo decreased rates of enzymatic activity (129-131). Some of these enzymes are well-characterized as hypomorphic variants, such as R154H-OGG1, while the intrinsic activity level of other variants is controversial. For example, there have been *in vitro* measurements of the activity of S326C-OGG1 showing a decreased enzymatic activity (132, 133), but other studies have shown that this variant undergoes decreased activity only under oxidative stress (134-138). Cys326 is postulated to undergo disulfide bridge formation, inducing dimerization of S326C-OGG1 enzymes (100). Presumably, such dimerization would inhibit the enzymatic activity of S326C-OGG1 by impairing the mobility of the enzyme. S326C-*Ogg1* has been associated with the development of several different forms of cancer (139-145), especially in the context of oxidative stress (146) or under loss of heterozygosity (147).

2.2. Nucleotide Excision Repair (NER)

Nucleotide excision repair (NER) primarily targets bulky, strand-distorting DNA lesions, which are not addressed within this work. However, one subpathway of NER is

of interest to these studies. NER typically involves the stalling of a polymerase as the site of a DNA lesion, which can cause the recruitment of alternative error-prone polymerases in a phenomenon known as Transcription-Coupled Repair (TCR) (**Figure 8**). TCR can thus cause the preferential repair of DNA damage on the transcribed strand of a gene (148). There are some DNA lesions that give rise to TM and are subject to TCR, such as cyclobutane pyrimidine dimers (149).

2.3. Mismatch Repair (MMR)

Another system by which DNA damage is repaired is mismatch repair (MMR), which removes bases paired incorrectly, as well as removing insertions and deletions arising from polymerase errors (150). In humans, MMR targets adenines mispaired with 8-oxodG via the MUTYH glycosylase (MYH) (151). MYH excises such adenines, and the resulting AP site is then repaired by canonical BER (115).

2.4. Alternative Excision Repair

In *E. coli*, excision repair occurs through a similar process as compared to mammals, but is carried out by a smaller cadre of enzymes. In *E. coli*, one enzyme that initiates excision repair is Endonuclease V (Endo V), an endoribonuclease enzyme, encoded by the *nfi* gene, of about 25 kDA. Unlike other DNA repair glycosylases, Endo V does not release free bases after cleaving its target, and instead cleaves the phosphodiester bond 3' to the DNA lesion. As such, the repair initiated by Endo V is categorized as alternative excision repair (152, 153). The enzyme cleaves single-stranded DNA, uracil in dsDNA, deaminated bases, and DNA damaged by alkali, acid, or UV

radiation (22). Endo V nicks DNA 3' to the lesion being targeted, allowing gap formation, filled in by DNA polymerase, after which the nick is sealed by DNA ligase (154).

2.5. DNA Damage Tolerance Pathways

In cells containing DNA damage that is not repaired during replication, cellular mechanisms exist by which the DNA damage can be tolerated in such a way that allows cellular survival. One such mechanism, homologous recombination (HR), allows cells to repair strand breaks and stalled replication forks (155). Such damage may arise when a cell's repair capacity is exceeded. However, less pernicious forms of DNA damage can also incur DNA damage tolerance pathways (110). Although many forms of non-helix distorting DNA damage can be bypassed by DNA or RNA polymerase, some lesions, when present in the template strand, stimulate the translesion synthesis (156) pathway. TLS involves the utilization of alternative DNA polymerases that can either assist the canonical DNA polymerase in synthesis past a lesion, or can directly synthesize nucleotides opposite a lesion. A variety of lesions stimulate TLS, including 8-oxodG, uracil, and AP sites *in vitro* (157).

2.6 DNA Repair in Euchromatin and Heterochromatin

Eukaryotic DNA exists within a multi-tiered structure of proteins that, together, is called chromatin. Chromatin is organized into nucleosomes, which encompass approximately 147 DNA base pairs wrapped around a network of proteins called histones (158). Different regions of chromatin undergo different levels of compaction, regulated

largely by post-translational modification of histones in concert with the SWI/SNF remodeling enzyme family (159). Highly compacted chromatin, called heterochromatin, tends to prevent proteins from accessing the DNA within. Conversely, loosely compacted chromatin, called euchromatin, tends to facilitate the interaction between proteins and the DNA therein. Euchromatin tends to more readily undergo interactions with DNA repair proteins (160).

Consistent with these generalizations, OGG1 has been found to be excluded from heterochromatin (161). Likewise, OGG1 repair complexes have been found to localize primarily to euchromatin, presumably because of the greater accessibility of the DNA therein (162). 8-oxodG is repaired much less efficiently when it is located within telomeric heterochromatin regions, increasing the likelihood of mutagenesis (163). Interestingly, SWI/SNF exposure can drive 8-oxodG cleavage activity in nucleosomal DNA at a rate similar to the level of non-nucleosomal DNA (164). Therefore, the genomic context in which 8-oxodG is found may have a profound effect on its biological consequences.

3. Determining Mechanisms by which Genotoxic Stress Hinders Genomic Stability

The defective DNA repair phenotypes described in these studies may lead to genetic instability, a decreased ability of cells to confer intact genetic material (165). The studies described in this work investigate the capacity of cells to limit genetic instability during defective 8-oxodG repair.

3.1. Measuring the Contribution of Transcriptional Mutagenesis to Adaptive Mutagenesis

The studies in bacterial cells described herein measure the degree to which DNA damage in non-dividing bacterial cells induces phenotypic reversion. Importantly, phenotypic reversion is typically thought to arise from mutation, which is thought to occur during replication. However, our studies support a model in which DNA damage gives rise to mutant transcripts, which allow a cell to undergo phenotypic reversion in the absence of replication (166). By understanding the contribution of genotoxic stress to mutagenesis in non-dividing cells, we may one day be able to better determine how to limit pathogenic mutagenesis in physiological and environmental bacteria. Genotoxic stress has been postulated to underpin adaptive mutagenesis (167). Furthermore, transcription has been illustrated to be essential to stress-induced mutagenesis in *E. coli* (168).

3.1.1. Transcription-Associated Mutagenesis

One of the primary structural configurations that limit damage to DNA is double-stranded conformation (169). Genomic DNA is typically found in a double-stranded conformation, but there are genomic loci that can be found in a single-stranded conformation, including telomeric DNA repeats (21). Also, the binding of RNA polymerase to DNA causes the DNA to adopt a single-stranded conformation (170), which causes the non-transcribed strand to undergo damage similar to that of single-stranded DNA. The damage to the non-transcribed strand causes mutagenesis, known as

Transcription-Associated Mutagenesis (TAM) (171). Transcription-associated mutagenesis is different from TM and has been shown to occur in both bacteria and in human genes (172), and gives rise to mutations on the non-transcribed strand of genes (173). The nature of these mutations varies depending on the locus at which transcription is induced (174).

TAM could be a confounding variable in the measurement of strand-specific mutations, as caused by retromutagenesis (see **Section 1.4.2**). However, the transcription necessary for TAM can be reduced by the supplementation of metabolites that limit the cellular demand for the expression of a given gene (175). The studies described in this work examine strand-specific mutations in *lacZ*, the gene encoding β-galactosidase, which is necessary to metabolize lactose (176). Transcription of *lacZ* can be repressed by supplementing bacteria with glucose, an alternative metabolite to lactose (177), limiting TAM.

3.1.2. Deamination-Induced Mutagenesis

As mentioned, one form of DNA damage occurs through deamination, which can be cause by mutagens such as $NaNO_2$. Following treatment with such nitrosative deaminating agents, the amino groups of nucleotides form *N*-nitroso derivatives that ultimately lead to deamination (178). Such deamination converts adenine into hypoxanthine, although guanine and cytosine can also be subjected to such deamination (179). Hypoxanthine causes AT>GC mutations during replication of cellular DNA (**Figure 9**) (180).

3.2. Studying the Inhibition of S326C-OGG1 Repair Activity

In mammalian cells, defects in enzymes that carry out each step of BER have been associated with disease, but the mechanistic contributions of only a few human BER enzyme variants to disease have been well-defined (**Figure 10**) (181). Presumably, repair enzyme SNPs typically cause deficiencies in enzymatic localization and/or function that, in the context of genotoxic agents (especially enriched during inflammation) contribute to the likelihood of tumorigenesis (182). By understanding whether oxidative stress causes dysfunction in to variant repair enzymes, we may one day be able to better understand how to manage genotoxic and inflammatory stresses in patients so as to minimize their cancer risk.

3.2.1. Tumor Necrosis Factor α (TNF-α)

Oxidative stress can be caused by exposure to TNF-α, an inflammatory cytokine implicated in every step of cancer development (183). TNF-α is notable for its ability to induce chronic oxidative stress through the JNK pathway (184). TNF-α stimulates several signaling pathways, which ultimately can contribute to apoptosis (185).

3.2.2. N-acetylcysteine (NAC)

Oxidative stress is counterbalanced in cells by antioxidant molecules that limit oxidative stress. N-acetylcysteine (NAC) is a precursor to the potent cellular antioxidant glutathione (GSH). Given enough time for NAC to stimulate GSH synthesis, NAC supplementation allows cells to produce enough GSH to buffer a cell against oxidative

stress (186). Therefore, NAC supplementation can limit, and potentially reverse, cellular oxidative stress (187).

4. Summary of Project Objectives

We have utilized *E. coli*, a powerful model organism that we have used to study the genetic changes underlying genotoxically induced phenotypic reversion. Objective 1 (Chapter 2) encompasses the measurement of the degree to which phenotypic reversion is driven by transcriptional mutagenesis (TM). Understanding the role of TM in phenotypic reversion may facilitate our understanding of how to limit mutagenesis in non-dividing cells that contributes to pathological phenotypes, such as the development of resistance to certain antibiotics. We also utilized mouse embryonic fibroblasts (MEFs) as a tool with which to study the degree to which the cellular oxidative stress environment of S326C-OGG1 affects a cell's DNA repair phenotype. Objective 2 (Chapter 3) is focused on measuring the effects of oxidative stressors on the enzymatic and repair activity of S326C-*Ogg1* MEFs. These studies may contribute to a better understanding about the degree to which oxidative stressors drive pathological dysfunction in redox-sensitive enzymes, and possibly help in the management of these stressors.

References

1. Friedberg EC, Aguilera A, Gellert M, Hanawalt PC, Hays JB, Lehmann AR, Lindahl T, Lowndes N, Sarasin A, Wood RD. 2006. DNA repair: from molecular mechanism to human disease. DNA repair 5:986-996.
2. Lindahl T. 1993. Instability and decay of the primary structure of DNA. Nature 362:709-715.
3. Roos WP, Kaina B. 2013. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Lett 332:237-248.
4. Cerutti PA. 1994. OXYRADICALS AND CANCER. Lancet 344:862-863.
5. Watson J. 2013. Oxidants, antioxidants and the current incurability of metastatic cancers. Open biology 3:120144.
6. Halliwell B. 1994. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? The Lancet 344:721-724.
7. Ames BN, Shigenaga MK, Gold LS. 1993. DNA lesions, inducible DNA repair, and cell division: three key factors in mutagenesis and carcinogenesis. Environ Health Perspect 101 Suppl 5:35-44.
8. Yakes FM, Van Houten B. 1997. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proceedings of the National Academy of Sciences of the United States of America 94:514-519.
9. Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408:239-247.

10. Nunez ME, Hall DB, Barton JK. 1999. Long-range oxidative damage to DNA: effects of distance and sequence. Chem. Biol. 6:85-97.

11. Barja G. 2002. Endogenous oxidative stress: relationship to aging, longevity and caloric restriction. Ageing research reviews 1:397-411.

12. Ray PD, Huang BW, Tsuji Y. 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cellular signalling 24:981-990.

13. Dizdaroglu M, Rao G, Halliwell B, Gajewski E. 1991. Damage to the DNA bases in mammalian chromatin by hydrogen peroxide in the presence of ferric and cupric ions. Archives of biochemistry and biophysics 285:317-324.

14. Friedberg EC, G.C.W., Wolfram Siede, Richard D. Wood, Roger A. Schultz,, Ellenberger T. 2006. DNA Repair and Mutagenesis, Second edition ed. ASM Press, Washington, D.C.

15. Wiseman H, Halliwell B. 1996. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. The Biochemical journal 313 (Pt 1):17-29.

16. Wang Y, Yang J, Yi J. 2012. Redox sensing by proteins: oxidative modifications on cysteines and the consequent events. Antioxid Redox Signal 16:649-657.

17. Grollman AP, Moriya M. 1993. Mutagenesis by 8-oxoguanine: an enemy within. Trends in genetics : TIG 9:246-249.

18. Saxowsky TT, Meadows KL, Klungland A, Doetsch PW. 2008. 8-Oxoguanine-mediated transcriptional mutagenesis causes Ras activation in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America 105:18877-18882.

19. Murata M, Bansho Y, Inoue S, Ito K, Ohnishi S, Midorikawa K, Kawanishi S. 2001. Requirement of glutathione and cysteine in guanine-specific oxidation of DNA by carcinogenic potassium bromate. Chem Res Toxicol 14:678-685.

20. Kavli B, Otterlei M, Slupphaug G, Krokan HE. 2007. Uracil in DNA--general mutagen, but normal intermediate in acquired immunity. DNA repair 6:505-516.

21. Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T. 1999. Mammalian telomeres end in a large duplex loop. Cell 97:503-514.

22. Guo G, Ding Y, Weiss B. 1997. nfi, the gene for endonuclease V in Escherichia coli K-12. Journal of bacteriology 179:310-316.

23. Mukai F, Hawryluk I, Shapiro R. 1970. The mutagenic specificity of sodium bisulfite. Biochem Biophys Res Commun 39:983-988.

24. Schouten KA, Weiss B. 1999. Endonuclease V protects Escherichia coli against specific mutations caused by nitrous acid. Mutat Res 435:245-254.

25. Nyska A, Kohen R. 2002. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol. 30:620-650.

26. Mitchell DL. 1988. The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol. Jul;48(1):51-7.

27. Oller AR, Fijalkowska IJ, Dunn RL, Schaaper RM. 1992. Transcription-repair coupling determines the strandedness of ultraviolet mutagenesis in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 89:11036-11040.

28. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. 2003. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17:1195-1214.

29. MacLean RC, Torres-Barcelo C, Moxon R. 2013. Evaluating evolutionary models of stress-induced mutagenesis in bacteria. Nat Rev Genet 14:221-227.

30. Hall BG. 1991. Spectrum of mutations that occur under selective and non-selective conditions in E. coli. Genetica 84:73-76.

31. De Paepe M, Gaboriau-Routhiau V, Rainteau D, Rakotobe S, Taddei F, Cerf-Bensussan N. 2011. Trade-off between bile resistance and nutritional competence drives Escherichia coli diversification in the mouse gut. PLoS genetics 7:e1002107.

32. Petrosino JF, Galhardo RS, Morales LD, Rosenberg SM. 2009. Stress-induced beta-lactam antibiotic resistance mutation and sequences of stationary-phase mutations in the Escherichia coli chromosome. Journal of bacteriology 191:5881-5889.

33. Hall BG. 1998. Adaptive mutagenesis: a process that generates almost exclusively beneficial mutations. Genetica 102-103:109-125.

34. Kivisaar M. 2003. Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. Environ Microbiol 5:814-827.

35. Rosenberg SM, Harris RS, Torkelson J. 1995. Molecular handles on adaptive mutation. Mol Microbiol 18:185-189.

36. Rosenberg SM, Hastings PJ. 2004. Adaptive point mutation and adaptive amplification pathways in the Escherichia coli Lac system: stress responses producing genetic change. Journal of bacteriology 186:4838-4843.

37. Foster PL. 2007. Stress-induced mutagenesis in bacteria. Crit Rev Biochem Mol Biol 42:373-397.

38. Galhardo RS, Hastings PJ, Rosenberg SM. 2007. Mutation as a stress response and the regulation of evolvability. Crit Rev Biochem Mol Biol 42:399-435.

39. McKenzie GJ, Lee PL, Lombardo MJ, Hastings PJ, Rosenberg SM. 2001. SOS mutator DNA polymerase IV functions in adaptive mutation and not adaptive amplification. Molecular cell 7:571-579.

40. Tenaillon O, Denamur E, Matic I. 2004. Evolutionary significance of stress-induced mutagenesis in bacteria. Trends Microbiol 12:264-270.

41. Cairns J, Overbaugh J, Miller S. 1988. The origin of mutants. Nature 335:142-145.

42. Bridges BA. 1993. Spontaneous mutation in stationary-phase Escherichia coli WP2 carrying various DNA repair alleles. Mutat Res 302:173-176.

43. Bridges BA, Sekiguchi M, Tajiri T. 1996. Effect of mutY and mutM/fpg-1 mutations on starvation-associated mutation in Escherichia coli: implications for the role of 7,8-dihydro-8-oxoguanine. Mol Gen Genet 251:352-357.

44. Bjedov I, Tenaillon O, Gerard B, Souza V, Denamur E, Radman M, Taddei F, Matic I. 2003. Stress-induced mutagenesis in bacteria. Science 300:1404-1409.

45. Vidales LE, Cardenas LC, Robleto E, Yasbin RE, Pedraza-Reyes M. 2009. Defects in the error prevention oxidized guanine system potentiate stationary-phase mutagenesis in Bacillus subtilis. Journal of bacteriology 191:506-513.

46. Viswanathan A, Liu J, Doetsch PW. 1999. E. coli RNA polymerase bypass of DNA base damage. Mutagenesis at the level of transcription. Annals of the New York Academy of Sciences 870:386-388.

47. Damsma GE, Alt A, Brueckner F, Carell T, Cramer P. 2007. Mechanism of transcriptional stalling at cisplatin-damaged DNA. Nat Struct Mol Biol 14:1127-1133.

48. Kuraoka I, Endou M, Yamaguchi Y, Wada T, Handa H, Tanaka K. 2003. Effects of endogenous DNA base lesions on transcription elongation by mammalian RNA polymerase II. Implications for transcription-coupled DNA repair and transcriptional mutagenesis. The Journal of biological chemistry 278:7294-7299.

49. Larsen E, Kwon K, Coin F, Egly JM, Klungland A. 2004. Transcription activities at 8-oxoG lesions in DNA. DNA repair 3:1457-1468.

50. Nakanishi N, Fukuoh A, Kang D, Iwai S, Kuraoka I. 2013. Effects of DNA lesions on the transcription reaction of mitochondrial RNA polymerase: implications for bypass RNA synthesis on oxidative DNA lesions. Mutagenesis 28:117-123.

51. Liu J, Zhou W, Doetsch PW. 1995. RNA polymerase bypass at sites of dihydrouracil: implications for transcriptional mutagenesis. Mol Cell Biol 15:6729-6735.

52. Zhou W, Doetsch PW. 1993. Effects of abasic sites and DNA single-strand breaks on prokaryotic RNA polymerases. Proceedings of the National Academy of Sciences of the United States of America 90:6601-6605.

53. Liu J, Doetsch PW. 1998. Escherichia coli RNA and DNA polymerase bypass of dihydrouracil: mutagenic potential via transcription and replication. Nucleic Acids Res 26:1707-1712.

54. Viswanathan A, Doetsch PW. 1998. Effects of nonbulky DNA base damages on Escherichia coli RNA polymerase-mediated elongation and promoter clearance. The Journal of biological chemistry 273:21276-21281.

55. Damsma GE, Cramer P. 2009. Molecular basis of transcriptional mutagenesis at 8-oxoguanine. The Journal of biological chemistry 284:31658-31663.

56. You C, Dai X, Yuan B, Wang Y. 2012. Effects of 6-thioguanine and s6-methylthioguanine on transcription in vitro and in human cells. The Journal of biological chemistry 287:40915-40923.

57. Viswanathan A, You HJ, Doetsch PW. 1999. Phenotypic change caused by transcriptional bypass of uracil in nondividing cells. Science 284:159-162.

58. Burns JA, Dreij K, Cartularo L, Scicchitano DA. 2010. O6-methylguanine induces altered proteins at the level of transcription in human cells. Nucleic Acids Res 38:8178-8187.

59. Charlet-Berguerand N, Feuerhahn S, Kong SE, Ziserman H, Conaway JW, Conaway R, Egly JM. 2006. RNA polymerase II bypass of oxidative DNA damage is regulated by transcription elongation factors. EMBO J 25:5481-5491.

60. Saxowsky TT, Doetsch PW. 2006. RNA polymerase encounters with DNA damage: transcription-coupled repair or transcriptional mutagenesis? Chem Rev 106:474-488.

61. Walmacq C, Cheung AC, Kireeva ML, Lubkowska L, Ye C, Gotte D, Strathern JN, Carell T, Cramer P, Kashlev M. 2012. Mechanism of translesion transcription by RNA polymerase II and its role in cellular resistance to DNA damage. Molecular cell 46:18-29.

62. Chen YH, Bogenhagen DF. 1993. Effects of DNA lesions on transcription elongation by T7 RNA polymerase. The Journal of biological chemistry 268:5849-5855.

63. Pages V, Mazon G, Naiman K, Philippin G, Fuchs RP. 2012. Monitoring bypass of single replication-blocking lesions by damage avoidance in the Escherichia coli chromosome. Nucleic Acids Res 40:9036-9043.

64. Kitsera N, Stathis D, Luhnsdorf B, Muller H, Carell T, Epe B, Khobta A. 2011. 8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1. Nucleic Acids Res 39:5926-5934.

65. Clauson CL, Oestreich KJ, Austin JW, Doetsch PW. 2010. Abasic sites and strand breaks in DNA cause transcriptional mutagenesis in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 107:3657-3662.

66. Bregeon D, Doddridge ZA, You HJ, Weiss B, Doetsch PW. 2003. Transcriptional mutagenesis induced by uracil and 8-oxoguanine in Escherichia coli. Molecular cell 12:959-970.

67. Brégeon D, Peignon PA, Sarasin A. 2009. Transcriptional mutagenesis induced by 8-oxoguanine in mammalian cells. PLoS Genet. Jul;5(7):e1000577. doi: 10.1371/journal.pgen.1000577.

68. Shockley AH, Doo DW, Rodriguez GP, Crouse GF. 2013. Oxidative damage and mutagenesis in Saccharomyces cerevisiae: genetic studies of pathways affecting replication fidelity of 8-oxoguanine. Genetics 195:359-367.

69. Bregeon D, Doetsch PW. 2011. Transcriptional mutagenesis: causes and involvement in tumour development. Nature reviews. Cancer 11:218-227.

70. Rodin SN, Rodin AS, Juhasz A, Holmquist GP. 2002. Cancerous hyper-mutagenesis in p53 genes is possibly associated with transcriptional bypass of DNA lesions. Mutat Res 510:153-168.

71. Morreall J, Petrova L, Doetsch PW. 2013. Transcriptional mutagenesis and its potential roles in the etiology of cancer and bacterial antibiotic resistance. J Cell Physiol.

72. Holmquist GP. 2002. Cell-selfish modes of evolution and mutations directed after transcriptional bypass. Mutat Res 510:141-152.

73. Singh R, Ledesma KR, Chang KT, Tam VH. 2010. Impact of recA on levofloxacin exposure-related resistance development. Antimicrobial agents and chemotherapy 54:4262-4268.

74. Hastings PJ, Slack A, Petrosino JF, Rosenberg SM. 2004. Adaptive amplification and point mutation are independent mechanisms: evidence for various stress-inducible mutation mechanisms. PLoS biology 2:e399.

75. Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, Holmes NE, Rhee HW, Hong JI, Hartland EL, Stinear TP, Howden BP. 2010. Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog 6:e1000944.

76. Martinez JL, Baquero F. 2000. Mutation frequencies and antibiotic resistance. Antimicrobial agents and chemotherapy 44:1771-1777.

77. Melancon P, Lemieux C, Brakier-Gingras L. 1988. A mutation in the 530 loop of Escherichia coli 16S ribosomal RNA causes resistance to streptomycin. Nucleic Acids Res 16:9631-9639.

78. Yamagishi J, Kojima T, Oyamada Y, Fujimoto K, Hattori H, Nakamura S, Inoue M. 1996. Alterations in the DNA topoisomerase IV grlA gene responsible for quinolone resistance in Staphylococcus aureus. Antimicrobial agents and chemotherapy 40:1157-1163.

79. Schmitz FJ, Jones ME, Hofmann B, Hansen B, Scheuring S, Luckefahr M, Fluit A, Verhoef J, Hadding U, Heinz HP, Kohrer K. 1998. Characterization of grlA, grlB, gyrA, and gyrB mutations in 116 unrelated isolates of Staphylococcus aureus and effects of mutations on ciprofloxacin MIC. Antimicrobial agents and chemotherapy 42:1249-1252.

80. Goswitz JJ, Willard KE, Fasching CE, Peterson LR. 1992. Detection of gyrA gene mutations associated with ciprofloxacin resistance in methicillin-resistant Staphylococcus aureus: analysis by polymerase chain reaction and automated direct DNA sequencing. Antimicrobial agents and chemotherapy 36:1166-1169.

81. Ferrero L, Cameron B, Crouzet J. 1995. Analysis of gyrA and grlA mutations in stepwise-selected ciprofloxacin-resistant mutants of Staphylococcus aureus. Antimicrobial agents and chemotherapy 39:1554-1558.

82. Hooper DC. 1999. Mechanisms of fluoroquinolone resistance. Drug Resist Updat 2:38-55.

83. Mwangi MM, Wu SW, Zhou Y, Sieradzki K, de Lencastre H, Richardson P, Bruce D, Rubin E, Myers E, Siggia ED, Tomasz A. 2007. Tracking the in vivo evolution of multidrug resistance in Staphylococcus aureus by whole-genome sequencing. Proceedings of the National Academy of Sciences of the United States of America 104:9451-9456.

84. Brakier-Gingras L, Phoenix P. 1984. The control of accuracy during protein synthesis in Escherichia coli and perturbations of this control by streptomycin, neomycin, or ribosomal mutations. Can J Biochem Cell Biol 62:231-244.

85. Dwyer DJ, Kohanski MA, Collins JJ. 2009. Role of reactive oxygen species in antibiotic action and resistance. Curr Opin Microbiol 12:482-489.

86. Kamada N, Seo SU, Chen GY, Nunez G. 2013. Role of the gut microbiota in immunity and inflammatory disease. Nature reviews. Immunology 13:321-335.

87. Kohanski MA, DePristo MA, Collins JJ. 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Molecular cell 37:311-320.

88. Riesenfeld C, Everett M, Piddock LJ, Hall BG. 1997. Adaptive mutations produce resistance to ciprofloxacin. Antimicrobial agents and chemotherapy 41:2059-2060.

89. Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. Cell 144:646-674.

90. Hakim J. 1993. [Reactive oxygen species and inflammation]. Comptes rendus des seances de la Societe de biologie et de ses filiales 187:286-295.

91. Yusuf R, Frenkel K. 2010. Morphologic transformation of human breast epithelial cells MCF-10A: dependence on an oxidative microenvironment and estrogen/epidermal growth factor receptors. Cancer cell international 10:30.

92. Barbuti G, Moschioni M, Fumarulo R, Censini S, Montemurro P. 2010. Streptococcus pneumoniae modulates the respiratory burst response in human neutrophils. FEMS Immunol Med Microbiol 60:57-62.

93. Ohshima H, Bartsch H. 1994. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. Mutat Res 305:253-264.

94. Halliwell B, Cross CE. 1994. Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect 102 Suppl 10:5-12.

95. Ferre F, Clote P. 2005. DiANNA: a web server for disulfide connectivity prediction. Nucleic Acids Res 33:W230-232.

96. Crump KE, Juneau DG, Poole LB, Haas KM, Grayson JM. 2012. The reversible formation of cysteine sulfenic acid promotes B-cell activation and proliferation. European journal of immunology 42:2152-2164.

97. Bravard A, Vacher M, Gouget B, Coutant A, de Boisferon FH, Marsin S, Chevillard S, Radicella JP. 2006. Redox regulation of human OGG1 activity in response to cellular oxidative stress. Mol Cell Biol 26:7430-7436.

98. Bravard A, Vacher M, Moritz E, Vaslin L, Hall J, Epe B, Radicella JP. 2009. Oxidation status of human OGG1-S326C polymorphic variant determines cellular DNA repair capacity. Cancer research 69:3642-3649.

99. Simonelli V, Camerini S, Mazzei F, Van Loon B, Allione A, D'Errico M, Barone F, Minoprio A, Ricceri F, Guarrera S, Russo A, Dalhus B, Crescenzi M, Hubscher U, Bjoras M, Matullo G, Dogliotti E. 2013. Genotype-phenotype analysis of S326C OGG1 polymorphism: a risk factor for oxidative pathologies. Free radical biology & medicine 63:401-409.

100. Hill JW, Evans MK. 2006. Dimerization and opposite base-dependent catalytic impairment of polymorphic S326C OGG1 glycosylase. Nucleic Acids Res 34:1620-1632.

101. Paz-Elizur T, Krupsky M, Blumenstein S, Elinger D, Schechtman E, Livneh Z. 2003. DNA repair activity for oxidative damage and risk of lung cancer. Journal of the National Cancer Institute 95:1312-1319.

102. De Bont R, van Larebeke N. 2004. Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19:169-185.

103. Paz-Elizur T, Sevilya Z, Leitner-Dagan Y, Elinger D, Roisman LC, Livneh Z. 2008. DNA repair of oxidative DNA damage in human carcinogenesis: potential application for cancer risk assessment and prevention. Cancer Lett 266:60-72.

104. Loft S, Poulsen HE. 1996. Cancer risk and oxidative DNA damage in man. J Mol Med (Berl) 74:297-312.

105. Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR. 2007. Patterns of somatic mutation in human cancer genomes. Nature 446:153-158.

106. Balkwill F. 2009. Tumour necrosis factor and cancer. Nature reviews. Cancer 9:361-371.

107. Cleaver JE, Lam ET, Revet I. 2009. Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity. Nat Rev Genet 10:756-768.

108. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annual review of biochemistry 73:39-85.

109. Siede W, Kow YW, Doetsch PW. 2005. DNA damage recognition. Taylor & Francis, Boca Raton.

110. Lindahl T, Wood RD. 1999. Quality control by DNA repair. Science 286:1897-1905.

111. Fu D, Calvo JA, Samson LD. 2012. Balancing repair and tolerance of DNA damage caused by alkylating agents. Nature reviews. Cancer 12:104-120.

112. Dalhus B, Laerdahl JK, Backe PH, Bjoras M. 2009. DNA base repair--recognition and initiation of catalysis. FEMS Microbiol Rev 33:1044-1078.

113. Sampath H, McCullough AK, Lloyd RS. 2012. Regulation of DNA glycosylases and their role in limiting disease. Free Radic Res 46:460-478.

114. Wood RD, Mitchell M, Sgouros J, Lindahl T. 2001. Human DNA repair genes. Science 291:1284-1289.

115. Parlanti E, Fortini P, Macpherson P, Laval J, Dogliotti E. 2002. Base excision repair of adenine/8-oxoguanine mispairs by an aphidicolin-sensitive DNA polymerase in human cell extracts. Oncogene 21:5204-5212.

116. Ohno M, Miura T, Furuichi M, Tominaga Y, Tsuchimoto D, Sakumi K, Nakabeppu Y. 2006. A genome-wide distribution of 8-oxoguanine correlates with the preferred regions for recombination and single nucleotide polymorphism in the human genome. Genome Res 16:567-575.

117. Sassa A, Beard WA, Prasad R, Wilson SH. 2012. DNA sequence context effects on the glycosylase activity of human 8-oxoguanine DNA glycosylase. The Journal of biological chemistry 287:36702-36710.

118. David SS, O'Shea VL, Kundu S. 2007. Base-excision repair of oxidative DNA damage. Nature 447:941-950.

119. Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, Epe B, Seeberg E, Lindahl T, Barnes DE. 1999. Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. Proceedings of the National Academy of Sciences of the United States of America 96:13300-13305.

120. Bruner SD, Norman DP, Fromme JC, Verdine GL. 2000. Structural and mechanistic studies on repair of 8-oxoguanine in mammalian cells. Cold Spring Harb Symp Quant Biol 65:103-111.

121. Kohno T, Shinmura K, Tosaka M, Tani M, Kim SR, Sugimura H, Nohmi T, Kasai H, Yokota J. 1998. Genetic polymorphisms and alternative splicing of the hOGG1 gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. Oncogene 16:3219-3225.

122. Bruner SD, Norman DP, Verdine GL. 2000. Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. Nature 403:859-866.

123. Nilsen H, Krokan HE. 2001. Base excision repair in a network of defence and tolerance. Carcinogenesis 22:987-998.

124. Wilson SH, Kunkel TA. 2000. Passing the baton in base excision repair. Nat Struct Biol 7:176-178.

125. Sakumi K, Tominaga Y, Furuichi M, Xu P, Tsuzuki T, Sekiguchi M, Nakabeppu Y. 2003. Ogg1 knockout-associated lung tumorigenesis and its suppression by Mth1 gene disruption. Cancer research 63:902-905.

126. Liao J, Seril DN, Lu GG, Zhang M, Toyokuni S, Yang AL, Yang GY. 2008. Increased susceptibility of chronic ulcerative colitis-induced carcinoma development in DNA repair enzyme Ogg1 deficient mice. Mol Carcinog 47:638-646.

127. Izumi T, Wiederhold LR, Roy G, Roy R, Jaiswal A, Bhakat KK, Mitra S, Hazra TK. 2003. Mammalian DNA base excision repair proteins: their interactions and role in repair of oxidative DNA damage. Toxicology 193:43-65.

128. Sunaga N, Kohno T, Shinmura K, Saitoh T, Matsuda T, Saito R, Yokota J. 2001. OGG1 protein suppresses G:C-->T:A mutation in a shuttle vector containing 8-hydroxyguanine in human cells. Carcinogenesis 22:1355-1362.

129. Audebert M, Radicella JP, Dizdaroglu M. 2000. Effect of single mutations in the OGG1 gene found in human tumors on the substrate specificity of the Ogg1 protein. Nucleic Acids Res 28:2672-2678.

130. Smart DJ, Chipman JK, Hodges NJ. 2006. Activity of OGG1 variants in the repair of pro-oxidant-induced 8-oxo-2'-deoxyguanosine. DNA repair 5:1337-1345.

131. Singh R, Swick MC, Ledesma KR, Yang Z, Hu M, Zechiedrich L, Tam VH. 2012. Temporal interplay between efflux pumps and target mutations in development of antibiotic resistance in Escherichia coli. Antimicrobial agents and chemotherapy 56:1680-1685.

132. Sidorenko VS, Grollman AP, Jaruga P, Dizdaroglu M, Zharkov DO. 2009. Substrate specificity and excision kinetics of natural polymorphic variants and phosphomimetic mutants of human 8-oxoguanine-DNA glycosylase. The FEBS journal 276:5149-5162.

133. Kasymov RD, Grin IR, Endutkin AV, Smirnov SL, Ishchenko AA, Saparbaev MK, Zharkov DO. 2013. Excision of 8-oxoguanine from methylated CpG dinucleotides by human 8-oxoguanine DNA glycosylase. FEBS letters 587:3129-3134.

134. Dherin C, Radicella JP, Dizdaroglu M, Boiteux S. 1999. Excision of oxidatively damaged DNA bases by the human alpha-hOgg1 protein and the polymorphic alpha-hOgg1(Ser326Cys) protein which is frequently found in human populations. Nucleic Acids Res 27:4001-4007.

135. Kershaw RM, Hodges NJ. 2012. Repair of oxidative DNA damage is delayed in the Ser326Cys polymorphic variant of the base excision repair protein OGG1. Mutagenesis 27:501-510.

136. Jensen A, Lohr M, Eriksen L, Gronbaek M, Dorry E, Loft S, Moller P. 2012. Influence of the OGG1 Ser326Cys polymorphism on oxidatively damaged DNA and repair activity. Free radical biology & medicine 52:118-125.

137. Moritz E, Pauly K, Bravard A, Hall J, Radicella JP, Epe B. 2014. hOGG1-Cys326 variant cells are hypersensitive to DNA repair inhibition by nitric oxide. Carcinogenesis.

138. Lee AJ, Hodges NJ, Chipman JK. 2005. Interindividual variability in response to sodium dichromate-induced oxidative DNA damage: role of the Ser326Cys polymorphism in the DNA-repair protein of 8-oxo-7,8-dihydro-2'-deoxyguanosine DNA glycosylase 1. Cancer Epidemiol Biomarkers Prev 14:497-505.

139. Elahi A, Zheng Z, Park J, Eyring K, McCaffrey T, Lazarus P. 2002. The human OGG1 DNA repair enzyme and its association with orolaryngeal cancer risk. Carcinogenesis 23:1229-1234.

140. Hung RJ, Christiani DC, Risch A, Popanda O, Haugen A, Zienolddiny S, Benhamou S, Bouchardy C, Lan Q, Spitz MR, Wichmann HE, LeMarchand L, Vineis P, Matullo G, Kiyohara C, Zhang ZF, Pezeshki B, Harris C, Mechanic L, Seow A, Ng DP, Szeszenia-Dabrowska N, Zaridze D, Lissowska J, Rudnai P, Fabianova E, Mates D, Foretova L, Janout V, Bencko V, Caporaso N, Chen C, Duell EJ, Goodman G, Field JK, Houlston RS, Hong YC, Landi MT, Lazarus P, Muscat J, McLaughlin J, Schwartz AG, Shen H, Stucker I, Tajima K, Matsuo K, Thun M, Yang P, Wiencke J, Andrew AS, Monnier S, Boffetta P, Brennan P. 2008. International Lung Cancer Consortium: pooled analysis of sequence variants in DNA repair and cell cycle pathways. Cancer Epidemiol Biomarkers Prev 17:3081-3089.

141. Hung RJ, Hall J, Brennan P, Boffetta P. 2005. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. Am J Epidemiol 162:925-942.

142. Duan WX, Hua RX, Yi W, Shen LJ, Jin ZX, Zhao YH, Yi DH, Chen WS, Yu SQ. 2012. The association between OGG1 Ser326Cys polymorphism and lung cancer susceptibility: a meta-analysis of 27 studies. PLoS One 7:e35970.

143. Goode EL, Ulrich CM, Potter JD. 2002. Polymorphisms in DNA repair genes and associations with cancer risk. Cancer Epidemiol Biomarkers Prev 11:1513-1530.

144. Huang M, Dinney CP, Lin X, Lin J, Grossman HB, Wu X. 2007. High-order interactions among genetic variants in DNA base excision repair pathway genes and smoking in bladder cancer susceptibility. Cancer Epidemiol Biomarkers Prev 16:84-91.

145. Shinmura K, Kohno T, Kasai H, Koda K, Sugimura H, Yokota J. 1998. Infrequent mutations of the hOGG1 gene, that is involved in the excision of 8-hydroxyguanine in damaged DNA, in human gastric cancer. Jpn J Cancer Res 89:825-828.

146. Lan Q, Mumford JL, Shen M, Demarini DM, Bonner MR, He X, Yeager M, Welch R, Chanock S, Tian L, Chapman RS, Zheng T, Keohavong P, Caporaso N, Rothman N. 2004. Oxidative damage-related genes AKR1C3 and OGG1 modulate risks for lung cancer due to exposure to PAH-rich coal combustion emissions. Carcinogenesis 25:2177-2181.

147. Blons H, Radicella JP, Laccourreye O, Brasnu D, Beaune P, Boiteux S, Laurent-Puig P. 1999. Frequent allelic loss at chromosome 3p distinct from genetic alterations of the 8-oxoguanine DNA glycosylase 1 gene in head and neck cancer. Mol Carcinog 26:254-260.

148. Hanawalt PC, Spivak G. 2008. Transcription-coupled DNA repair: two decades of progress and surprises. Nature reviews. Molecular cell biology 9:958-970.

149. Brueckner F, Hennecke U, Carell T, Cramer P. 2007. CPD damage recognition by transcribing RNA polymerase II. Science 315:859-862.

150. Hayashi H, Tominaga Y, Hirano S, McKenna AE, Nakabeppu Y, Matsumoto Y. 2002. Replication-associated repair of adenine:8-oxoguanine mispairs by MYH. Curr Biol 12:335-339.

151. Clauson CL, Saxowsky TT, Doetsch PW. 2010. Dynamic flexibility of DNA repair pathways in growth arrested Escherichia coli. DNA repair 9:842-847.

152. Parish T, Brown A. 2009. Mycobacterium : genomics and molecular biology. Caister Academic Press, Norfolk, UK.

153. Kow YW. 2002. Repair of deaminated bases in DNA. Free radical biology & medicine 33:886-893.

154. Cao W. 2013. Endonuclease V: an unusual enzyme for repair of DNA deamination. Cell Mol Life Sci 70:3145-3156.

155. Carr AM, Lambert S. 2013. Replication stress-induced genome instability: the dark side of replication maintenance by homologous recombination. Journal of molecular biology 425:4733-4744.

156. Lambert G, Estevez-Salmeron L, Oh S, Liao D, Emerson BM, Tlsty TD, Austin RH. 2011. An analogy between the evolution of drug resistance in bacterial communities and malignant tissues. Nature reviews. Cancer 11:375-382.

157. Doetsch PW. 2002. Translesion synthesis by RNA polymerases: occurrence and biological implications for transcriptional mutagenesis. Mutation research 510:131-140.

158. Jiang C, Pugh BF. 2009. Nucleosome positioning and gene regulation: advances through genomics. Nat Rev Genet 10:161-172.

159. Euskirchen G, Auerbach RK, Snyder M. 2012. SWI/SNF chromatin-remodeling factors: multiscale analyses and diverse functions. The Journal of biological chemistry 287:30897-30905.

160. Cann KL, Dellaire G. 2011. Heterochromatin and the DNA damage response: the need to relax. Biochemistry and cell biology = Biochimie et biologie cellulaire 89:45-60.

161. Amouroux R, Campalans A, Epe B, Radicella JP. 2010. Oxidative stress triggers the preferential assembly of base excision repair complexes on open chromatin regions. Nucleic Acids Res 38:2878-2890.

162. Campalans A, Kortulewski T, Amouroux R, Menoni H, Vermeulen W, Radicella JP. 2013. Distinct spatiotemporal patterns and PARP dependence of XRCC1 recruitment to single-strand break and base excision repair. Nucleic Acids Res 41:3115-3129.

163. Rhee DB, Ghosh A, Lu J, Bohr VA, Liu Y. 2011. Factors that influence telomeric oxidative base damage and repair by DNA glycosylase OGG1. DNA repair 10:34-44.

164. Menoni H, Gasparutto D, Hamiche A, Cadet J, Dimitrov S, Bouvet P, Angelov D. 2007. ATP-dependent chromatin remodeling is required for base excision repair in conventional but not in variant H2A.Bbd nucleosomes. Mol Cell Biol 27:5949-5956.

165. Nemec AA, Wallace SS, Sweasy JB. 2010. Variant base excision repair proteins: contributors to genomic instability. Seminars in cancer biology 20:320-328.

166. Rosenberg SM, Thulin C, Harris RS. 1998. Transient and heritable mutators in adaptive evolution in the lab and in nature. Genetics 148:1559-1566.

167. Aubron C, Glodt J, Matar C, Huet O, Borderie D, Dobrindt U, Duranteau J, Denamur E, Conti M, Bouvet O. 2012. Variation in endogenous oxidative stress in Escherichia coli natural isolates during growth in urine. BMC Microbiol 12:120.

168. Cohen SE, Walker GC. 2010. The transcription elongation factor NusA is required for stress-induced mutagenesis in Escherichia coli. Curr Biol 20:80-85.

169. Yakovchuk P, Protozanova E, Frank-Kamenetskii MD. 2006. Base-stacking and base-pairing contributions into thermal stability of the DNA double helix. Nucleic Acids Res 34:564-574.

170. Fix DF, Glickman BW. 1987. Asymmetric cytosine deamination revealed by spontaneous mutational specificity in an Ung- strain of Escherichia coli. Mol Gen Genet 209:78-82.

171. Kim H, Lee BS, Tomita M, Kanai A. 2010. Transcription-associated mutagenesis increases protein sequence diversity more effectively than does random mutagenesis in Escherichia coli. PLoS One 5:e10567.

172. Polak P, Arndt PF. 2008. Transcription induces strand-specific mutations at the 5' end of human genes. Genome Res 18:1216-1223.

173. Beletskii A, Bhagwat AS. 1998. Correlation between transcription and C to T mutations in the non-transcribed DNA strand. Biol Chem 379:549-551.

174. Klapacz J, Bhagwat AS. 2005. Transcription promotes guanine to thymine mutations in the non-transcribed strand of an Escherichia coli gene. DNA repair 4:806-813.

175. MacPhee DG, Ambrose M. 2010. Catabolite repression of SOS-dependent and SOS-independent spontaneous mutagenesis in stationary-phase Escherichia coli. Mutat Res 686:84-89.

176. Jacob F, Monod J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. Journal of molecular biology 3:318-356.

177. Pastan I, Perlman RL. 1969. Repression of beta-galactosidase synthesis by glucose in phosphotransferase mutants of Escherichia coli. Repression in the absence of glucose phosphorylation. The Journal of biological chemistry 244:5836-5842.

178. Hartman Z, Henrikson EN, Hartman PE, Cebula TA. 1994. Molecular models that may account for nitrous acid mutagenesis in organisms containing double-stranded DNA. Environ Mol Mutagen 24:168-175.

179. Weiss B. 2006. Evidence for mutagenesis by nitric oxide during nitrate metabolism in Escherichia coli. Journal of bacteriology 188:829-833.

180. Moe A, Ringvoll J, Nordstrand LM, Eide L, Bjoras M, Seeberg E, Rognes T, Klungland A. 2003. Incision at hypoxanthine residues in DNA by a mammalian homologue of the Escherichia coli antimutator enzyme endonuclease V. Nucleic Acids Res 31:3893-3900.

181. Wilson DM, 3rd, Kim D, Berquist BR, Sigurdson AJ. 2011. Variation in base excision repair capacity. Mutat Res 711:100-112.

182. Raetz AG, Xie Y, Kundu S, Brinkmeyer MK, Chang C, David SS. 2012. Cancer-associated variants and a common polymorphism of MUTYH exhibit reduced repair of oxidative DNA damage using a GFP-based assay in mammalian cells. Carcinogenesis 33:2301-2309.

183. Komori A, Yatsunami J, Suganuma M, Okabe S, Abe S, Sakai A, Sasaki K, Fujiki H. 1993. Tumor-Necrosis-Factor Acts as a Tumor Promoter in BALB/3T3 Cell Transformation. Cancer Res. 53:1982-1985.

184. Kim JJ, Lee SB, Park JK, Yoo YD. 2010. TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-X(L). Cell Death Differ 17:1420-1434.

185. Jamaluddin M, Wang S, Boldogh I, Tian B, Brasier AR. 2007. TNF-alpha-induced NF-kappaB/RelA Ser(276) phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway. Cellular signalling 19:1419-1433.

186. Duthie SJ, Ma AG, Ross MA, Collins AR. 1996. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. Cancer Res. 56:1291-1295.

187. Aruoma OI, Halliwell B, Hoey BM, Butler J. 1989. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free radical biology & medicine 6:593-597.

188. Cairns J, Foster PL. 1991. Adaptive reversion of a frameshift mutation in Escherichia coli. Genetics 128:695-701.

189. Pedraza-Reyes M, Yasbin RE. 2004. Contribution of the mismatch DNA repair system to the generation of stationary-phase-induced mutants of Bacillus subtilis. Journal of bacteriology 186:6485-6491.

190. Ross C, Pybus C, Pedraza-Reyes M, Sung HM, Yasbin RE, Robleto E. 2006. Novel role of mfd: effects on stationary-phase mutagenesis in Bacillus subtilis. Journal of bacteriology 188:7512-7520.

191. Farr SB, Kogoma T. 1991. Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiological reviews 55:561-585.

192. Taddei F, Hayakawa H, Bouton M, Cirinesi A, Matic I, Sekiguchi M, Radman M. 1997. Counteraction by MutT protein of transcriptional errors caused by oxidative damage. Science 278:128-130.

193. Taddei F, Halliday JA, Matic I, Radman M. 1997. Genetic analysis of mutagenesis in aging Escherichia coli colonies. Mol Gen Genet 256:277-281.

194. Foster PL, Cairns J. 1994. The occurrence of heritable Mu excisions in starving cells of Escherichia coli. EMBO J 13:5240-5244.

195. Harris RS, Longerich S, Rosenberg SM. 1994. Recombination in adaptive mutation. Science 264:258-260.

196. Bull HJ, Lombardo MJ, Rosenberg SM. 2001. Stationary-phase mutation in the bacterial chromosome: recombination protein and DNA polymerase IV dependence. Proceedings of the National Academy of Sciences of the United States of America 98:8334-8341.

197. Bhamre S, Gadea BB, Koyama CA, White SJ, Fowler RG. 2001. An aerobic recA-, umuC-dependent pathway of spontaneous base-pair substitution mutagenesis in Escherichia coli. Mutat Res 473:229-247.

198. Hall BG. 1998. Adaptive mutagenesis at ebgR is regulated by PhoPQ. Journal of bacteriology 180:2862-2865.

199. Rosenberg SM. 2001. Evolving responsively: adaptive mutation. Nat Rev Genet 2:504-515.

200. Sweasy JB, Lang T, DiMaio D. 2006. Is base excision repair a tumor suppressor mechanism? Cell cycle 5:250-259.

201. Baute J, Depicker A. 2008. Base excision repair and its role in maintaining genome stability. Crit Rev Biochem Mol Biol. Jul-Aug;43(4):239-76. doi: 10.1080/10409230802309905.

202. Wang Y, Schlick T. 2007. Distinct energetics and closing pathways for DNA polymerase beta with 8-oxoG template and different incoming nucleotides. BMC Struct Biol. Feb 21;7:7.

203. Wilson DM 3rd, Bohr VA. 2007. The mechanics of base excision repair, and its relationship to aging and disease. DNA Repair (Amst). Apr 1;6(4):544-59. Epub 2006 Nov 16.

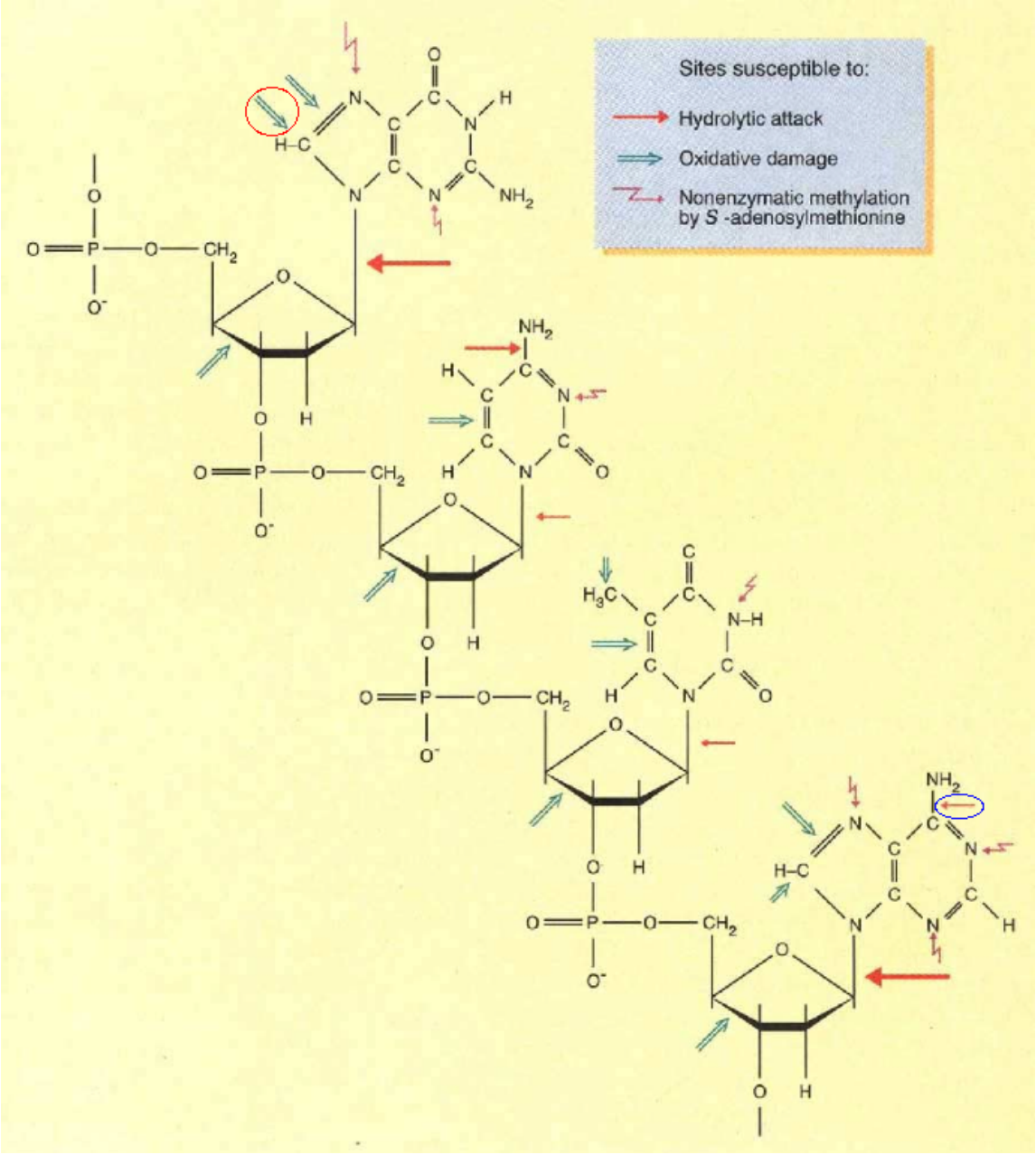

Figure 1. Sites of damage and modification on nucleotides. DNA undergoes decay by different mechanisms at different sites in each nucleotide. Shown from top to bottom are guanine, cytosine, thymine, and adenine. Larger arrows indicate more common sites of decay. Red arrows indicate sites of hydrolytic attack, blue arrows show sites of oxidative damage, and zig-zag arrows mark sites of methylation. The blue circle indicates the site

of adenine deamination important to the bacterial studies, and the red circle indicates the site of guanine oxidation important to the mammalian studies. Adapted from Lindahl (2).

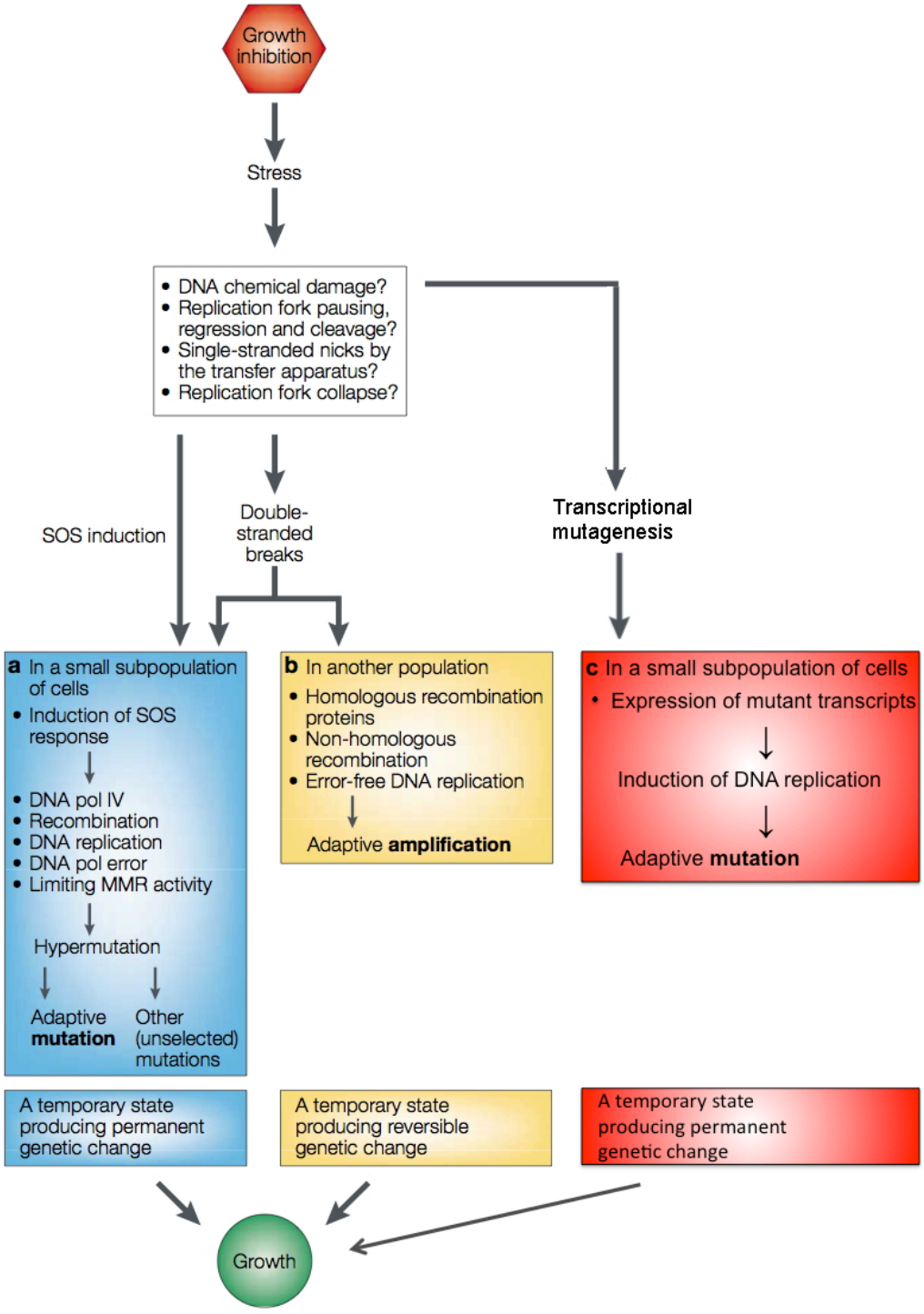

Figure 2. Prokaryotic mechanisms of adaptive mutagenesis. Adaptive mutagenesis occurs when non-dividing cells are subjected to stress. The nature of this stress dictates its genotoxic consequences, and the subsequent mechanisms by which cells mitigate such

consequences, either by (**a** & **c**) adaptive mutation or (**b**) adaptive amplification. After adaptive mutagenesis, a cell can then divide under selection. Adapted from Rosenberg (199) with permission from the publisher.

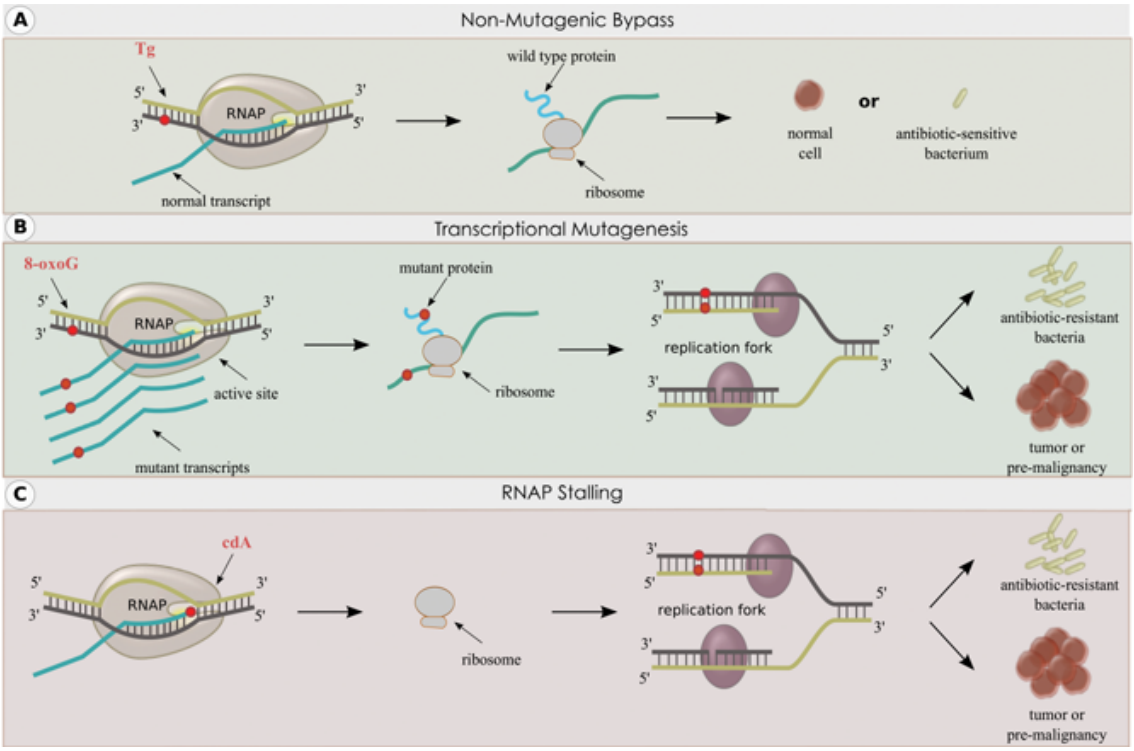

Figure 3. RNA polymerase can mediate TM after encountering DNA lesions. RNA polymerase (RNAP) can undergo different interactions with DNA lesions, depending on the nature of the lesion. **(A)** When RNAP encounters a relatively non-mutagenic lesion, such as thymine glycol (Tg), it will generate a normal wild-type transcript. **(B)** When RNAP encounters a non-bulky mutagenic lesion, such as 8-oxoguanine (8-oxoG), it will generate mutant transcripts that can encode mutant proteins, which may confer antibiotic resistance or increased tumorigenicity. **(C)** When RNAP encounters a bulky mutagenic lesion, such as cyclo-dA (cdA), it will stall and undergo limited bypass of the lesion, but will also generate mutant transcripts that can encode mutant proteins, which can confer antibiotic resistance or increased tumorigenicity. Reprinted from Morreall & Petrova (71) with permission from the publisher.

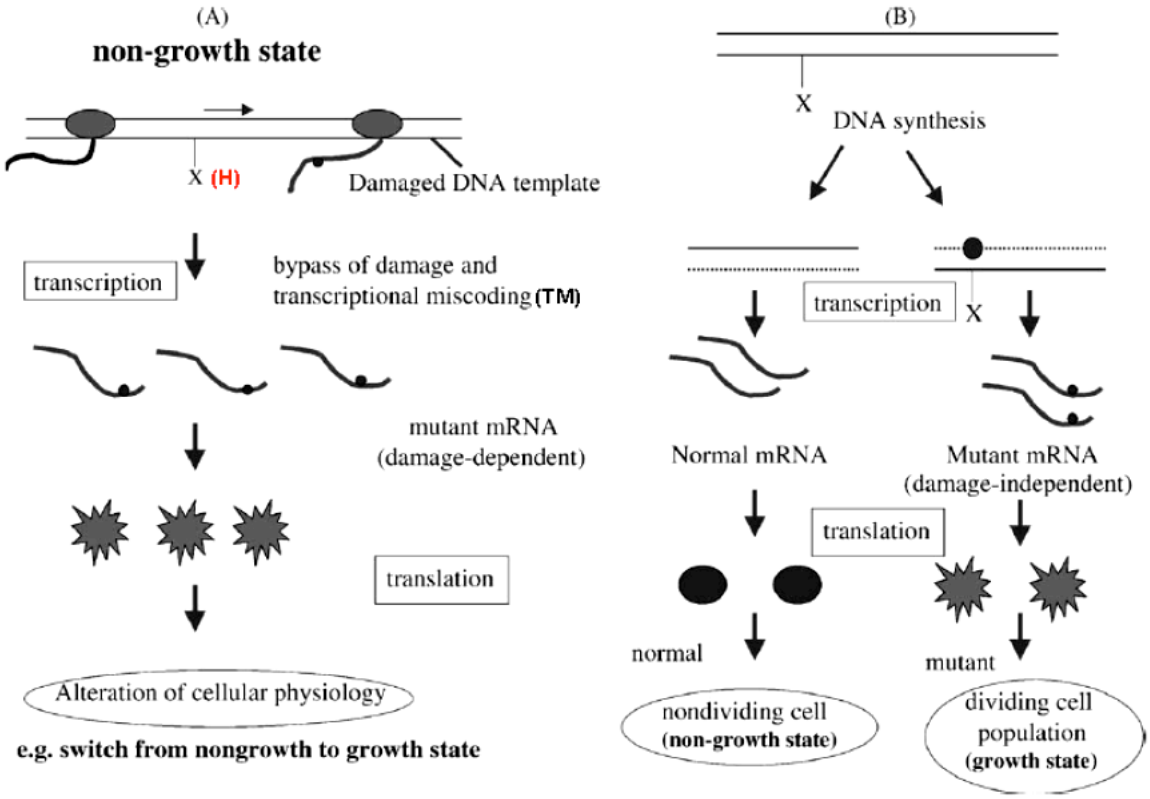

Fig. 4. Transcriptional mutagenesis could cause retromutagenesis that allows non-growing cells to switch to a pro-growth state. (**A**) Transcriptional mutragenesis (TM) occurs when transcription bypasses DNA damage, such as hypoxanthine (H), and gives rise to a mutant transcript. This mutant transcript can encode a mutant protein that allows cells to switch to a pro-growth state. (**B**) Replication initiated by TM could bypass the causative DNA lesion, establishing an analogous mutation in a daughter cell (retromutagenesis) that goes on to establish a dividing cell population. Adapted from (157).

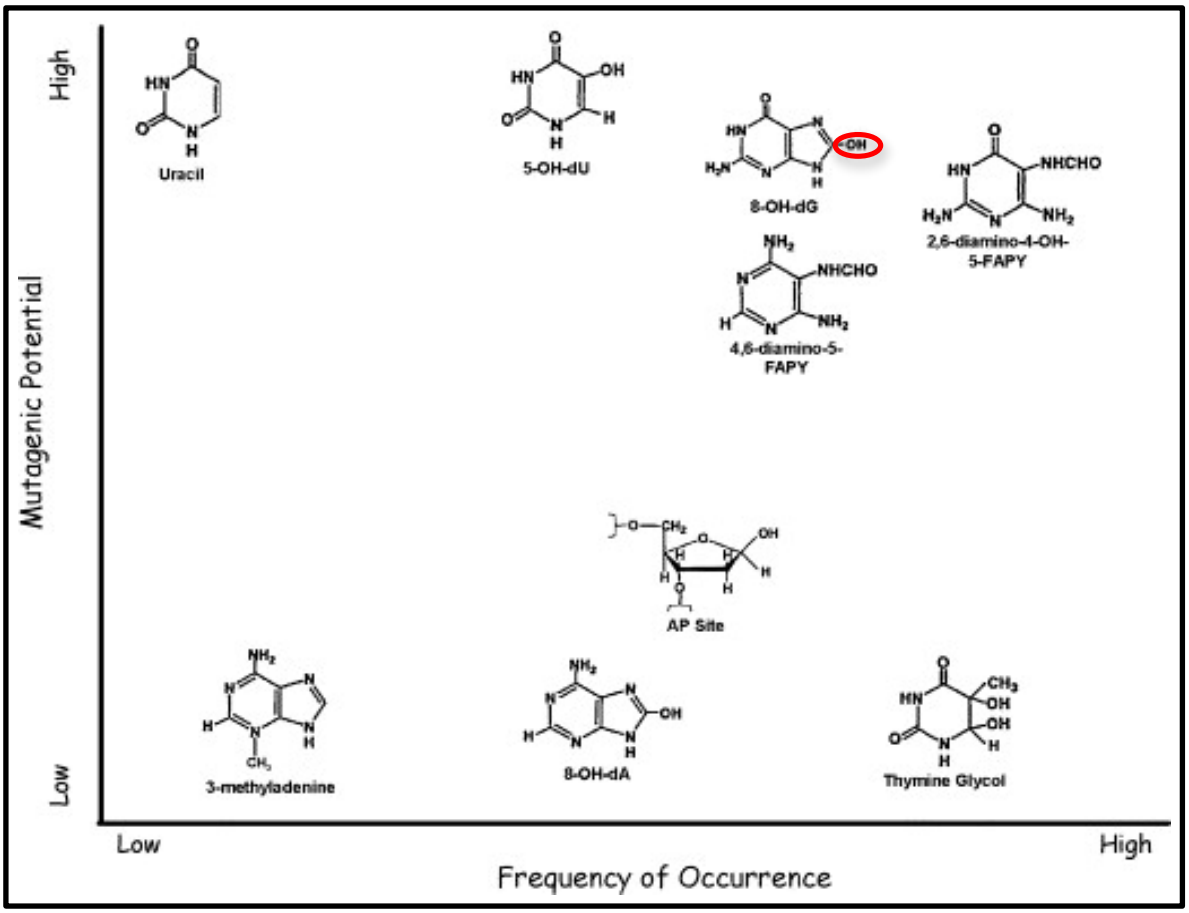

Figure 5. Frequency vs. mutagenicity of nucleotide lesions. DNA damage can take a number of different forms. Different DNA lesions have different levels of mutagenicity, depending on the base-miscoding properties of each. Some mutagenic lesions, such as 8-oxoguanine (8-OH-dG), also occur with a high frequency. 8-oxoguanine arises from the addition of a –OH group (circled). Frequently-occurring mutagenic lesions have the greatest impact on rates of physiological mutations. Adapted from Wilson & Bohr (203) with permission from the publisher.

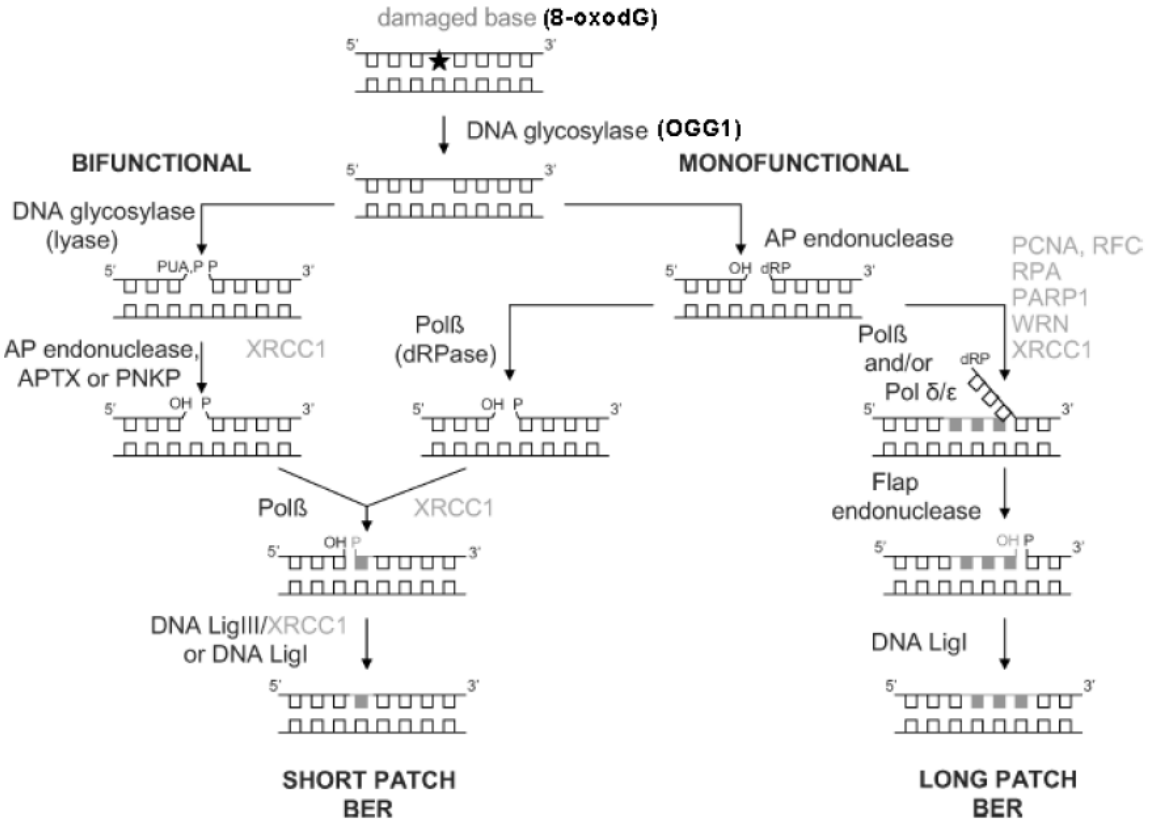

Figure 6. Model of base excision repair in mammals. Base excision repair (BER) is initiated by a DNA glycosylase, which recognizes and excises a specific lesion (star). Bifunctional DNA glycosylases, such as OGG1, have AP lyase activity, which cleaves the sugar-phosphate backbone and leaves a 5' phosphate (P) and a 3' phosphate or 3' polyunsaturated aldehyde (PUA), depending on the glycosylase involved. Monofunctional DNA glycosylases remove the lesion, after which AP endonuclease cleaves the damaged strand, leaving a 3' hydroxyl (OH) and 5' deoxyribose-phosphate (dRP). The dRPase activity of DNA polymerase (Pol) *ß* removes the dRP group, while AP endonuclease removes the 3' PUA group, and the phosphate group (P) is removed by polynucleotide kinase phosphatase (PNKP) or aprataxin (APTX). Repair is mediated by

short-patch or long-patch BER Short-patch repair involves Pol β incorporating a single nucleotide, after which the nick is sealed by the XRCC1/LigIIIα complex or LigI. Long-patch BER occurs if Pol β is incapable of cleaving the 5' lesion. Long-patch BER involves Pol β and/or δ/ε displacing the damaged strand by incorporating nucleotides, after which Flap endonuclease removes the 5' DNA flap, and the nick is sealed by LigI. Adapted from (201).

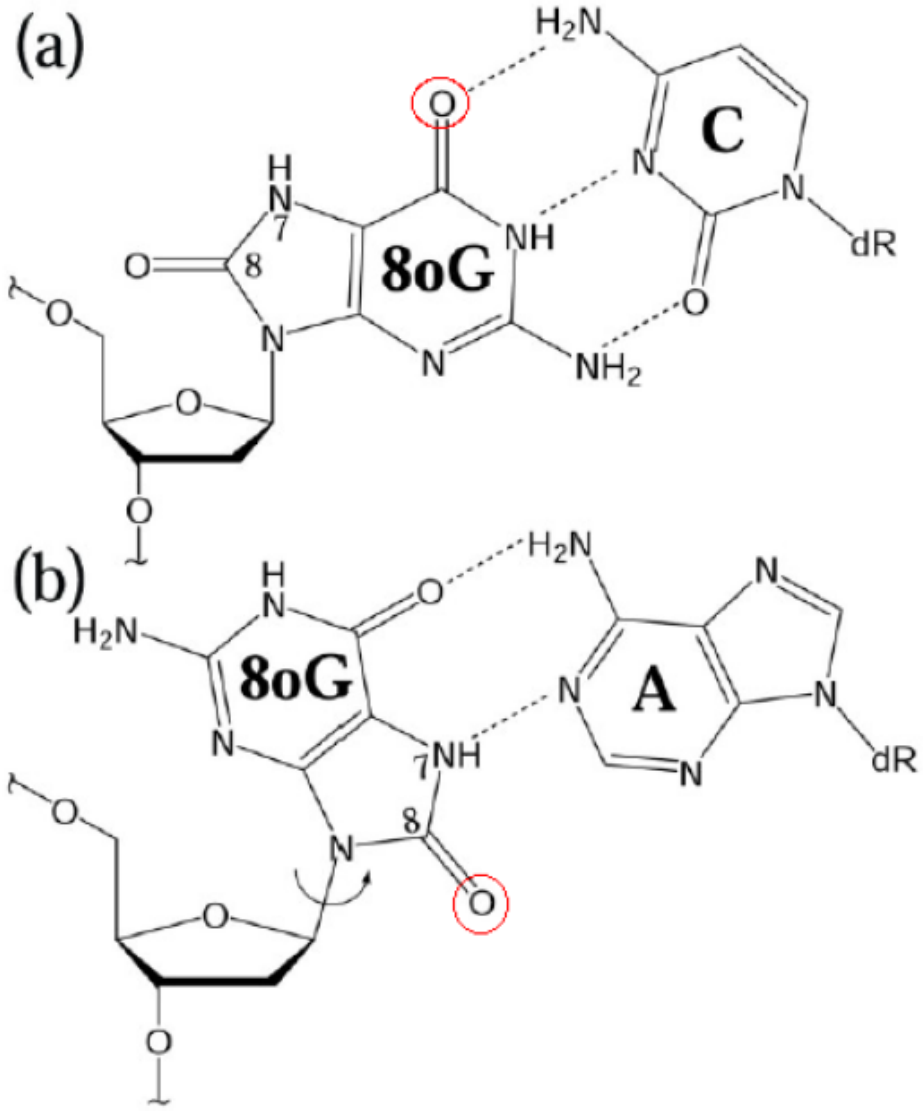

Fig. 7. 8-oxoguanine undergoes base mispairing with adenine. 8-oxoguanine (8oG) contains an oxygen in a double-bond with the 8' carbon. Instead of the typical *anti* conformation (**a**) that allows base pairing with cytosine (C), 8-oxoguanine adopts a *syn* conformation (**b**) allowing Hoogsteen base mispairing with adenine (A). Adapted from (202) with permission from the publisher.

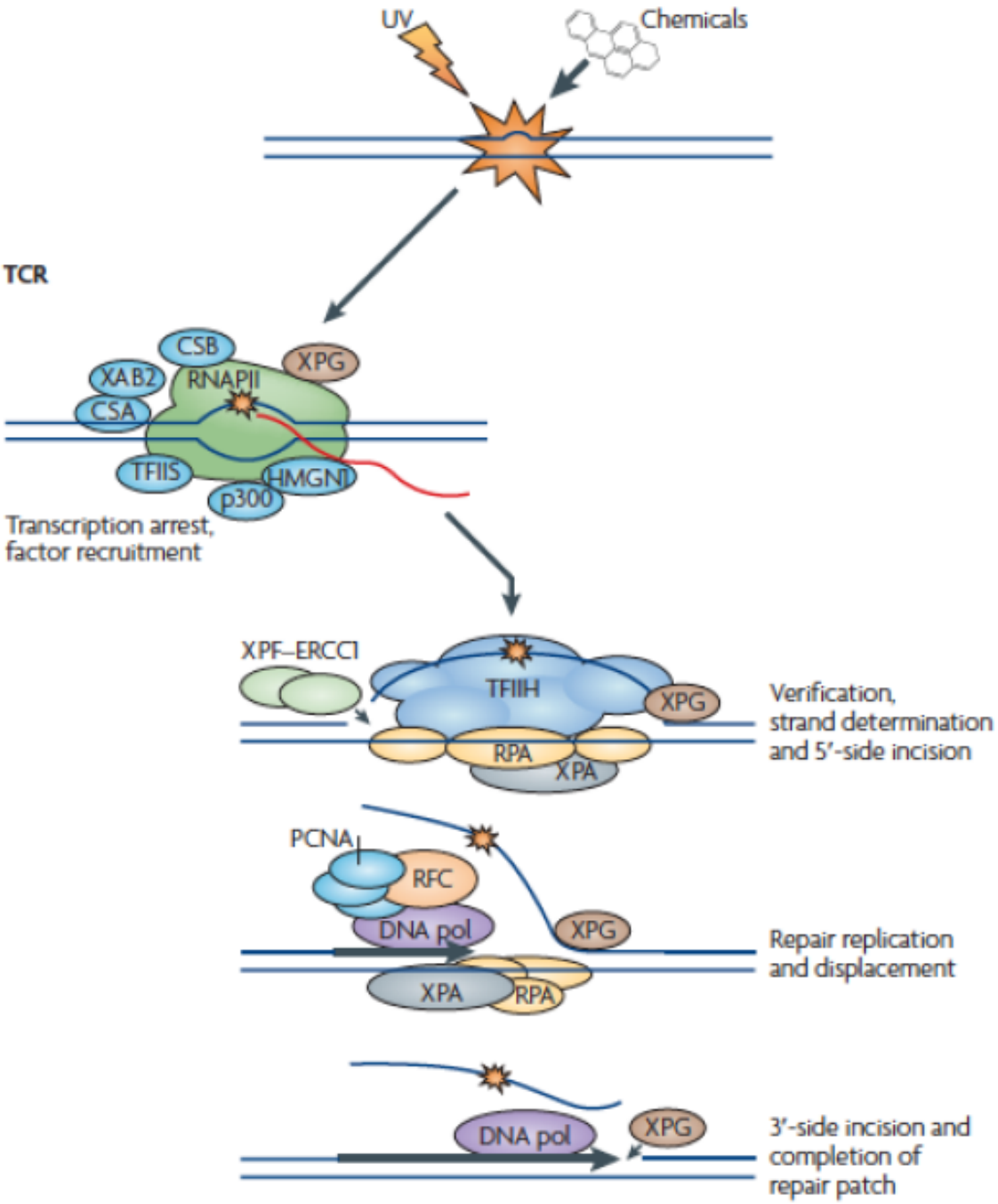

Fig. 8. Model of mammalian transcription-coupled repair. Transcription-coupled repair (TCR) involves the recognition of a lesion by RNA polymerase (RNAP). Afterwards, transcription factor TFIIH and XPG are recruited. Replication protein A (RPA) and XPA may assist in protecting single-stranded DNA and stabilizing the pre-

incision complex. Next, the XPF-ERCC1 (excision repair cross complementing-1) endonuclease is recruited to cleave the damaged strand of DNA. Replication factor C (RFC) recruits proliferating cell nuclear antigen (PCNA) to assist in polymerase processivity. Finally, ligation is carried out by ligase-I and flap endonuclease-1 or by the ligase-III-XRCC1 compex. Adapted from (148) with permission from the publisher.

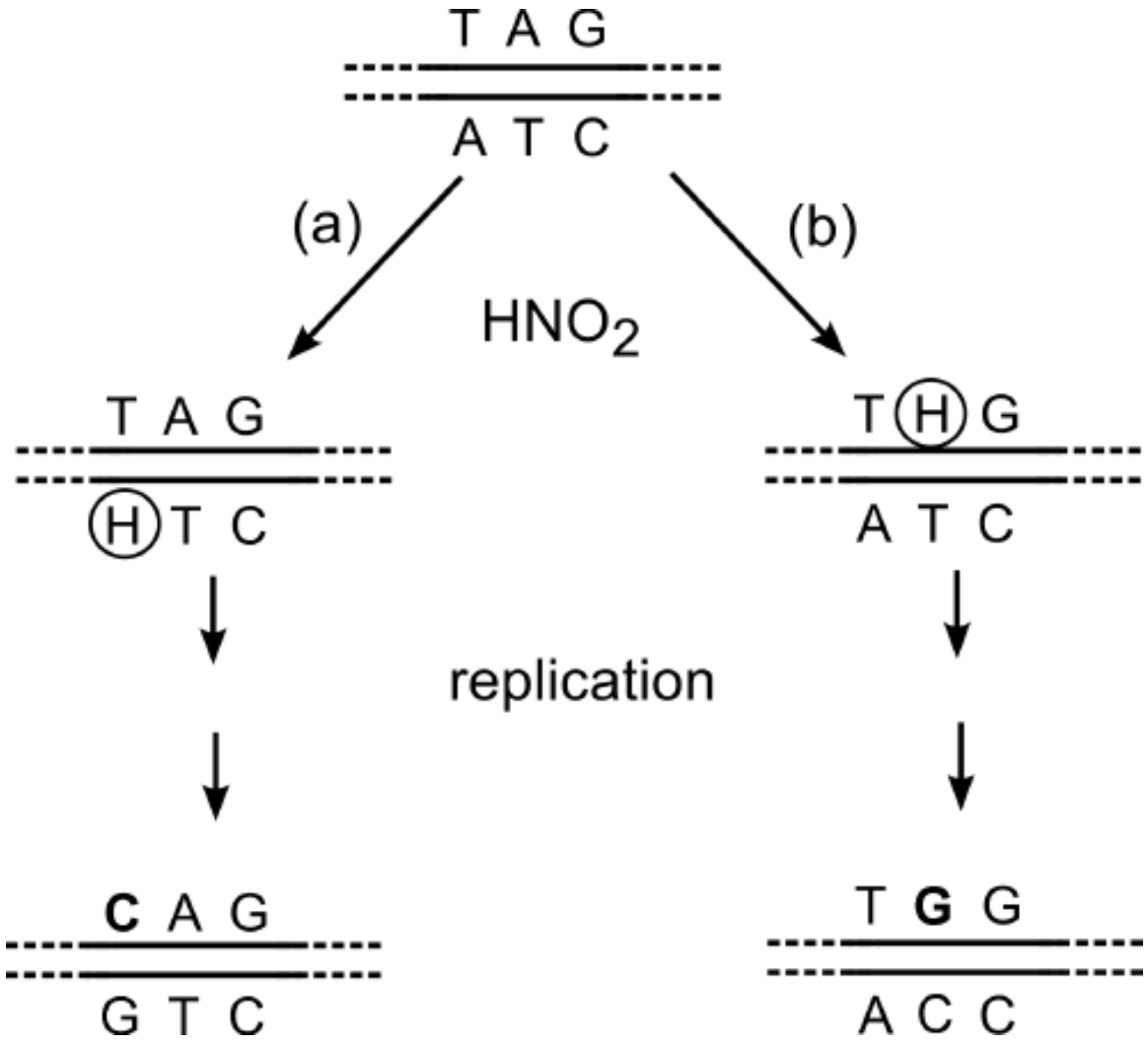

Fig. 9. Hypoxanthine causes AT>GC mutations. Hypoxanthine (H) arises from the deamination of adenine (A), which can be caused by exposure to deaminating agents such as HNO_2. While A canonically pairs with thymine (T), H undergoes mispairing with cytosine (C), causing AT>GC mutations after replication.

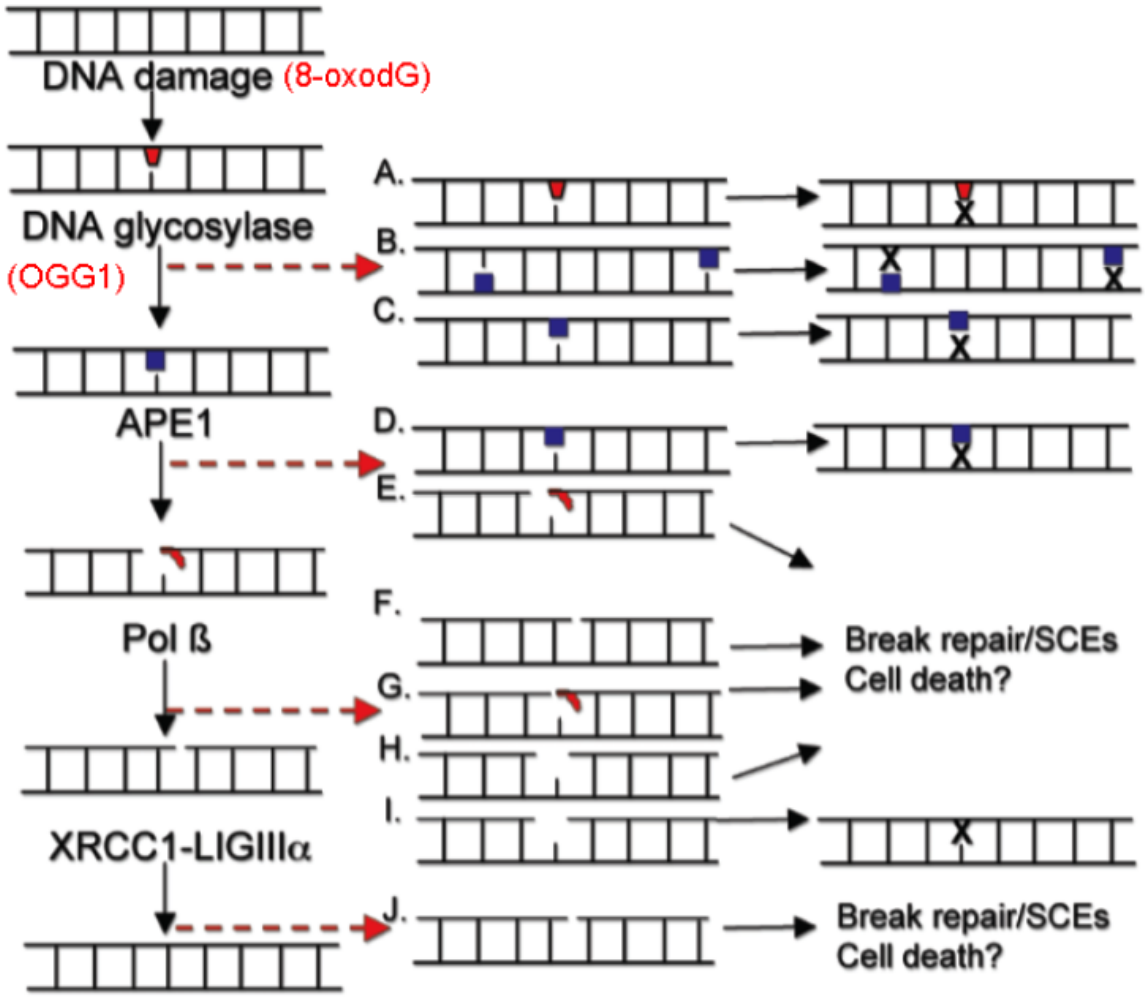

Figure 10. Variant BER proteins can cause a variety of deleterious outcomes. DNA damage, such as 8-oxodG, can be repaired efficiently by the canonical BER pathway, shown on the left. Red trapezoids represent DNA damage (8-oxodG), blue squares represent abasic sites, red scribbles represent dRP moieties, and X represent mutations. (**A**) However, DNA damage like 8-oxodG cause mutagenesis when bound by an inactive DNA glycosylase (Ogg1) variant. (**B**) The variant may also erroneously excise normal base pairs, causing mutagenesis at the resulting abasic sites. (**C**) Glycosylase variants may also not be able to undergo stimulation by APE1, causing decreased incision and increased mutagenesis. (**D**) Variant APE1 may have decreased activity, causing less

abasic site incision and more mutagenesis. (**E**) APE1 may be unable to load polymerase β onto DNA, which can cause mutagenic break repair or cell death. (**F**) Polymerase β variants may be unable to load XRCC1 complexes onto DNA, causing mutagenic break repair or cell death. (**G**) Polymerase β variant enzymes may have decreased dRP lyase activity, which could cause mutagenic break repair or cell death. (**H**) Inefficient polymerase β variants may also cause mutagenic break repair or cell death. (**I**) Polymerase β variants may also undergo mutagenic gap filling. (**J**) Variant XRCC1 complexes may have decreased activity, causing mutagenic break repair or cell death. Adapted from Sweasy (200) with permission from the publisher.

Table 1. Examples of Stress-Induced Mutagenesis

Type of Mutagenesis	Organism	Mutation	Contributing Genes	Selection	Ref.s
Adaptive frameshift mutation	*E. coli*	Frameshift	*recA*	Growth on lactose	(188)
Stationary-phase mutagenesis	*B. subtilis*	Base substitutions	*Mfd*	Amino acid prototrophy	(189, 190)
SOS mutagenesis	*E. coli, S. typhimurium*	Base substitutions	*recA*	Carbon source starvation	(191)
"Resting organisms in a structured environment"	*E. coli*	Base substitutions	*recA, lexA, uvrB, polA*	Rifampicin resistance	(192, 193)
Starvation-induced Mu-mediated fusions	*E. coli*	Transpositions	*uvrB*	Prototrophy on lactose with arabinose	(41, 194)
Homologous recombination	*E. coli*	Recombination	*recA, recB*	Growth on lactose	(195)
Recombination protein-dependent stationary-phase mutation	*E. coli*	Deletions	*dinB, recA, ruvC*	Tetracycline resistance	(196)
SOS-dependent spontaneous mutagenesis	*E. coli*	Base substitutions	*recA*, *umuC*	Tryptophan reversion	(197)
Starvation response	*E. coli, S. typhimurium*	Insertions	*phoP, phoQ*	Growth on lactulose	(198)
Translesion synthesis	*E. coli*	Base substitutions	*dinB*	Growth on lactose	(39)

CHAPTER 2

RETROMUTAGENESIS IS A MECHANISM FOR ADAPTIVE MUTATION IN *ESCHERICHIA COLI*

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Author contributions: JFM, ND, BW, and PWD designed the experiments and analyzed the data. JFM performed the experiments. AK carried out preliminary experiments. YL carried out statistical analysis of preliminary data. JFM and BW wrote the manuscript. JFM, ND, BW, and PWD edited the manuscript. PWD supervised the entire project.

Abstract

Adaptive mutation refers to the continuous outgrowth of new mutants from a non-dividing population during selection, in apparent violation of the neo-Darwinian principle that mutation precedes selection. One explanation is that of retromutagenesis, in which a DNA lesion causes a transcriptional mutation that yields a mutant protein, allowing escape from selection. This enables a round of DNA replication that establishes heritability. Because the model requires that gene expression precede DNA replication, it uniquely predicts that during selection, new mutants will arise from damage only to the transcribed DNA strand. As a test, we used a *lacZ* amber mutant of *Escherichia coli* that can revert by nitrous acid-induced deamination of adenine residues on either strand of the TAG stop codon, each causing different DNA mutations. When stationary-phase, mutagenized cells were grown in rich broth before being plated on lactose-selective media, only non-transcribed strand mutations appeared in the revertants. This result was consistent with the known high sensitivity of deamination of the single-stranded DNA in a transcription bubble. With the other results, it demonstrated the ability of the system to detect mutational bias toward either Strand. When residual *lacZ* transcription was blocked beforehand by catabolite repression, both strands were mutated about equally, but if revertants were selected immediately after nitrous acid exposure, transcribed-strand mutations predominated among the revertants, implicating retromutagenesis as the mechanism. This result was not affected by gene orientation. Retromutagenesis is apt to be a universal method of evolutionary adaptation, which enables the emergence of new mutants from mutations acquired during counterselection rather than beforehand, and it

may have roles in processes as diverse as the development of antibiotic resistance and neoplasia.

1. Introduction

A central tenet of genetics is that mutation precedes selection rather than the reverse. This principle was verified by isolation, through sibling selection, of mutants from cultures that had never been exposed to the growth pressure of a selective agent (1, 2). In apparent contradiction to this principle, it was later observed that new mutants would arise continuously from an ostensibly non-growing population of cells for many days after being plated on a selective medium (3, 4, 5). The phenomenon has been termed "adaptive mutation" or "stationary-phase mutagenesis." Much of our knowledge of the underlying mechanisms has come from studies of reversions of frameshift mutations in the *lacZ* (β-galactosidase) gene of *Escherichia coli* in a system refined by Cairns and Foster (6). As reviewed by Roth (7), the mechanisms include: occult growth of a sub-population aided by the residual activity of the defective gene product and amplification of the gene by homologous recombination, both of which increase the number of mutational targets, and stress-related amplification, and possibly induction of *dinB*, which encodes an error-prone DNA polymerase that may produce mutations during gene amplification. Thus, the apparent Lamarckian adaptations are actually due to occult mechanisms that still conform to the neo-Darwinian principle of mutation preceding selection. Controversy still exists around the contributions of residual growth and enhanced mutation rates, and so adaptive mutagenesis is best defined simply as the process by which new mutations arise under selective conditions regardless of the mechanism (7).

Davis (8) proposed a unique mechanism to explain reversion of *lacZ* mutants in a non-growing population. The lactose in the selection medium induces transcription of

the *lac* operon, and the non-transcribed strand (NTS) is displaced by mRNA in a transcription bubble. Because the NTS is now single-stranded, it is unusually susceptible to environmental damage. Before the reversion mutation specified by the DNA damage can be expressed, however, a round of DNA replication is required to copy the defect onto the transcribed strand (TS). The energy for this DNA synthesis was proposed to come from the breakdown of cellular constituents, particularly ribosomes, which is expected to occur after three days of incubation on growth media. Davis's model predicts that the new mutations will arise from the NTS.

An alternative mechanism, which we explore in this paper, was proposed by Bridges (3) and has come to be known as "retromutagenesis" (9). It differs from most others in that expression of a damaged gene precedes its replication. Therefore, it will only yield mutants from damage to the TS of a gene. If the DNA damage escapes repair before DNA replication, the altered gene may be transcribed and translated to produce a mutant protein that enables the cell to undergo at least one round of DNA replication during selection (**Figure 1**). If the DNA polymerase then misreads the lesion in the same way that the RNA polymerase did, the mutation will be permanently established in a daughter cell. In contrast, lesions on the NTS will not enable the outgrowth of mutants under stringent selection because, before the mutant gene can be expressed, the mutation specified by the lesion must be copied onto the TS by a round of DNA replication.

The first requirement for the retromutagenesis model is that a damaged gene must produce an altered protein in the absence of DNA synthesis, a process called transcriptional mutagenesis. A second requirement for retromutagenesis is that the RNA and DNA polymerases of a cell incorporate analogous nucleoside triphosphates opposite

a lesion. As reviewed by Bregeon and Doetsch (10), these requirements have been fulfilled for several types of DNA lesions in both prokaryotes and eukaryotes. However, for the model to be verified, it must be demonstrated that adaptive mutation favors the TS of a gene. Two reported findings are consistent with this prediction. During selection for tryptophan prototrophy in strains with the *trp5*-A149C missense mutation, an *ogg1* mutant of *Saccharomyces cerevisiae,* defective in the repair 8-oxoguanine oxidative lesions in DNA, displayed a high frequency of late-arising Trp^+ revertants resulting from G:C → T:A transversions (11). The results were consistent with an implied mispairing of an 8-oxoguanine in the TS. In another experiment, 8-oxo-dGTP that was electroporated into a *trp5* mutant could cause reversions when incorporated into the NTS, but such revertants were only found after replication, and not after immediate selection under non-growth conditions (12). Unfortunately, these studies could not test both TS and NTS strand mutations in the same experimental system.

In this paper, we describe an experimental system in *Escherichia coli* that can distinguish between mutations arising from damage to either DNA strand. We use it to test the hypothesis that retromutagenesis plays a role in the appearance of new bacterial mutants during stationary phase, and we discuss its implications for higher organisms.

4. **Materials and Methods**

4.1 *Strain construction*

The *E. coli* K-12 $F^-\lambda^-$ strains used in this study and the details of their construction are listed in **Table S1.** We constructed two tester strains that had amber mutations in *lacZ* genes in opposite orientations on the chromosome. The amber mutation in codon 17 of *lacZ* was previously generated by oligonucleotide-mediated transformation. Subsequent gene transfers were by generalized transduction with phage P1 *dam rev6* (13). To orient the genes differently, we used *attλ* elements, which were plasmid-derived DNA segments that had integrated into the bacterial chromosome in opposite directions at the attachment site of phage λ. They contained a selectable ampicillin resistance marker and a *lac* operon with a *lacZ* missense mutation. They were transduced into an amber suppressor strain, BW5660, to create strains Z122 and Z123. Then the *lacZ* amber allele was transduced into these strains, crossing out the recipient's *lac* missense mutation. (The chromosomal *lac* deletions encompassed a genomic region too large to undergo P1-mediated transduction.) Selection was for the Lac^+ phenotype specified by the suppressed *lacZ* amber allele. The *lacI* (constitutive) mutations in the recipients were crossed out at the same time, as determined by scoring with X-Gal and IPTG (14). Finally, the *attλ* elements were transduced into BW1181, a non-suppressing strain that had an *nfi* mutation and a *lac* deletion, thereby creating strains Z126 and Z127, which had the following relevant genotype: Δ*lac nfi-1*::*cat attλ*::[*lacZ*(Am)Y^+Z^+]. In strain Z126, the *lacZ* gene on the *attλ* element is codirectional with the leading strand during DNA synthesis, and in strain Z127, it is in the opposite orientation. The *lac* point mutations

were confirmed by DNA sequencing. The *nfi-1*::*cat* insertion and the orientations of the *attλ* elements were confirmed by PCR with primers listed in **Table S2**.

4.2 *Bacteriological media*

LB media were the rich media used used for routine growth. Lactose-minimal agar was Medium E (15) supplemented with 0.1% lactose (Sigma Aldrich, > 99% pure by gas chromatography) and thiamine at 1 μg/ml. It was pretreated with a Δ*lac* scavenger strain, BW5660, (**Table S1**) to remove traces of carbon sources other than lactose (16): a saturated culture of strain BW5660 grown in LB broth was centrifuged and resuspended in one-tenth its original volume of 10 mm $MgSO_4$, and 0.1 ml was spread on each plate and incubated for 48 h at 37°C.

4.3 *Mutagenesis*

Strains Z126 and Z127 were grown overnight with aeration at 37ºC in LB broth containing 0.4% glucose. The inocula contained less than 10^4 cells so that they would be unlikely to contain spontaneous revertants. The saturated cultures were centrifuged, and the cells were washed and resuspended in 10 mM $MgSO_4$, then starved by incubation with shaking for 30 min at 37°C. They were separated into twenty-eight 1-mL aliquots and centrifuged. The cells were resuspended in 0.5 mL of 0.1 M sodium acetate buffer (pH 4.6) with or without 80 mM $NaNO_2$ and incubated for 9 min at 37ºC. The reactions were stopped by the addition of 5.0 mL of cold Medium A buffer (14). The remaining cells were centrifuged and resuspended in 0.4 mL of 10 mM $MgSO_4$. From each treated sample, 0.2 mL were spread on lactose-minimal agar, and 0.05 mL were added to each of

4 tubes containing 1.8 mL of LB broth and incubated overnight with shaking at 37°C. The untreated samples were handled similarly, except that the intermediate growth was performed in two tubes each. The saturated cultures were centrifuged, and the cells were resuspended in 10 mM $MgSO_4$ and plated on lactose-minimal media. Before and after overnight growth, two samples each of the treated and untreated cells were diluted and plated on LB agar to determine cell survival and cell concentration. After 48 h of growth at 37ºC, random, uniformly large Lac^+ colonies were picked for DNA sequencing.

4.4 *Other methods*

Colony diameters were measured with a loupe containing a reticle (Edmund Scientific Co.). The *5′*-terminal *lacZ* region of each Lac^+ colony to be sequenced was amplified by colony PCR (17) with primers lacZ-249F and lacZ+178R (**Table S2**). Sequencing was carried out by Beckman-Coulter Genomics (Danvers, MA) via dye termination capillary electrophoresis on an ABI 3730XL (Applied Biosystems, Inc.) instrument, using the lacZ-249F primer. Statistical significances were determined by the two-tailed Fisher's exact test (18) using an online interface (http://www.langsrud.com/fisher.htm), and the Cochran–Mantel–Haenszel test (19) using a published spreadsheet (http://www.biostathandbook.com/cmh.xls).

5. Results

5.1 *Rationale and experimental design*

We have constructed an experimental system by which mutations on both strands can be separately measured. We used nitrous acid (HNO_2), a deaminating agent that attacks adenine in an A:T base pair. The resulting hypoxanthine (20) pairs with cytosine and produces A:T→G:C transition mutations after replication. Our target was a premature TAG (amber) stop codon that replaced codon 17 (TGG) of the *lacZ* (ß-galactosidase) gene (http://www.ncbi.nlm.nih.gov/nuccore/NC_000913.3). The amber mutants were Lac^-, that is, unable to grow on lactose as the sole carbon source, but they could undergo HNO_2-induced reversion to Lac^+. The amber codon contains adenines on both the transcribed and the non-transcribed strands. From the DNA sequence of each Lac^+ revertant, we can determine which strand was mutated (**Figure 2**).

The tester strains contained a disrupted allele of *nfi*, which encodes endonuclease V (21, 22). Endonuclease V catalyzes the first step in the excision repair of hypoxanthine-containing DNA (23), and *nfi* mutants have an enhanced frequency of HNO_2-induced A:T→G:C transition mutations (24). The tester strains also contained a chromosomal deletion for the *lac* operon. The *lacZ* amber mutant alleles were on an *attλ* element (25), which is a non-replicating DNA segment inserted at the bacterial attachment site for bacteriophage λ. Because gene orientation affects strand bias for mutagenesis, two tester strains were constructed such that the *attλ* elements (and therefore the *lacZ* genes) were in opposite orientations: the transcribed strand of *lacZ* was either the leading or the lagging strand during DNA replication.

Steps were taken to minimize DNA replication during selection because it would reduce any strand bias. The mutagenesis was performed on stationary phase cells that were starved by being washed and incubated in a nutrient-free solution, and the agar selection medium was purged of carbon sources (other than lactose) by prior growth of a Δ*lac* mutant (BW5660) on its surface. In addition, we eliminated the possibility of a sampling bias based on colony size. After 48 h of growth on lactose-minimal media the mean colony diameters (± 1 SD) were the same [1.1 (± 0.1) mm] for the both the TGG and CAG revertants, based on random samples of fifty colonies each.

5.2 *HNO_2 treatment and outgrowth of mutants*

A preliminary experiment was performed to determine whether the transcribed and non-transcribed strands were equally susceptible to damage by HNO_2. A culture that was grown to saturation without added glucose was exposed to HNO_2. Before being plated on lactose-minimal media, the cells were grown overnight in LB broth to allow the propagation of mutants containing lesions in either strand. Lac reversion was induced greater than 20-fold among cells treated with HNO_2. Thirty Lac^+ revertants were picked, all of which had mutations in the non-transcribed strand. This result was consistent with the suggestion of Davis (8) that the single-stranded region of an NTS in a transcription bubble is highly sensitive to DNA-damaging agents. To observe the effects of retromutagenesis, we sought conditions under which both strands of DNA are about equally damaged. Therefore, in subsequent experiments, we grew the cells in the presence of glucose before mutagenesis to enable catabolite repression of *lac*

transcription (26). This resulted in a more balanced distribution of mutations between the two strands, as will be shown below.

The strand specificity for mutagenesis was determined as detailed under **Methods** and outlined in **Figure 3**. Briefly, the amber mutant tester strains were grown to saturation with glucose, washed, and starved. Multiple samples were treated with $NaNO_2$ in an acidic buffer, with a survival of 29 to 33%. After the treatment was stopped with a neutral buffer, each sample was divided in two. One portion was spread immediately on the selective medium; the other was grown in LB broth before selection (**Figure 3**). If reversion occurred entirely by retromutagenesis, direct plating would reveal that only TS mutations were selected, whereas after intermediate growth, both TS and NTS mutations would appear among the revertants.

Treatment with HNO_2 increased the frequency of Lac^+ revertants by 19- to 49-fold (**Table S3**). Because of the relatively low frequency of spontaneous mutants and their statistically small sample size, the sequencing data for the HNO_2-induced revertants (**Table 1**) were not corrected for those of the untreated samples. When the mutagenized cultures were allowed to grow in LB broth before selection, they yielded CAG and TGG Lac^+ revertants in almost equal number (**Table 1**), indicating that both strands were about equally mutagenized ($p = 0.7$, Fisher exact test). However, when the mutagenized cells were plated directly on selective medium, without intermediate growth, CAG mutants predominated, indicating a preference for TS mutations ($p < 0.01$, Fisher's exact test) in strains with either gene orientation. Therefore, during selection, most of mutations of the NTS were not replicated, allowing those of the TS to prevail. The results were not significantly affected by gene orientation (**Table 1**) ($p < 0.01$, Cochran-Mantel-Haenszel

test), which is consistent with our expectation that at the time of treatment with HNO_2, our stationary phase cells did not contain a significant number of replication forks. These findings support the transcription-mediated mechanism of retromutagenesis for the inheritance of new mutations under selective conditions.

6. Discussion

Retromutagenesis provides a simple, direct explanation for adaptive mutation. However, it is not as obvious as it first seems because it relies on several tacit assumptions. Retromutagenesis requires that RNA polymerases bypass lesions in DNA and incorporate mutations analogous to those caused by DNA polymerase bypass (described in **Introduction**). Our experimental demonstration relied on a further assumption, which we verified. During selection, the starved cells had to be able to harvest enough energy to make functional protein (β-galactosidase) from the damaged gene before they have enough energy for DNA synthesis. In addition, our results provide evidence, albeit indirect, that hypoxanthine can be added to the list of abnormal bases (10) that undergo similar base mispairing when bypassed by either DNA or RNA polymerase in *E. coli.*

For many years, adaptive mutation has been operationally defined by the observation of mutagenesis during selection as evidenced by the continuous outgrowth of new mutants many days after selective plating, during which there is no observable background growth of the parental cells. In our experiments, however, DNA damage was induced before selection, and the mutant colonies appeared by 48 h of growth. The rapid, exclusive growth of revertants under selection enabled us to avoid interference from growth-dependent mechanisms that may contribute to adaptive mutation. Most of the previous experiments on adaptive mutation have attempted to rule out growth of the parent cells during selection, which is almost impossible to do with the necessary rigor. Although there may be no observable overall growth, there might still be growth of a sub-population that gives rise to new mutants. Consequently, it is generally acknowledged

that much apparent adaptive mutation stems from occult growth of at least some parental cells, or at least of parts of their chromosomes through gene amplification (7). In our approach, however, there is no need to rule out DNA replication occurring before selection, because it would only have reduced the observed strand bias. Instead of measuring overall DNA replication via cellular growth in the parental population during selection as in previously published experiments, we are able to gauge DNA replication specifically in the revertants via NTS mutations. The low frequency of NTS mutations found after direct selection indicates there was little DNA synthesis before gene expression and strongly supports the model of retromutagenesis.

Our studies employed an amber mutation in our tester strains. The expected low residual activity of the protein containing the amber mutation minimized growth of the parental cells during selection. Its position in a dispensable part of the *lacZ* gene (27) meant that unlike most other mutation indicators, it should be able to revert by more than one type of amino acid substitution, enabling detection of mutations at different positions in the codon. However, there is a caveat to using this method as a general approach. Although the early region of the gene can be deleted without significant loss of function, it is possible that some amino acid substitutions may cause a deleterious alteration of protein structure. In a preliminary experiment, we found that we were unable to use an amber mutation in codon 3 of *lacZ*. Following mutagenesis and intermediate growth, the revertants displayed AT → GC mutations at only the first position of the codon, which restored the wild type sequence (data not shown).

When the cells were grown without added glucose, exposed to HNO_2, and then grown before selection, all of the new revertants studied were NTS mutants. Although

transcription-coupled repair (28) could create such a strand bias by selectively repairing the TS, it acts only on lesions that obstruct RNA polymerase and that are repaired by the UvrABC system, both of which are not features of hypoxanthine in DNA. Our subsequent demonstration of a high frequency of TS mutations during both direct and delayed selection (after catabolite repression) provides direct experimental evidence that transcription-coupled repair is not functioning on these lesions. NTS mutations in the absence of catabolite repression are best explained by "transcription-associated mutagenesis" (not to be confused with "transcriptional mutagenesis"), reviewed by Jinks-Robertson and Bhagwat (29). This mechanism provides the basis for an alternative model of adaptive mutation, proposed by Davis (8), which was prompted by the widespread observation that single-stranded DNA, such as that in a transcription bubble, is unusually sensitive to many types of DNA damage. This susceptibility has been specifically confirmed for nitrosative deamination *in vitro*, which is ten times faster in single-stranded than in double-stranded DNA (30). In contrast to retromutagenesis, the Davis model states that selectable mutations result from damage to the NTS, and a round of DNA replication is required to encode a mutation in the TS for gene expression to begin. Our results indicated that our experimental system can detect mutational bias toward the NTS as well as toward the TS, although we did not specifically test the contribution of this transcription-associated mutagenesis to adaptive mutation. However, we may have gained some insight into its significance. The extent of this NTS bias surprised us for several reasons. First, previous reports had indicated only a fourfold to tenfold increase in spontaneous mutations of the NTS during transcription, although mutations on the TS were not studied at the same time (31, 32). Second, we did not

expect persistent transcripts of the *lac* operon in our starved, stationary phase cells at the time of mutagenic treatment. Our strains had been constructed with a tested, repressible *lac* operon and they had been grown without an added inducer. The explanation may lie in the spontaneous cAMP-dependent derepression of the *lac* operon observed in cells entering the stationary phase (33) and in the possible contamination, by lactose, of tryptone (digested casein) in the medium (34). Third, it seemed unlikely that DNA-bound RNAs should persist in stationary phase cells. They should be digested by ribonuclease HI, or else they could serve as primers for aberrant ("constitutive stable") DNA replication (35). It appears that stationary-phase cells may be primed for transcription-associated mutagenesis and adaptive mutation by the induction of cAMP-regulated genes and the persistence of transcription bubbles.

How widespread is retromutagenesis in nature? Although it does not apply to growing cells, most organisms in nature and most cells in our body exist in a state of limited growth. Retromutagenesis applies only to dominant mutations, but this should be true of most adaptive mutations, which are usually due to a gain of function. Theoretically, retromutagenesis cannot provide resistance to inhibitors of transcription or of protein synthesis because it relies on gene expression to enable the initial DNA synthesis that establishes heritability. It could, however, mediate resistance to DNA synthesis inhibitors or to tumor suppressors. For example, adaptive mutation to ciprofloxacin resistance was observed in *E. coli*; new mutants arose continuously days after plating in the presence of the antibiotic (36). During this time there was no measurable growth of the parental cells; ciprofloxacin is an inhibitor of gyrase and therefore of DNA synthesis. Remarkably, in a parallel experiment, stationary phase cells

did not become resistant to rifampin, an inhibitor of RNA polymerase. Although it was not appreciated at the time, these combined findings point to retromutagenesis as the adaptive mechanism. Similarly, retromutagenesis could be involved in resistance to the antimicrobial agent trimethoprim or the antitumor compound methotrexate, both based on dominant mutations that block the binding of the drugs to dihydrofolate reductase (37, 38), which would otherwise ultimately inhibit DNA synthesis. Other possible examples in eukaryotes are the common mutations in *p53* that turn this tumor suppressor gene into a dominant transforming oncogene (39) and dominant gain-of-function mutations in *JAK2* that result in myeloproliferative disorders (40). Similarly, TM can induce single-base changes in Ras transcripts that cause pro-growth increases in phosphorylated ERK protein (41).

Retromutagenesis is a transcription-mediated process of adaptive mutation that requires a damaged gene to be expressed before it can be replicated. In light of previous evidence, it is probably one of several mechanisms by which cells can undergo directed selection in the absence of apparent growth.

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References

1. Lederberg J, Lederberg EM. 1952. Replica plating and indirect selection of bacterial mutants. Journal of bacteriology 63(3):399-406.

2. Luria SE, Delbruck M. 1943. Mutations of Bacteria from Virus Sensitivity to Virus Resistance. Genetics 28(6):491-511.

3. Bridges BA. 1994. Starvation-associated mutation in Escherichia coli: a spontaneous lesion hypothesis for "directed" mutation. Mutat Res 307(1):149-156.

4. Cairns J, Overbaugh J, Miller S. 1988. The origin of mutants. Nature 335(6186):142-145.

5. Ryan FJ, Okada T, Nagata T. 1963. Spontaneous mutation in spheroplasts of Escherichia coli. J Gen Microbiol. 1963 Feb;30:193-9.

6. Cairns J, Foster PL. 1991. Adaptive reversion of a frameshift mutation in Escherichia coli. Genetics 128(4):695-701.

7. Roth JR, Kugelberg E, Reams AB, Kofoid E, Andersson DI. 2006. Origin of mutations under selection: the adaptive mutation controversy. Annu Rev Microbiol 60:477-501.

8. Davis BD. 1989. Transcriptional bias: a non-Lamarckian mechanism for substrate-induced mutations. Proceedings of the National Academy of Sciences of the United States of America 86(13):5005-5009.

9. Doetsch PW. 2002. Translesion synthesis by RNA polymerases: occurrence and biological implications for transcriptional mutagenesis. Mutat Res 510(1-2):131-140.

10. Bregeon D, Doetsch PW. 2011. Transcriptional mutagenesis: causes and involvement in tumour development. Nature reviews Cancer 11(3):218-227.

11. Shockley AH, Doo DW, Rodriguez GP, Crouse GF. 2013. Oxidative damage and mutagenesis in Saccharomyces cerevisiae: genetic studies of pathways affecting replication fidelity of 8-oxoguanine. Genetics 195(2):359-367.

12. Rodriguez GP, Romanova NV, Bao G, Rouf NC, Kow YW, Crouse GF. 2012. Mismatch repair-dependent mutagenesis in nondividing cells. Proceedings of the National Academy of Sciences of the United States of America 109(16):6153-6158.

13. Sternberg NL, Maurer R. 1991. Bacteriophage-mediated generalized transduction in Escherichia coli and Salmonella typhimurium. Methods Enzymol 204:18-43.

14. Miller J. 1993. A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria. Trends in Biochemical Sciences-Library Compendium 18:193.

15. Vogel HJ, Bonner DM. 1956. Acetylornithinase of Escherichia coli: partial purification and some properties. The Journal of biological chemistry 218(1):97-106.

16. Smith TF, Sadler JR. 1971. The nature of lactose operator constitutive mutations. J Mol Biol. Jul 28;59(2):273-305.

17. Sheu DS, Wang YT, Lee CY. 2000. Rapid detection of polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. Microbiology 146 (Pt 8):2019-2025.

18. Agresti A. 1992. A Survey of Exact Inference for Contingency Tables. Statistical Science,7,131-153.

19. McDonald, J.H. 2014. Handbook of Biological Statistics (3rd ed.). Sparky House Publishing, Baltimore, Maryland.

20. Shapiro R, Pohl SH. 1968. The reaction of ribonucleosides with nitrous acid. Side products and kinetics. Biochemistry 7(1):448-455.

21. Guo G, Ding Y, Weiss B. 1997. nfi, the gene for endonuclease V in Escherichia coli K-12. J Bacteriol. Jan;179(2):310-6.

22. Yao M, Kow YW. 1996. Cleavage of insertion/deletion mismatches, flap and pseudo-Y DNA structures by deoxyinosine 3'-endonuclease from Escherichia coli. J Biol Chem. Nov 29;271(48):30672-6.

23. Yao M, Hatahet Z, Melamede RJ, Kow YW. 1994. Purification and characterization of a novel deoxyinosine-specific enzyme, deoxyinosine 3' endonuclease, from Escherichia coli. J Biol Chem. Jun 10;269(23):16260-8.

24. Schouten KA, Weiss B. 1999. Endonuclease V protects Escherichia coli against specific mutations caused by nitrous acid. Mutat Res 435(3):245-254.

25. Diederich L, Rasmussen LJ, Messer W. 1992. New cloning vectors for integration in the lambda attachment site attB of the Escherichia coli chromosome. Plasmid 28(1):14-24.

26. Pastan I, Perlman RL. 1969. Repression of beta-galactosidase synthesis by glucose in phosphotransferase mutants of Escherichia coli. Repression in the absence of glucose phosphorylation. The Journal of biological chemistry 244(21):5836-5842.

27. Miller JH, Albertini AM. 1983. Effects of surrounding sequence on the suppression of nonsense codons. Journal of molecular biology 164(1):59-71.

28. Hanawalt PC, Spivak G. 2008. Transcription-coupled DNA repair: two decades of progress and surprises. Nat Rev Mol Cell Biol. Dec;9(12):958-70.

29. Jinks-Robertson S, Bhagwat AS. 2014. Transcription-associated mutagenesis. Annu Rev Genet. 48:341-59.

30. Caulfield JL, Wishnok JS, Tannenbaum SR. 1998. Nitric oxide-induced deamination of cytosine and guanine in deoxynucleosides and oligonucleotides. J Biol Chem. May 22;273(21):12689-95.

31. Beletskii A, Bhagwat AS. 1996. Transcription-induced mutations: increase in C to T mutations in the nontranscribed strand during transcription in Escherichia coli. Proc Natl Acad Sci U S A. Nov 26;93(24):13919-24.

32. Beletskii A, Bhagwat AS. 1998. Correlation between transcription and C to T mutations in the non-transcribed DNA strand. Biol Chem. Apr-May;379(4-5):549-51.

33. Grossman TH, Kawasaki ES, Punreddy SR, Osburne MS. 1998. Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. Gene. Mar 16;209(1-2):95-103.

34. Studier FW. 2005. Protein production by auto-induction in high density shaking cultures. Protein expression and purification 41(1):207-234.

35. Kogoma T. 1997. Stable DNA replication: interplay between DNA replication, homologous recombination, and transcription. Microbiology and molecular biology reviews : MMBR 61(2):212-238.

36. Riesenfeld C, Everett M, Piddock LJ, Hall BG. 1997. Adaptive mutations produce resistance to ciprofloxacin. Antimicrobial agents and chemotherapy 41(9):2059-2060.

37. Dale GE, Broger C, D'Arcy A, Hartman PG, DeHoogt R, Jolidon S, Kompis I, Labhardt AM, Langen H, Locher H, Page MG, Stuber D, Then RL, Wipf B, Oefner C. 1997. A single amino acid substitution in Staphylococcus aureus dihydrofolate reductase determines trimethoprim resistance. Journal of molecular biology 266(1):23-30.

38. Simonsen CC, Levinson AD. 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proceedings of the National Academy of Sciences of the United States of America 80(9):2495-2499.

39. Gannon JV, Greaves R, Iggo R, Lane DP. 1990. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J 9(5):1595-1602.

40. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, Tichelli A, Cazzola M, Skoda RC. 2005. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 352(17):1779-1790.

41. Saxowsky TT, Meadows KL, Klungland A, Doetsch PW. 8-Oxoguanine-mediated transcriptional mutagenesis causes Ras activation in mammalian cells. Proc Natl Acad Sci U S A. 2008 Dec 2;105(48):18877-82.

42. Weiss B. 2008. Removal of deoxyinosine from the Escherichia coli chromosome as studied by oligonucleotide transformation. DNA Repair (Amst). Feb 1;7(2):205-12.

43. Wanner BL. 1986. Novel regulatory mutants of the phosphate regulon in Escherichia coli K-12. J Mol Biol. Sep 5;191(1):39-58.

44. Fijalkowska IJ, Jonczyk P, Tkaczyk MM, Bialoskorska M, Schaaper RM. 1998. Unequal fidelity of leading strand and lagging strand DNA replication on the Escherichia coli chromosome. Proc Natl Acad Sci U S A. Aug 18;95(17):10020-5.

45. Schouten KA, Weiss B. 1999. Endonuclease V protects Escherichia coli against specific mutations caused by nitrous acid. Mutat Res 435(3):245-254.

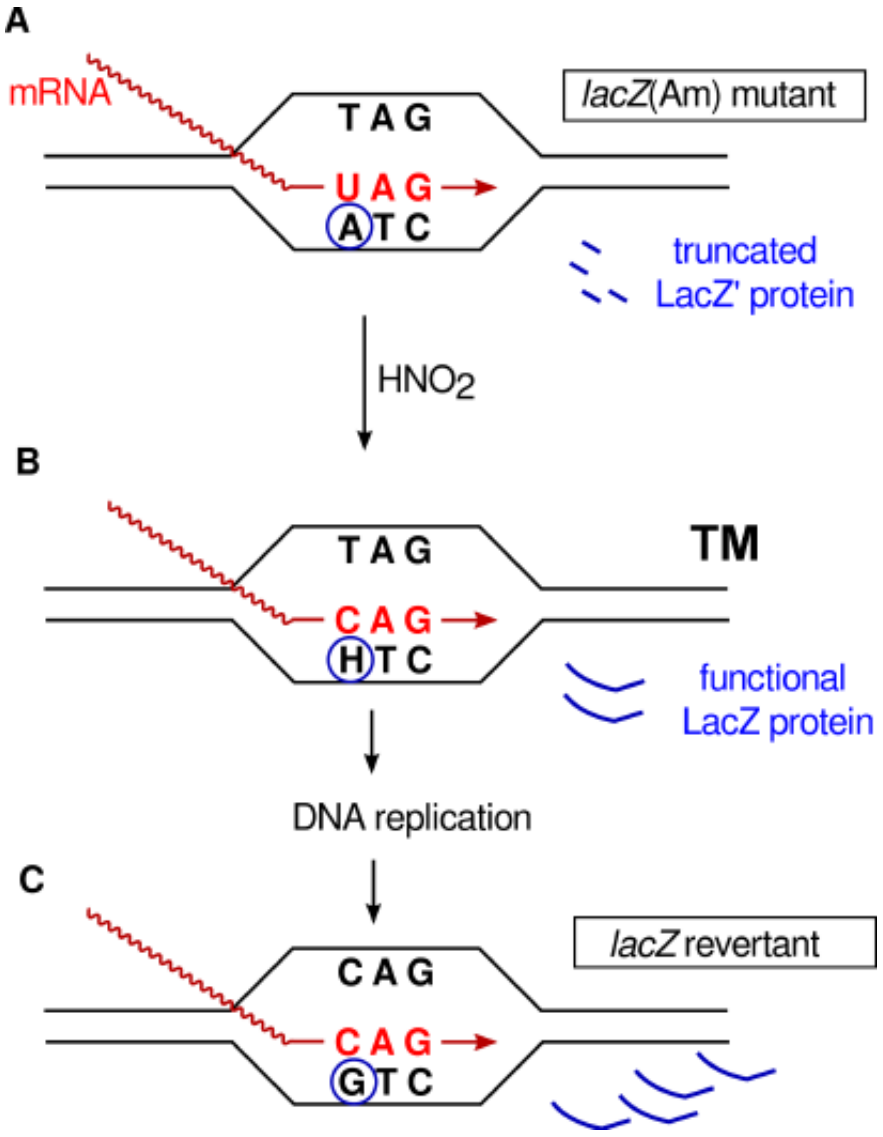

Fig. 1. Example of retromutagenesis. Shown are schematic representations of a transcription bubble that containing an amber (TAG) stop codon from which is produced inactive, truncated LacZ' polypeptide. (**A**) An adenine in the TS is deaminated by HNO_2 to hypoxanthine (H). (**B**) RNA polymerase pairs the hypoxanthine with cytidine instead of the original uracil, leading to the translation of a full-length functional β-galactosidase (*LacZ*), which allows the cell to grow on selective media. (**C**) This gene expression

enables DNA replication, which establishes the A:T → G:C mutation producing the Lac^+ phenotype.

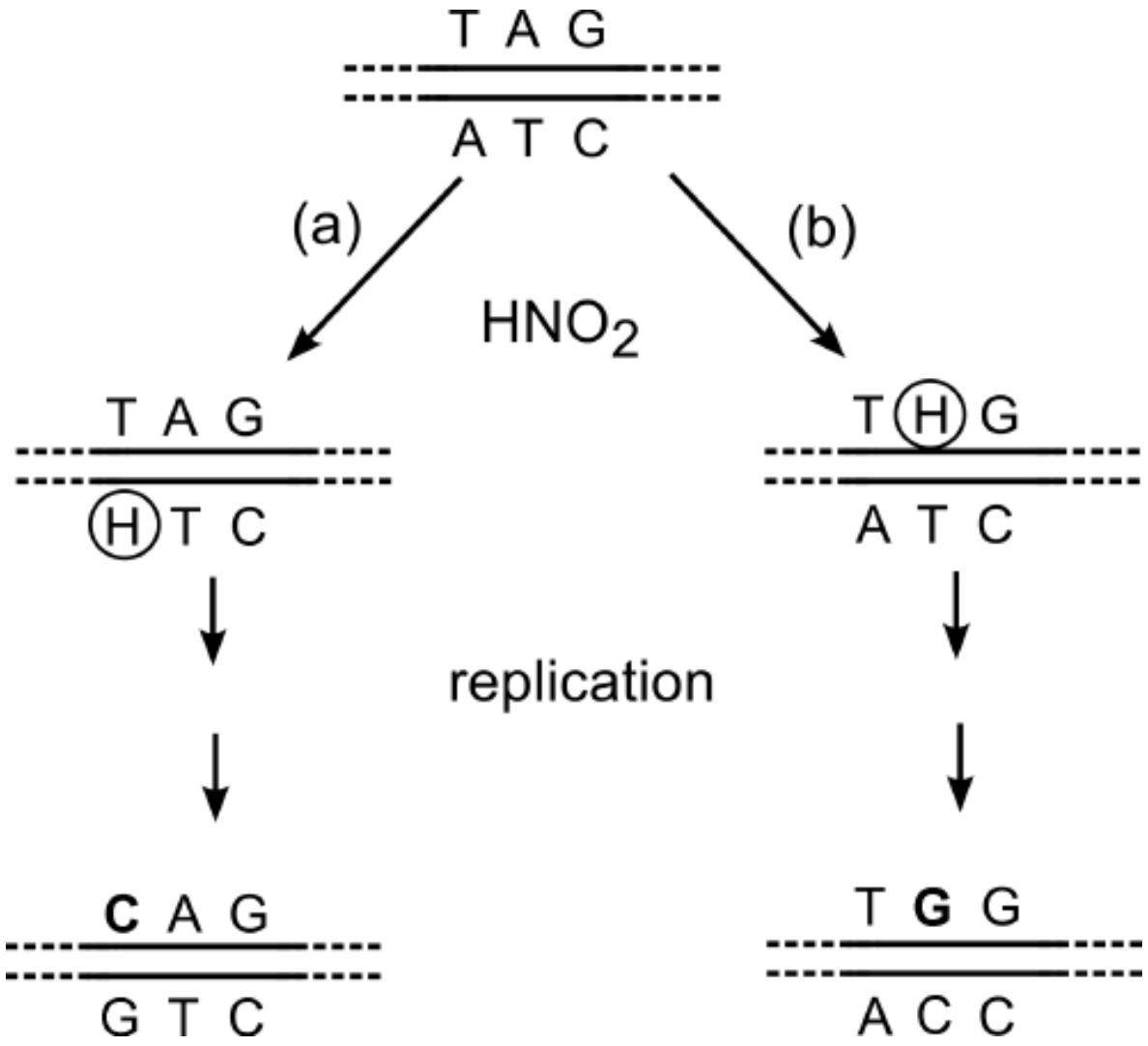

Fig. 2. Different sequences produced by nitrosative deamination of an adenine on the (a) TS or (b) NTS. Although TGG (Trp) is the wild type codon and CAG (Gln) is a missense codon, they both restore functionality (see **Results**), and can be distingushed through DNA sequencing of Lac^+ revertants. H, hypoxanthine.

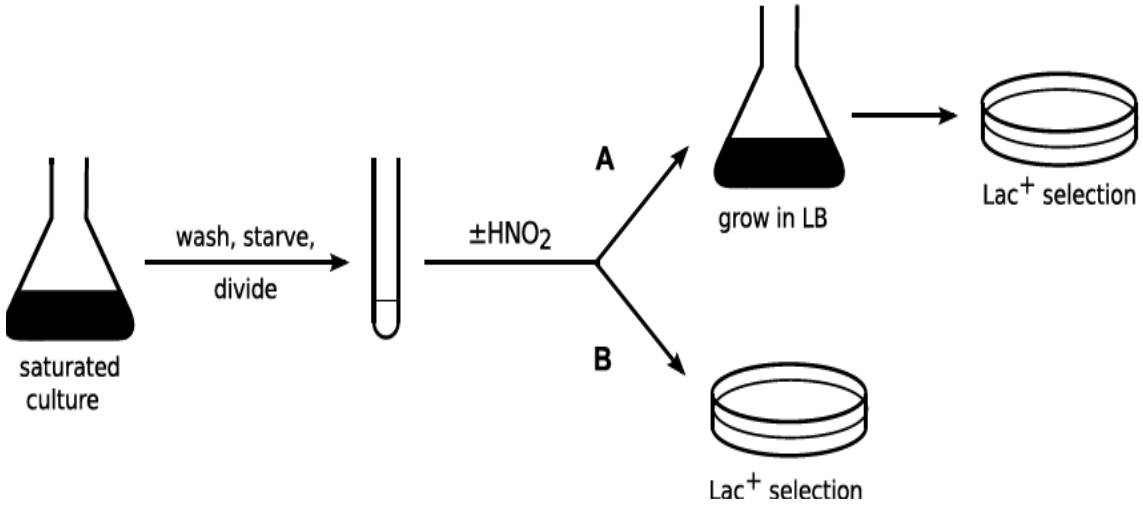

Fig. 3. **Experimental scheme**. A stationary phase, saturated culture of lac mutants in LB broth + glucose is divided into many samples, washed by centrifigation, starved by incubation in 10 mM $MgSO_4$, resuspended in acidic buffer, and incubated with or without added $NaNO_2$. Each sample is divided in half. One part (**A**) is grown in LB broth before being plated on lactose-minimal agar. The other (**B**) is plated directly on the lactose-minimal agar. After 48 h, Lac^+ revertant colonies were picked for DNA sequencing.

Table 1. Spectrum of mutations in a *lacZ* amber codon after HNO_2 mutagenesis.

Gene orientation[a] (strain)	Lac$^+$ selection	Lac$^+$ revertants		
		CAG (TS)	TAG (NTS)	Other[b]
Leading strand (Z126)	Immediate	47	6	6
	After growth	22	23	9
Lagging strand (Z127)	Immediate	49	5	7
	After growth	21	24	9

a) Gene orientation refers to the directionality of the transcribed strand at the replication fork.

b) Other mutations consisted of in-codon transversions and uncharacterized suppressor mutations (see **Table S4**).

Table S1. Bacterial strains used.

Strain[a]	Relevant genotype[b]	Reference or source[c]
BW1181	*nfi-1*::*cat* Δ(*argF-lac*)*169*	B. Weiss
BW1948	Δ(*cro-bioA*) *lacZ*(Am) (nt 50, with G→A)	42
BW5660	Δ(*gpt-lac*)5 *glnX44*(AS)	43
EC3150	*attλ*::[*lacI lacZ*(*CC106*) *bla*]Left	44
EC3209	*attλ*::[*lacI lacZ*(*CC106*) *bla*]Right	44
Z122	*attλ*::[*lacI lacZ(CC106) bla*]Right Δ(*gpt-lac*)5 *glnX44*(AS)	P1(EC3209) × BW5660 → Amp^r
Z123	*attλ*::[*lacI lacZ*(*CC106*) *bla*]Left Δ(*gpt-lac*)5 *glnX44*(AS)	P1(EC3150) × BW5660 → Amp^r
Z124	*attλ*::[*lacZ*(Am)(nt 50, G→A) *bla*]Right Δ(*gpt-lac*)5 *glnX44*(AS)	P1(BW1948) × Z122 → Lac$^+$, inducible
Z125	*attλ*::[*lacZ*(Am)(nt 50, G→A) *bla*]Left Δ(*gpt-lac*)5 *glnX44*(AS)	P1(BW1948) × Z123 → Lac$^+$, inducible
Z126	*attλ*::[*lacZ*(Am) (nt 50, G→A) *bla*]Right Δ(*argF-lac*)*169 nfi-1*::*cat*	P1(Z124) × BW1181 → Amp^r
Z127	*attλ*::[*lacZ*(Am) (nt 50, G→A) *bla*]HLeft Δ(*argF-lac*)*169 nfi-1*::*cat*	P1(Z125) × BW1181 → Amp^r

a) All strains were derivatives of *E. coli* K-12 F$^-$λ$^-$.

b) The *attλ* insertion elements contain the entire *lac* operon, with mutations in the *lacI* and *lacZ* genes where indicated, and a functional β-lactamase (*bla*) gene specifying ampicillin resistance (Amp^r). "Left" and "Right" refer to the orientation of the *lac* operon in the *attλ* elements with respect to the origin o DNA replication (44). The *lacZ*(*CC106*) allele has as missense mutation at nucleotide (nt) 1384. Non-standard abbreviations: Am, amber mutation; AS, amber suppressor; nt, nucleotide.

c) Transductions with phage P1 are described as follows: P1(donor) × recipient → selected phenotype. Amp^r, ampicillin resistance.

Table S2. Oligonucleotide primers used.

Primers	Sequence (5' → 3')	Target
lacZ-249F	TCAGCTGTTGCCCGTCTCAC	*lacZ*(Am) codon 17
lacZ+178R	CGCCATTCGCCATTCAGG	*lacZ*(Am) codon 17
nfi::catF	ATGGATCTCGCGTCATTAC	*nfi-1*::*cat* insertion
nfi::catR	CAGTTTACCTGAATTAGGG	*nfi-1*::*cat* insertion
pLDR9 MCS 5′F	AGGTGCCTCACTGATTAAGC	*attλ* element orientation, with lac insert 5′ B
lac insert 5′ B	CCCGTTATAGGAGTGC	*attλ* element orientation, with pLDR9 MCS 5′F
PstI 5′ F	ATGGATAAGCTTGGGCTGCA	*attλ* element orientation, with PstI 5′ F
PstI 3′ R	GGGATCCTCTAGAGTCGACC	*attλ* element orientation, with PstI 5′ F

Table S3. Reversion frequencies after exposure to nitrous acid.

Gene orientation[a] and strain	Lac^+ Selection	$NaNO_2$ exposure	Lac^+ revertants per 10^8 viable cells	Induced/spontaneous revertants
Leading strand (strain Z126)	Immediate	-	0.9	33
		+	30	
	After non-selective growth	-	1.3	40
		+	50	
Lagging strand (strain Z127)	Immediate	-	1.1	19
		+	20	
	After non-selective growth	-	1.0	49
		+	51	

a) Gene orientation refers to the direction of the transcribed strand at the replication fork.

Table S4. Spectrum of unanticipated sequences in a *lacZ*$^+$ *revertants at* amber codon after nitrous acid mutagenesis[a]

Gene orientation[b]	**Lac$^+$ selection**	**Lac$^+$ revertants**				
		AAG	**TAT**	**TTG**	**GAG**	**TAG**[c]
Leading strand (strain Z126)	Immediate	2	1	0	0	3
	After intermediate growth	3	1	1	0	4
Lagging strand (strain Z127)	Immediate	2	2	1	0	2
	After intermediate growth	1	2	0	2	4

a) The table shows the breakdown of revertants in the category "Other" in **Table 1** of Results.
b) Gene orientation refers to the directionality of the transcribed strand at the replication fork.
c) Revertants that retained the original TAG codon had unmapped suppressor mutations, by definition. The other mutations are transversions, the frequencies of which are enhanced by HNO_2 in both wild type and *nfi* mutant cells (45). TAA, which should have resulted from the deamination of guanine or cytosine at the third position of the codon (45), was not found in the revertants because it is a stop codon.

CHAPTER 3

INACTIVATION OF A COMMON OGG1 VARIANT BY TNF-ALPHA IN MAMMALIAN CELLS

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Abstract

Reactive oxygen species threaten genomic integrity by inducing oxidative DNA damage. One common form of oxidative DNA damage is the mutagenic lesion 8-oxoguanine (8-oxodG). One driver of oxidative stress that can induce 8-oxodG is inflammation, which can be initiated by the cytokine tumor necrosis factor alpha (TNF-α). Oxidative DNA damage is primarily repaired by the base excision repair pathway, initiated by glycosylases targeting specific DNA lesions. 8-oxodG is excised by 8-oxoguanine glycosylase 1 (OGG1). A common *Ogg1* allelic variant is S326C-*Ogg1*, prevalent in Asian and Caucasian populations. S326C-*Ogg1* is associated with various forms of cancer, and S326C-OGG1 is inactivated by oxidation. However, whether oxidative stress caused by inflammatory cytokines compromises OGG1 variant repair activity remains unknown. We addressed whether TNF-α causes oxidative stress that both induces DNA damage and inactivates S326C-OGG1 via cysteine 326 oxidation. In mouse embryonic fibroblasts, we found that S326C-OGG1 was inactivated only after exposure to H_2O_2 or TNF-α. Treatment with the antioxidant N-acetylcysteine prior to oxidative stress rescued S326C-OGG1 activity, demonstrated by *in vitro* and cellular repair assays. In contrast, S326C-OGG1 activity was unaffected by potassium bromate, which induces oxidative DNA damage without causing oxidative stress, and presumably cysteine oxidation. This study reveals that Cys326 is vulnerable to oxidation that inactivates S326C-OGG1. Physiologically relevant levels of TNF-α simultaneously induce 8-oxodG and inactivate S326C-OGG1. These results suggest a mechanism that could contribute to increased risk of cancer among S326C-*Ogg1* homozygous individuals.

1. Introduction

Mammalian cells undergo continuous oxidative DNA damage resulting from reactive oxygen species (ROS) that are generated endogenously from sources such as normal cellular metabolism (1) and exogenously from sources such as radiation (2). Oxidative stress occurs when the concentration of ROS exhausts the cellular antioxidant capacity, leaving ROS free to damage proteins, lipids, and nucleic acids (3). ROS-induced DNA damage can drive mutagenesis if DNA damage is left unrepaired (4). One of the major physiological contexts of oxidative stress is inflammation (5). Inflammation is one of the hallmarks of cancer (6), and is a known risk factor for cancer development (7). Inflammation in tumors is driven by cytokines such as TNF-α produced by tumor-associated cells, which induces ROS (8). Moreover, tumor cells actually produce TNF-α (9), which could cause chronic oxidative stress within the tumor microenvironment. TNF-α also promotes cancer by increasing cellular transformation, measured using a focus-formation assay (10). Similarly, stem cells treated with TNF-α underwent ROS-dependent genetic instability, and such cells injected into mice caused acute myelogenous leukemia (11).

Base excision repair (BER) is the major mechanism for repairing oxidative DNA damage. BER is initiated by *N*-glycosylases, each of which excise specific subsets of base damage (12). A common oxidative lesion is the mutagenic 8-oxoguanine (8-oxodG), which causes GC→TA transversions if left unrepaired (13). In mammalian cells, OGG1 is the major BER glycosylase that initiates repair of 8-oxodG (14). S326C-*Ogg1* (rs1052133) is an allelic variant found in 40-60% of Asian and 13-38% of

Caucasian individuals, and is associated with various forms of cancer, including lung cancer (15), gastric cancer (16), orolaryngeal cancer (16), and lung adenocarcinoma (17). The contribution of S326C-OGG1 to tumorigenesis may be due to inefficient repair activity. Decreased OGG1 repair activity would explain why S326C-OGG1 is deficient in suppressing spontaneous mutagenesis (18), but the physiological conditions influencing S326C-OGG1 activity remain poorly understood and controversial.

ROS can also impair the activities of proteins by oxidizing several different amino acids. Cysteine oxidation can cause disulfide bridges that can inactivate proteins (19). *In vitro* studies suggest that S326C-OGG1 may undergo decreased activity under oxidative stress (20-22). The mechanism by which variant Cys326 oxidation causes a loss in activity has been suggested to be S326C-OGG1 dimerization, as demonstrated with EMSA following an *in vitro* reaction (23). Importantly, Cys326 oxidation has been shown to impede glycosylase activity through cysteine disulfide bridge formation via MS analysis of clinical samples (24). S326C-OGG1 has also been shown to undergo dimerization after treatment with diamide *in vitro* (20). However, such dimerization has yet to be demonstrated under conditions of physiologically relevant oxidative stress, and thus the mechanism of Cys326 oxidation resulting in loss of activity is still unclear. In contrast, several studies indicate that S326C-OGG1 enzymatic activity is equivalent to that of WT OGG1 (25-27), while others have reported constitutively reduced activity in the variant OGG1 (23,28,29). While the methodologies employed in such studies vary, each used purified enzymes under conditions that may alter the redox status of S326C-OGG1. Therefore, a relevant question remains whether the repair activity of S326C-OGG1 is affected by physiologically relevant oxidative stressors, such as low

concentrations of H_2O_2 or TNF-α, to determine whether the resulting levels of ROS are sufficient to inactivate S326C-OGG1 in mammalian cells. In contrast to previous studies, we employed physiologically relevant oxidative stressors to induce DNA damage and compare the DNA repair capability of cells expressing the wild-type and S326C-*Ogg1* variant alleles.

2. Materials & Methods

2.1. Cell Culture & Plasmid Generation

Ogg1-/- and F11.1 WT mouse embryonic fibroblasts (MEFs) were a gift from Arne Klungland (University of Oslo, Norway). *Ogg1-/-* and F11.1 WT MEFs were authenticated by Southern hybridization analysis (14), and by Western blot. MEFs were cultured as previously described (30), except at 5% CO_2. Immortalized *Ogg1-/-* MEFs (KO) stably transfected with WT *Ogg1* or S326C-*Ogg1* and expressing similar levels of glycosylase were obtained as a gift from Nikolas J. Hodges (University of Birmingham, Birmingham, UK) and were cultured as previously described (22). WT and S326C-*Ogg1* stably transfected cells were authenticated by sequencing, and the OGG1 expression levels in these cells as well as in *Ogg1 -/-* KO MEFs were tested using anti-OGG1 antibody (Abcam) (31). The WT *HA*-tagged *Ogg1* allele on the pRVY-Tet expression vector was a gift from Joanne Sweasy (Yale University, New Haven, CT). *Ogg1-HA* was subcloned into *pcDNA-3.1* in order to increase expression to levels detectable in our *in vitro* system. PCR was used to introduce at the *BamHI* and *XhoI* sites to the HA-*Ogg1* construct, employing OS1(F) (5'-GGATCCATGCCTGCCCGCGCGCTTC-3') and OS1(R) (5'-CTCGAGTCAGAGGCTAGCGTAATCC-3'). The resulting product was sequenced (Beckman Coulter Genomics, Inc.). The S326C mutation was generated employing point mutagenesis with complementary primers SC1(F) (5'-CCGACCTGCGCCAATGCCGCCATG-3') and SC2(R) (5'-CATGGCGGCATTGGCGCAGGTCGG-3'), using the WT *Ogg1-HA* construct as a template, and verified by sequencing (Beckman Coulter Genomics, Inc.).

2.2. Cell Lysate Preparation

MEFs were transfected as previously described (30), using 2 µg DNA per transfection. Cells were treated with 0-100 µM H_2O_2 in PBS for 15 min on ice, or 10 ng/mL TNF-α (R&D Systems) for 1 or 2 h in complete media. In some experiments, cells were pre-incubated for 2.5h with 1 mM N-acetylcysteine (Sigma-Aldrich Co. LLC). Cells were released with trypsin, washed twice with PBS, and resuspended in 10 mM Tris pH 8.0, 2 mM EDTA, containing Complete™ protease inhibitor (Hoffmann-La Roche Ltd.). Glass beads were added to the cell solution, vortexed at maximum speed for 7 min, and centrifuged 10 min at 13,000 rpm. The supernatant protein concentration was measured using the DC™ Protein Assay (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions.

2.3. In Vitro OGG1 Activity Assays

Experimental lysates were incubated with 5'-5' ^{32}P-labeled 8-oxodG oligonucleotides, which was prepared as previously described (32). Lysates were brought to an equal volume by the addition of *Ogg1-/-* MEFs lysate, and incubated in a reaction with 50 fmol labeled oligonucleotide, 10 mM Tris pH 8.0, 50 mM NaCl, and 2 mM EDTA. Lysate reactions were incubated for 60 min at 37°C, and were stopped by adding an equal volume of 0.05% bromophenol blue, 0.05% xylene, and 10 mM EDTA solution and heated to 100°C for 8 min. From each reaction, 7 µL were resolved in a 12.5% polyacrylamide UreaGel (National Diagnostics), which was then run at 2000V, 100mA, 125W for 90 min. The dried gel was then exposed overnight to a phosphorimager screen (GE Healthcare), which was then scanned using a Typhoon ™ Trio+ device (GE

Healthcare) (33). To calculate OGG1 activity, the 3' product and the uncut substrate bands in the autoradiograms as well as OGG1 expression and beta-actin levels measured by western blot were quantified by densitometry using ImageQuantTL software (GE Healthcare Life Sciences). The activity was corrected for transfection efficiency and protein loading among the different samples using the formula outlined below (34).

$$\text{Adjusted enzymatic activity} = \frac{\text{3' fragment}}{\text{3' fragment + uncut substrate}} \Big/ \frac{\text{OGG1-HA band}}{\text{Average OGG1-HA band}} \Big/ \frac{\text{β-actin band}}{\text{Average β-actin band}}$$

2.4. Western Blots

Western blots were carried out according to the Amersham ECL Plex western blotting system (GE Healthcare) per the manufacturer's recommendations. Resolution of 30 µg of protein from each lysate was achieved using Novex 10% NuPAGE gels (Life Technologies). Blots were probed with mouse monoclonal HA.11 16B12 (Covance Inc.) (36) overnight at 4˚C with shaking, or with mouse monoclonal anti-ß-actin antibody, clone AC-15 (Sigma-Aldrich) (37). Secondary antibody ECL Plex goat-α-mouse IgG Cy5 (GE Healthcare) (38) was used at a dilution of 1:2500. Laser scanning of blots was carried out using the Typhoon™ Trio+ (GE Healthcare Life Sciences), then quantified using ImageQuantTL software (GE Healthcare Life Sciences).

2.5. ROS & Cell Viability Assays

To measure cellular ROS, we employed the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies Corp). Cells were plated at 2.0 x 10^5 cells/well in 12-well plates, washed in PBS, then treated with 300 µL 10 ng/mL TNF-α in

complete media for 60 min at 5% CO_2 at 37°C. Concurrently, cells were incubated with 0.1 mM Amplex® Red (AR) + 0.1 U/mL horseradish peroxidase (HRP) with or without 131 µg/mL bovine liver catalase (Sigma-Aldrich Co. LLC) for 30 min. Fluorescence was measured in 200 µL of the supernatant in black special-optics Costar 96-well plates (Thermo Fisher Scientific) using a SpectraMax M5 spectrophotometer (Molecular Dynamics) and SoftMax Pro software (Molecular Dynamics) at 550 nm excitation and 590 nm emission frequency. Specific H_2O_2-induced AR oxidation was calculated by subtracting the reading from the corresponding catalase-treated samples. After ROS determination, to adjust Amplex® Red fluorescent signals for cell density, the cells remaining on the plate were fixed in 65% methanol for 5 min, then 100% methanol for 15 min, 0.1% crystal violet was added to cells for 5 min, and 200 µL 2% Na deoxycholate was used to solubilize cell-associated color. The crystal violet staining in 200 µL of the supernatant was measured using the SpectraMax M5 spectrophotometer (Molecular Dynamics) and SoftMax Pro software (Molecular Dynamics) set to 595 nm. Cell viability was measured using the MTT assay (39). After treatment, 10 µL 0.5% MTT (Invitrogen) was added to 100 µL cell media, then incubated at 37°C at 5% CO_2 for 5 h. Media was removed, then 100 µL DMSO was added to each well, and incubated with shaking at RT in the dark until dye crystals dissolved. Cell viability was expressed as a percentage of surviving cells (average 595 nm absorption) using untreated cells as a reference.

2.6. Comet Assays

For comet assays, 3.0 x 10^6 cells were plated in 10 cm-diameter tissue culture plates the day before. Cells were collected by trypsinization, after which they were subjected to an OGG1-modified alkaline comet assay as described (40), except that 10 µL of cells were mixed with 80 µL 1% low melting-point agarose (Life Technologies Corp.). Damage repair was allowed by incubating in DMEM media at 37°C in 5% CO_2 for 1 h. Lysis solution included 88 µg/mL N-*tert*-Butyl-α-phenylnitrone (Calbiochem, Merck KGaA), and lysis proceeded for 2 h at 4°C. OGG1 enzyme (New England Biolabs) was incubated at 1067 U/mL in 40 mM Hepes pH 8, 100 mM KCl, 0.5 mM EDTA, and 0.02% BSA for 30 min at 37°C. DNA was separated by electrophoresis in 21 V, 0.45 mA at 4°C for 30 min in alkaline buffer supplemented with 88 µg/mL N-*tert*-Butyl-α-phenylnitrone (Calbiochem). DNA was stained with 1 µg/mL SYBR® Green (Life Technologies Corporation) in PBS for 20 min in darkness at RT and mounted in VECTASHIELD® (Vector Laboratories, Inc.). Cells were photographed using the Hamamatsu C4742-95-12HR camera (Hamamatsu Photonics) on an Olympus IX81 microscope (Olympus Corp. of the Americas). Photographs were taken of ≥ 100 cell comets, whose average % tail DNA was then scored using CometScore (TriTek Corp.).

2.7. Cysteine Oxidation Modeling

In order to predict the likelihood that OGG1 Cys326 undergoes disulfide bridge formation, the DiANNA software program was used. This program is a neural network that predicts the likelihood of disulfide bridge formation among cysteines based on a library of secondary interaction data (41). The amino acid sequence of S326C-OGG1

was generated by downloading the 8-oxoguanine DNA-glycosylase 1 isoform 1a [Homo sapiens] (NCBI accession number NP_002533.1), and substituting Cys for Ser at amino acid 326. This sequence was then entered in the DiANNA online interface. The cysteine-cysteine bond score and associated information was recorded for the highest-scoring interactions predicted.

2.8. Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, Inc.). In order to calculate statistical differences among discrete data points, *p* values were calculated by paired Student's t-test, while *p* values for comparing multiple values simultaneously were calculated using one-way ANOVA. All results shown are the average of three independent experiments ± standard error, unless stated otherwise.

3. Results & Conclusions

3.1. H_2O_2 Exposure Decreases S326C-OGG1 Cleavage Activity In Vitro

In order to determine the intrinsic activity of S326C-OGG1 in the absence of exogenous oxidative stress, *Ogg1*-/- MEFs were transfected with WT or S326C-*Ogg1* (see **Materials & Methods**). Cell lysates were incubated with ^{32}P-labeled oligonucleotides containing 8-oxodG for 1h, sufficient to allow the oligonucleotide cleavage to proceed to the maximum extent possible (**Fig. S1**). The amount of cleaved versus uncleaved oligonucleotide was used to measure the intrinsic N-glycosylase/AP lyase activity of S326C-OGG1 compared to WT OGG1, using reactions without lysate as a control (**Fig. 1A**). No significant difference was observed between the enzymatic activity of S326C-OGG1 and WT OGG1 in non-treated cells (both with ~30% oligonucleotide cleavage, **Fig. 1B**). The expression of MTH1 was not an issue in these experiments because MTH1 hydrolyzes 8-oxo-dGTP, and the 1h time course for the experiment was insufficient for the occurrence of DNA replication necessary for dNTP incorporation. The expression level of OGG1 was used to normalize the measurement of 8-oxodG glycosylase activity. The expression of MUTYH, another BER enzyme, was not of interest because it excises A paired with 8-oxodG, while the oligonucleotide in these experiments was paired with C (35). To assess whether S326C-OGG1 undergoes a loss of activity under oxidative stress, *Ogg1* -/- MEFs were transfected with WT or S326C-*Ogg1* and exposed to a dose range of 0-100 μM H_2O_2 for 15 min, which caused no cytotoxicity (**Fig. S2A**). S326C-OGG1 activity decreased following treatment with increasing doses of H_2O_2. Treatment with 100 μM H_2O_2 caused a significant loss of 8-

oxoguanine cleavage activity in S326C-OGG1 compared to WT OGG1, while treatment with 25 μM H_2O_2 did not ($p = 0.055$) (**Fig. 1C**). Oxidative stress resulting from exposure to H_2O_2 therefore causes inactivation of the enzymatic activity of S326C-OGG1, but not WT OGG1.

3.2. S326C-OGG1 Activity Decreases after Cellular Exposure to TNF-α Due to Oxidative Stress

Because of the substantial loss in activity of S326C-OGG1 following exposure to H_2O_2, we tested whether intracellular ROS induced by an inflammatory cytokine, such as TNF-α, are also capable of inactivating S326C-OGG1. We employed *Ogg1* knockout (KO) MEFs stably expressing wild-type (42) or S326C-OGG1 at comparable levels (**Fig. S3A**) that express receptors for TNF-α and respond with increased intracellular ROS levels as part of the TNF-α-mediated signal transduction cascade (43). Cellular ROS increases following treatment with TNF-α were measured by employing Amplex Red (AR), which is a reporter for H_2O_2, and added catalase to duplicate wells to ensure that the fluorescent signal in the assay specifically reported levels of H_2O_2 (**Fig. 2A**). We exposed cells to 10 ng/mL TNF-α because it was the lowest dose at which maximal cleavage activity loss was observed (**Fig. S4A**), is not cytotoxic (**Fig. S2B**), and approximates reported plasma concentrations under pathological pro-inflammatory states (5 ng/mL) (44). Importantly, neither H_2O_2 (**Fig. S5A**) nor TNF-α (**Fig. S5B**) cause OGG1 degradation after the exposure conditions tested. TNF-α treatment causes an increase in cellular H_2O_2 levels, which is abolished in N-acetylcysteine (NAC) -treated cells (**Fig. 2A**). Cells were exposed to TNF-α in the presence or absence of the

antioxidant NAC (45) to measure the degree to which ROS induced by TNF-α could be scavenged and reduced. These results are consistent with previously reported measurements of increased ROS following TNF-α treatment (8,46). TNF-α also caused inactivation of S326C-OGG1 after just 1h of treatment, as measured *in vitro* (**Fig. 2B**). The inactivation of S326C-OGG1 was prevented by treatment with NAC, while NAC treatment alone does not affect activity (**Fig. 2B**). WT OGG1 displayed no loss in activity after treatment with TNF-α, indicating that the activity of S326C-OGG1 is sensitive to cellular oxidative stress induced by TNF-α (**Fig. 2B**). The distinct sensitivity of the S326C-OGG1 variant to the redox environment may explain the disparities among measurements of S326C-OGG1 activity under different conditions previously reported in the literature (26-28).

3.3. TNF-α Exposure Causes Increases in 8-oxodG and Inactivates S326C-OGG1 Variant via Oxidation

After establishing the inactivation of S326C-OGG1 following cellular exposure to TNF-α, we tested next whether such inactivation would lead to the accumulation of 8-oxodG in nuclear DNA. We measured the 8-oxodG repair activity of S326C-OGG1 variant under oxidative stress employing the comet assay. After exposing stably-transfected *Ogg1* KO, WT, and S326C-*Ogg1* MEFs to 10 ng/mL TNF-α for 1h, the accumulation of 8-oxodG was determined by incubating the cells embedded in the agarose with recombinant OGG1. OGG1 treatment increases the number of single stand breaks detected in the alkaline comet assay by its associated lyase activity, as it primarily recognizes 8-oxodG in the nuclear DNA (40). TNF-α exposure resulted in a significant

increase in 8-oxodG levels in each *Ogg1* genotype detected immediately following treatment (**Fig. 3A**). However, S326C-*Ogg1* variant cells and *Ogg1* KO cells were unable to repair 8-oxodG within a 60-minute recovery period following TNF-α exposure, while WT *Ogg1* cells were able to completely repair the damage. In order to exclude the possibility that S326C-OGG1 variant is constitutively inactive in this cell system, we addressed whether S326C-OGG1 possesses repair activity, employing $KBrO_3$ as a DNA-damaging agent which causes oxidative DNA damage without increasing cellular ROS levels (**Fig. 3B**) and therefore without affecting the oxidation status of cysteine residues (47). S326C-OGG1 variant was able to repair 8-oxodG arising from treatment with $KBrO_3$ (**Fig. 3C**), indicating that S326C-OGG1 is functional, but it is inactivated by cysteine oxidation during oxidative stress.

3.4. Cys326 Oxidation May Cause S326C-OGG1 Dimerization through Disulfide Bridge Formation

To address the mechanism by which S326C-OGG1 variant oxidation causes loss of activity, the role of Cys326 disulfide bridge formation was investigated by employing the DiANNA software program that predicts the likelihood of disulfide bridge formation from cysteine oxidation states using a predictive pattern recognition network based upon known secondary structure interaction data (41). DiANNA predicted that S326C-OGG1 undergoes disulfide bond formation, either inter- or intra-molecularly, between Cys326 and Cys140, Cys241, and Cys146 (**Fig. S6A**). While the software predicts S326C-OGG1 will form a total of six possible disulfide bonds, WT OGG1 has the potential to form only three predicted disulfide bonds (**Fig. S6B**), suggesting that the presence of Cys326

substantially increases the likelihood of disulfide bond formation. These results are based on the score calculated from the likelihood of oxidation of a given cysteine (compared to a database of known oxidized cysteines), the likelihood of secondary interactions between any two cysteines, and the likelihood of disulfide bridge formations between the two cysteines.

4. Discussion

We have shown for the first time that oxidative stress caused by exposure to TNF-α or H_2O_2 not only generates potentially mutagenic 8-oxodG lesions, but also inactivates S326C-OGG1 variant in the repair of 8-oxodG. The impact of cellular oxidative damage is therefore predicted to be greater in individuals harboring this oxidation-sensitive glycosylase variant, because they may be at a greater risk for persistent DNA damage as they remain unrepaired for extended periods of time, which may result in mutations. When S326C-*Ogg1* cells are exposed to oxidative stressors, the Cys326 residues will undergo oxidation, which may cause the S326C-OGG1 enzymes to dimerize. After undergoing oxidation, S326C-OGG1 enzymes carry out inefficient repair of 8-oxodG lesions induced by oxidative stress. Because of this increased DNA damage and decreased repair, S326C-*Ogg1* carriers may have an increased potential for cancer development under oxidative stress by a mechanism such as the one proposed in **Figure 4**. Our findings stand in contrast to studies showing that the S326C-OGG1 variant has constitutively reduced activity compared to the WT protein (23,28,29). The results of this study do not contradict recent findings that S326C-OGG1 undergoes aggregation *in vivo* under conditions of oxidative stress (48). However, since S326C-OGG1 dimerization has been shown in previous studies (20,23,24) and is predicted *in silico* (**Fig. S6**), dimerization is believed to underpin the S326C-OGG1 repair activity loss seen under oxidative stress in this study.

Consistent with this model, although only S326C-*Ogg1* homozygosity is associated with cancer among people not exposed to a particular oxidative stressor (15), both S326C-*Ogg1* variant heterozygotic and homozygotic individuals are at a

significantly increased risk of developing lung cancer after exposure to the oxidative stressors polycyclic aromatic hydrocarbons (PAHs) (49). PAHs (components of coal and tobacco smoke) cause both oxidative damage to DNA as well as the accumulation of ROS and are examples of environmental oxidative stressors that could inactivate S326C-OGG1 (49) in addition to their role as mutagens (50). Decreased activity of S326C-OGG1 may contribute to the development of lung cancer, as proposed in a study that found that low OGG1 activity is associated with lung cancer among patients who smoke tobacco (51). Such studies suggest that oxidative stress is a mechanism by which cellular microenvironments inactivate S326C-OGG1 and thereby contribute to tumorigenesis. In S326C-*Ogg1* heterozygous blood cells, increased DNA damage has been measured (52), suggesting that even a single allele of S326C-*Ogg1* may be sufficient to cause decreased 8-oxodG repair capacity. One contributor to the association between S326C-*Ogg1* and tumorigenesis may be loss of heterozygosity (LOH) (53), which is one of the most common DNA alterations in cancer (54). Although only S326C-*Ogg1* homozygosity is associated with cancer (15), S326C-*Ogg1* cells that underwent LOH would be expected to be at increased tumorigenic risk. Moreover, oxidative lesions such as 8-oxodG arise in non-random patterns that are consistent among individuals (55), suggesting there may be hotspots for oxidative damage. If such a hotspot exists in an oncogene, TNF-α could induce 8-oxodG formation, while simultaneously inactivating S326C-OGG1 via Cys326 oxidation, increasing the likelihood for the emergence of a mutation with functional consequences. Exposure to TNF-α can cause oncogenic transformation *ex vivo* in repair-compromised cells (11), suggesting that TNF-α could drive oncogenic transformation via

the induction of DNA damage and through repair enzyme inactivation as shown in this study.

Collectively, the results in this study show that oxidative stress caused by TNF-α not only induces DNA damage, but for the first time that TNF-α exposure causes inefficient repair in S326C-*Ogg1* variant cells. Chronic oxidative stress, such as that caused by exposure to TNF-α (56) or by additional mechanisms involved in cell transformation (57) could cause persistent dysfunction of S326C-OGG1 and continuous induction of 8-oxodG that results in increased mutation rates. Tumor cells secrete TNF-α themselves, illustrated in a model of ovarian cancer (9), which could cause chronic oxidative stress in a signaling loop leading to tumorigenesis. Cytokine signaling contributes to the chronic oxidative stress associated with carcinogenesis, caused by oxidative damage to both proteins and DNA (58,59). S326C-OGG1 inactivation similar to the findings in these studies (~50%) was observed after S326C-*Ogg1* variant cells were treated by the inflammatory modulator nitric oxide (60). Nitric oxide can be upregulated in response to TNF-α signaling, which could contribute to a chronic oxidative stress phenotype (61). Likewise, the decreased repair capacity of BER glycosylase NEIL1 variant S82C may involve cysteine dimerization (62). Modulating the redox environment of S326C-*Ogg1* tumors could cause increased sensitivity to DNA damage-dependent cancer therapies, such as radiation therapy and cisplatin chemotherapy, which has been demonstrated to exert toxicity partly through inducing oxidative stress (58,59). Decreased S326C-OGG1 activity is likely due to cysteine disulfide bond formation and future studies should address the mechanism of S326C-OGG1 oxidation, as well as measuring the predicted increase in mutagenesis expected

under such conditions. By modeling the redox regulation of S326C-OGG1 under oxidative stress, a better understanding of the induction and management of dysfunctional DNA repair phenotypes that can contribute to tumorigenesis is likely to emerge. Ultimately, biomarker screening for S326C-*Ogg1* may be a valuable predictor of cancer risk, guiding S326C-*Ogg1* individuals toward limiting oxidative stress and inflammation.

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References

1. Caldecott, K.W. (2004) *Eukaryotic DNA damage surveillance and repair*. Landes Bioscience/Eurekah.com; Kluwer Academic/Plenum Publishers, Georgetown, Tex. New York, N.Y.
2. Ames, B.N.*, et al.* (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A*, **90**, 7915-22.
3. Halliwell, B.*, et al.* (1994) Oxygen-derived species: their relation to human disease and environmental stress. *Environ Health Perspect*, **102 Suppl 10**, 5-12.
4. Inoue, M.*, et al.* (1998) Induction of chromosomal gene mutations in Escherichia coli by direct incorporation of oxidatively damaged nucleotides. New evaluation method for mutagenesis by damaged DNA precursors in vivo. *J Biol Chem*, **273**, 11069-74.
5. Reuter, S.*, et al.* (2010) Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med*, **49**, 1603-16.
6. Hanahan, D.*, et al.* (2011) Hallmarks of cancer: the next generation. *Cell*, **144**, 646-74.
7. Ohshima, H.*, et al.* (1994) Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res*, **305**, 253-64.
8. Jamaluddin, M.*, et al.* (2007) TNF-alpha-induced NF-kappaB/RelA Ser(276) phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway. *Cell Signal*, **19**, 1419-33.

9. Charles, K.A.*, et al.* (2009) The tumor-promoting actions of TNF-alpha involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *J Clin Invest*, **119**, 3011-23.

10. Komori, A.*, et al.* (1993) Tumor necrosis factor acts as a tumor promoter in BALB/3T3 cell transformation. *Cancer Res*, **53**, 1982-5.

11. Li, J.*, et al.* (2007) TNF-alpha induces leukemic clonal evolution ex vivo in Fanconi anemia group C murine stem cells. *J Clin Invest*, **117**, 3283-95.

12. De Bont, R.*, et al.* (2004) Endogenous DNA damage in humans: a review of quantitative data. *Mutagenesis*, **19**, 169-85.

13. Bruner, S.D.*, et al.* (2000) Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature*, **403**, 859-866.

14. Klungland, A.*, et al.* (1999) Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc Natl Acad Sci U S A*, **96**, 13300-5.

15. Hung, R.J.*, et al.* (2005) Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. *Am J Epidemiol*, **162**, 925-42.

16. Elahi, A.*, et al.* (2002) The human OGG1 DNA repair enzyme and its association with orolaryngeal cancer risk. *Carcinogenesis*, **23**, 1229-34.

17. Hung, R.J.*, et al.* (2008) International Lung Cancer Consortium: pooled analysis of sequence variants in DNA repair and cell cycle pathways. *Cancer Epidemiol Biomarkers Prev*, **17**, 3081-9.

18. Kohno, T.*, et al.* (1998) Genetic polymorphisms and alternative splicing of the hOGG1 gene, that is involved in the repair of 8-hydroxyguanine in damaged DNA. *Oncogene*, **16**, 3219-25.

19. Wang, Y.*, et al.* (2012) Redox sensing by proteins: oxidative modifications on cysteines and the consequent events. *Antioxid Redox Signal*, **16**, 649-57.

20. Bravard, A.*, et al.* (2009) Oxidation status of human OGG1-S326C polymorphic variant determines cellular DNA repair capacity. *Cancer Res*, **69**, 3642-9.

21. Smart, D.J.*, et al.* (2006) Activity of OGG1 variants in the repair of pro-oxidant-induced 8-oxo-2'-deoxyguanosine. *DNA Repair (Amst)*, **5**, 1337-45.

22. Kershaw, R.M.*, et al.* (2012) Repair of oxidative DNA damage is delayed in the Ser326Cys polymorphic variant of the base excision repair protein OGG1. *Mutagenesis*, **27**, 501-10.

23. Hill, J.W.*, et al.* (2006) Dimerization and opposite base-dependent catalytic impairment of polymorphic S326C OGG1 glycosylase. *Nucleic Acids Res*, **34**, 1620-32.

24. Simonelli, V.*, et al.* (2013) Genotype-phenotype analysis of S326C OGG1 polymorphism: a risk factor for oxidative pathologies. *Free Radic Biol Med*, **63**, 401-9.

25. Dherin, C.*, et al.* (1999) Excision of oxidatively damaged DNA bases by the human alpha-hOgg1 protein and the polymorphic alpha-hOgg1(Ser326Cys) protein which is frequently found in human populations. *Nucleic Acids Res*, **27**, 4001-7.

26. Audebert, M.*, et al.* (2000) Effect of single mutations in the OGG1 gene found in human tumors on the substrate specificity of the Ogg1 protein. *Nucleic Acids Res*, **28**, 2672-8.

27. Blons, H.*, et al.* (1999) Frequent allelic loss at chromosome 3p distinct from genetic alterations of the 8-oxoguanine DNA glycosylase 1 gene in head and neck cancer. *Mol Carcinog*, **26**, 254-60.

28. Sidorenko, V.S.*, et al.* (2009) Substrate specificity and excision kinetics of natural polymorphic variants and phosphomimetic mutants of human 8-oxoguanine-DNA glycosylase. *FEBS J*, **276**, 5149-62.

29. Kasymov, R.D.*, et al.* (2013) Excision of 8-oxoguanine from methylated CpG dinucleotides by human 8-oxoguanine DNA glycosylase. *FEBS Lett*, **587**, 3129-34.

30. Saxowsky, T.T.*, et al.* (2008) 8-Oxoguanine-mediated transcriptional mutagenesis causes Ras activation in mammalian cells. *Proc Natl Acad Sci U S A*, **105**, 18877-82.

31. Du, H.*, et al.* (2012) Oxidative damage and OGG1 expression induced by a combined effect of titanium dioxide nanoparticles and lead acetate in human hepatocytes. *Environ Toxicol*, **27**, 590-7.

32. Kisby, G.E.*, et al.* (2009) Oxidative stress and DNA damage in agricultural workers. *J Agromedicine*, **14**, 206-14.

33. La Maestra, S.*, et al.* (2011) Cigarette smoke induces DNA damage and alters base-excision repair and tau levels in the brain of neonatal mice. *Toxicol Sci*, **123**, 471-9.

34. von dem Bussche, A.*, et al.* (2010) Hepatitis C virus NS2 protein triggers endoplasmic reticulum stress and suppresses its own viral replication. *J Hepatol*, **53**, 797-804.

35. Nakabeppu, Y.*, et al.* (2006) Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. *Biol Chem*, **387**, 373-9.

36. Pecot, M.Y.*, et al.* (2004) Golgi membranes remain segregated from the endoplasmic reticulum during mitosis in mammalian cells. *Cell*, **116**, 99-107.

37. Nishida, Y.*, et al.* (2009) Depletion of vitamin E increases amyloid beta accumulation by decreasing its clearances from brain and blood in a mouse model of Alzheimer disease. *J Biol Chem*, **284**, 33400-8.

38. Chretien, A.*, et al.* (2008) Upregulation of annexin A2 in H(2)O(2)-induced premature senescence as evidenced by 2D-DIGE proteome analysis. *Exp Gerontol*, **43**, 353-9.

39. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, **65**, 55-63.

40. Smith, C.C.*, et al.* (2006) hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII. *Mutagenesis*, **21**, 185-90.

41. Ferre, F.*, et al.* (2005) DiANNA: a web server for disulfide connectivity prediction. *Nucleic Acids Res*, **33**, W230-2.

42. Shah, S.P.*, et al.* (2009) Mutation of FOXL2 in granulosa-cell tumors of the ovary. *N Engl J Med*, **360**, 2719-29.

43. Nishimura, S.*, et al.* (2012) In vivo imaging visualizes discoid platelet aggregations without endothelium disruption and implicates contribution of inflammatory cytokine and integrin signaling. *Blood*, **119**, e45-56.

44. Turner, D.A.*, et al.* (2010) Physiological levels of TNFalpha stimulation induce stochastic dynamics of NF-kappaB responses in single living cells. *J Cell Sci*, **123**, 2834-43.

45. Aruoma, O.I.*, et al.* (1989) The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med*, **6**, 593-7.

46. Kim, J.J.*, et al.* (2010) TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-X(L). *Cell Death Differ*, **17**, 1420-34.

47. Murata, M.*, et al.* (2001) Requirement of glutathione and cysteine in guanine-specific oxidation of DNA by carcinogenic potassium bromate. *Chem Res Toxicol*, **14**, 678-85.

48. Kaur, M.P.*, et al.* (2014) Cellular accumulation of Cys326-OGG1 protein complexes under conditions of oxidative stress. *Biochem Biophys Res Commun*, **447**, 12-8.

49. Lan, Q.*, et al.* (2004) Oxidative damage-related genes AKR1C3 and OGG1 modulate risks for lung cancer due to exposure to PAH-rich coal combustion emissions. *Carcinogenesis*, **25**, 2177-81.

50. Pashin, Y.V.*, et al.* (1979) Mutagenic and carcinogenic properties of polycyclic aromatic hydrocarbons. *Environ Health Perspect*, **30**, 185-9.

51. Paz-Elizur, T.*, et al.* (2003) DNA repair activity for oxidative damage and risk of lung cancer. *J Natl Cancer Inst*, **95**, 1312-9.

52. Jensen, A.*, et al.* (2012) Influence of the OGG1 Ser326Cys polymorphism on oxidatively damaged DNA and repair activity. *Free Radic Biol Med*, **52**, 118-25.

53. Lu, R.*, et al.* (1997) A mammalian DNA repair enzyme that excises oxidatively damaged guanines maps to a locus frequently lost in lung cancer. *Curr Biol*, **7**, 397-407.

54. Thiagalingam, S.*, et al.* (2001) Mechanisms underlying losses of heterozygosity in human colorectal cancers. *Proc Natl Acad Sci U S A*, **98**, 2698-702.

55. Ohno, M.*, et al.* (2006) A genome-wide distribution of 8-oxoguanine correlates with the preferred regions for recombination and single nucleotide polymorphism in the human genome. *Genome Res*, **16**, 567-75.

56. Shen, H.M.*, et al.* (2006) TNF receptor superfamily-induced cell death: redox-dependent execution. *FASEB J*, **20**, 1589-98.

57. Nakamura, H.*, et al.* (1997) Redox regulation of cellular activation. *Annu Rev Immunol*, **15**, 351-69.

58. Miyajima, A.*, et al.* (1997) Role of reactive oxygen species in cis-dichlorodiammineplatinum-induced cytotoxicity on bladder cancer cells. *Br J Cancer*, **76**, 206-10.

59. Marullo, R.*, et al.* (2013) Cisplatin induces a mitochondrial-ROS response that contributes to cytotoxicity depending on mitochondrial redox status and bioenergetic functions. *PLoS One*, **8**, e81162.

60. Moritz, E.*, et al.* (2014) hOGG1-Cys326 variant cells are hypersensitive to DNA repair inhibition by nitric oxide. *Carcinogenesis*, **35**, 1426-33.

61. Fonseca, S.G.*, et al.* (2003) TNF-alpha mediates the induction of nitric oxide synthase in macrophages but not in neutrophils in experimental cutaneous leishmaniasis. *Eur J Immunol*, **33**, 2297-306.

62. Prakash, A.*, et al.* (2014) Genome and cancer single nucleotide polymorphisms of the human NEIL1 DNA glycosylase: activity, structure, and the effect of editing. *DNA Repair (Amst)*, **14**, 17-26.

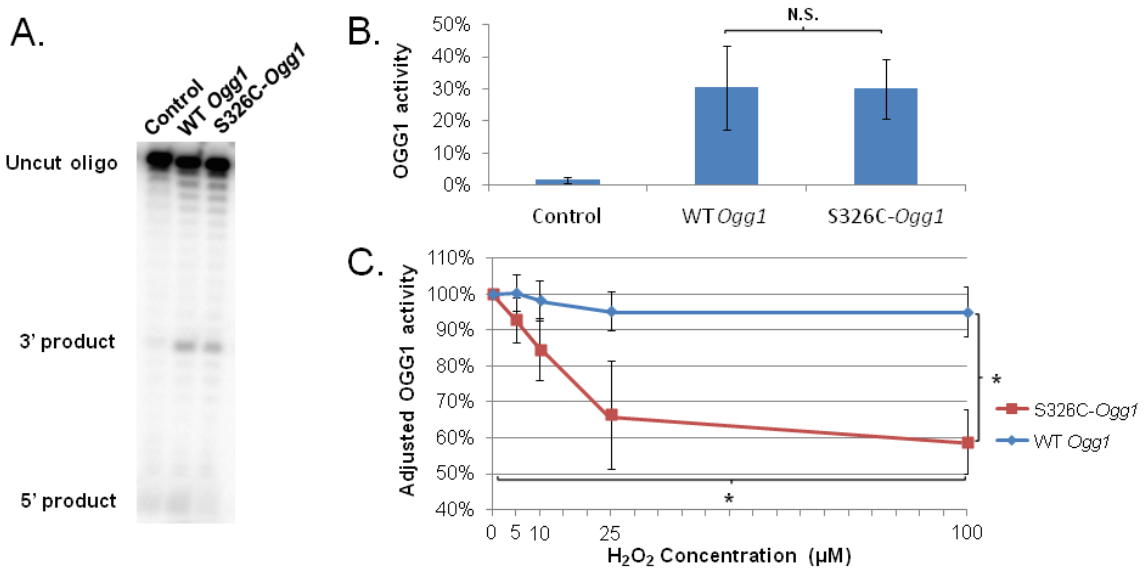

Fig. 1. The activity of S326C-OGG1 and WT OGG1 is equivalent in the absence of oxidative stress, and only S326C-OGG1 activity decreases following treatment with an oxidative stressor. **A**. ^{32}P-labeled 8-oxodG-containing oligonucleotide fragments were resolved in a polyacrylamide urea gel generated after incubation with whole-cell lysates from *Ogg1*-/- MEFs subjected to mock transfection (control), or transfected with WT *Ogg1* or S326C-*Ogg1*. Only the portion of the gel showing uncut oligo, 3' product, and 5' product is shown. **B.** Cleavage activity from three experiments was averaged following adjustment for OGG1 expression as described in Materials & Methods. S326C-OGG1 and WT OGG1 have similar levels of activity in the absence of oxidative stress. The statistical comparison between the activity of WT and S326C-*Ogg1* samples was carried out using a paired Student's t-test. **C.** Treatment with 100 μM H_2O_2 inhibits S326C-OGG1, but not WT OGG1 activity. Activity was measured by exposing cells to 0-100 μM H_2O_2 for 15 min, then measuring cleavage activity as described in A, except

expressed as a percentage of the mean activity of the untreated samples. Values shown represent the average of ≥3 independent experiments ± SE. Significant differences are represented by (*) $p < 0.05$ according to one-way ANOVA.

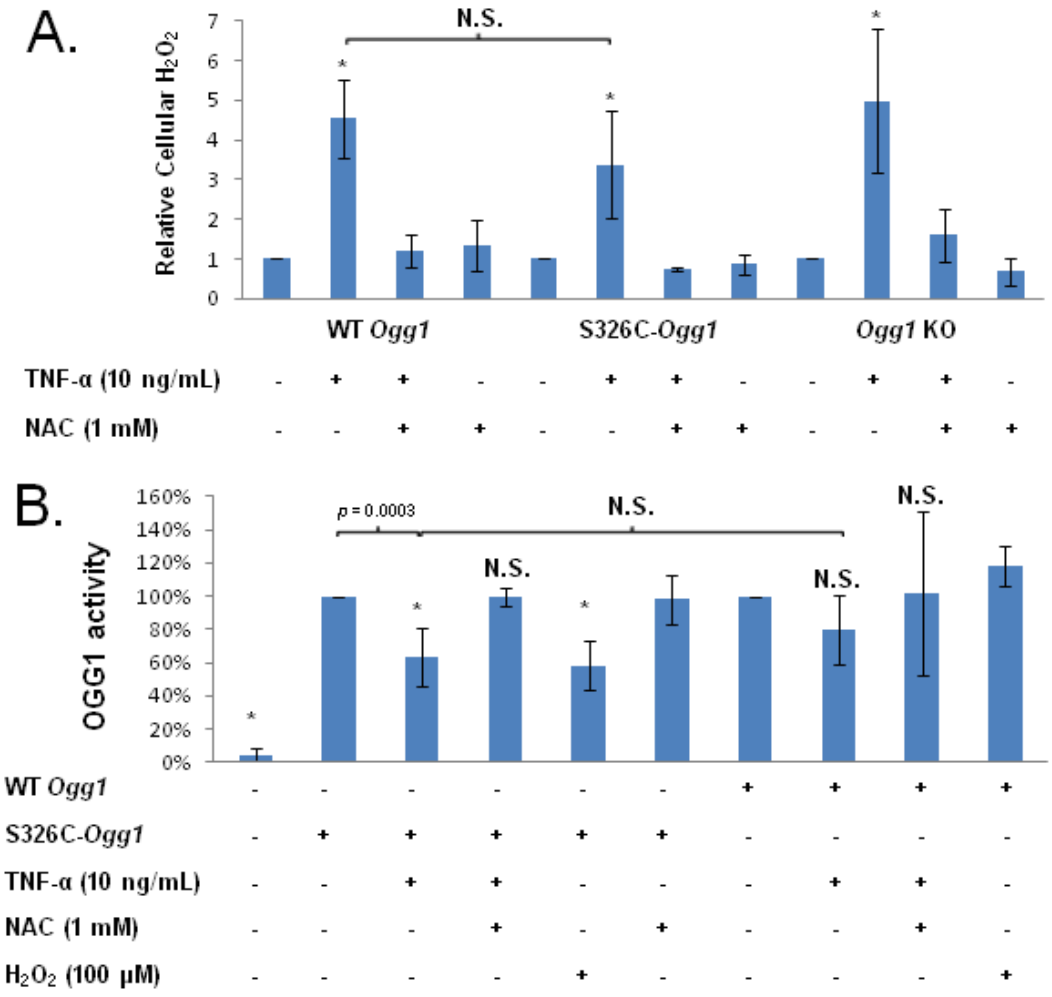

Fig. 2. S326C-OGG1 variant is inactivated by cellular exposure to TNF-α. **A.** TNF-α exposure increases H_2O_2 levels in mouse embryonic fibroblasts. WT, S326C-*Ogg1*, and *Ogg1* KO MEFs were treated with 10 ng/mL TNF-α in media for 1h, then subjected to treatment with Amplex Red for 30 min. Results shown are the average of 3 independent experiments, all ± SE. **B.** TNF-α inhibits S326C-OGG1 through oxidation. Activity assay results indicate that N-acetylcysteine (NAC) treatment reverses the activity inhibition caused by TNF-α treatment in S326C-*Ogg1* cells. *Ogg1*-/- MEFs were transfected with WT or S326C-*Ogg1*, treated with 10 ng/mL TNF-α for 1h ± pre-

treatment with 1 mM NAC for 2.5h or 100 μM H_2O_2 for 15 min, then subjected to the oligonucleotide cleavage assay described previously. All values represent the average of ≥ 3 independent experiments ± SE. Significant differences are represented by (*) $p < 0.05$ according to one-way ANOVA. "N.S." represents a non-significant difference.

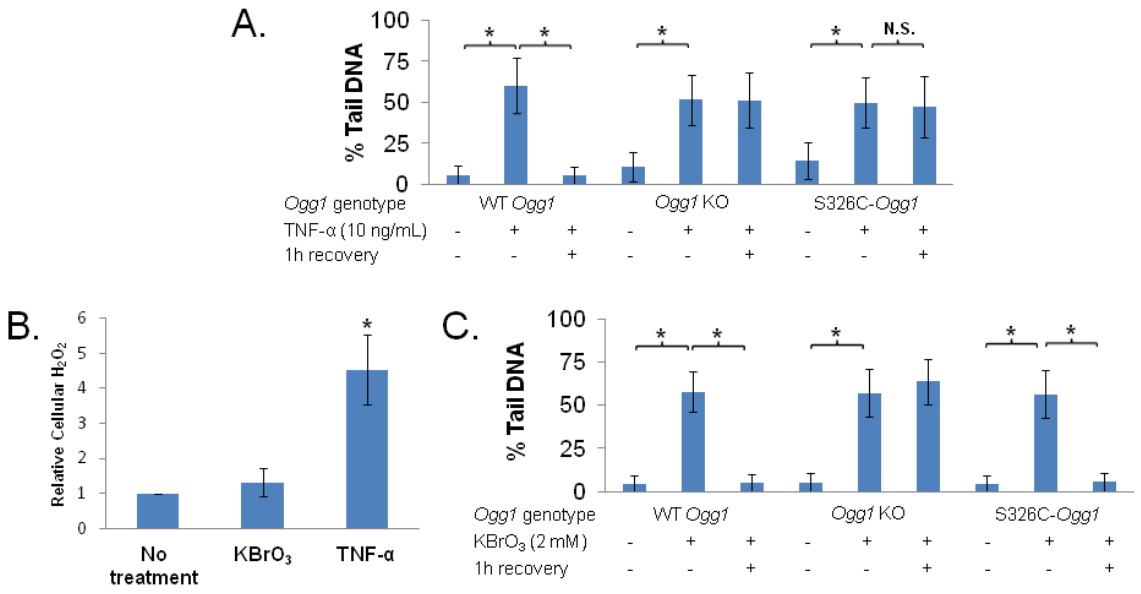

Fig. 3. S326C-*Ogg1* variant cells display a loss in DNA repair activity under oxidative stress. **A.** S326C-*Ogg1* variant cells display delayed repair activity following exposure to TNF-α. Cells were immobilized in agarose, treated with 10 ng/mL TNF-α for 1h, lysed, the DNA was digested with OGG1, then subjected to alkaline electrophoresis. The percentage of DNA that migrated beyond the nuclear area was quantified after fluorescent microscopy in ≥100 cells per cell line per treatment. **B**. $KBrO_3$ treatment causes oxidative DNA damage without increasing cellular H_2O_2. WT MEFs were treated with 2 mM $KBrO_3$ or 10 ng/mL TNF-α for 1h. MEFs were then subjected to Amplex Red (AR) treatment, and the fluorescent signal was measured. The results shown are the average of three independent experiments ± SE. **C**. S326C-OGG1 repair activity of nuclear DNA damage is comparable to WT OGG1 when oxidative stress is not imposed. Cells were subjected to OGG1-modified alkaline comet assay as in **A**, except that cells were treated with 2mM $KBrO_3$ for 1h instead of TNF-α. All values represent the average of ≥ 3 independent experiments ± SE. Significant differences are represented by (*) $p < 0.05$ according to one-way ANOVA. "N.S." represents a non-significant difference.

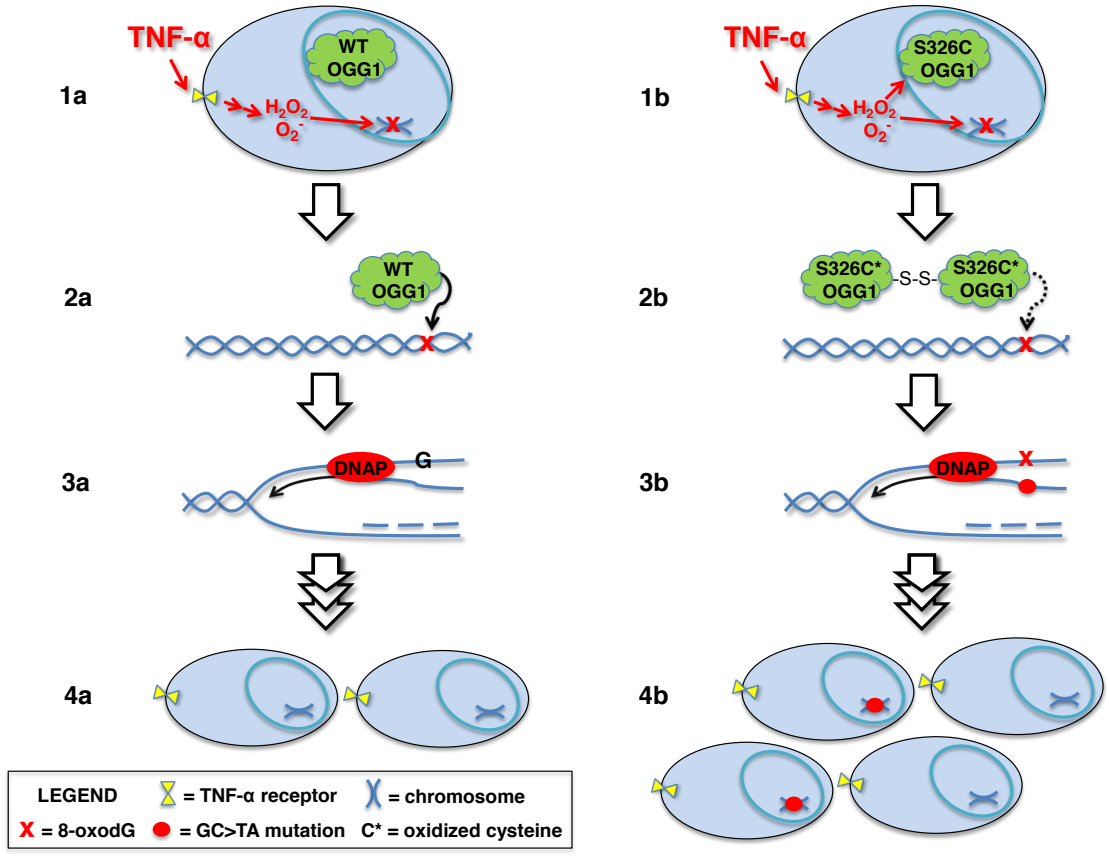

Fig. 4. Proposed biological model for increased tumorigenic potential in S326C-*Ogg1* variant cells. (**1a** & **1b**) H_2O_2 and TNF-α cause cellular oxidative stress that induces DNA damage in WT and S326C-*Ogg1* cells. (**2a**) In WT *Ogg1* cells, 8-oxodG damage is efficiently repaired. (**2b**) However, H_2O_2 and TNF-α exposure diminishes S326C-OGG1 activity *in vivo* (presumably via Cys326 oxidation that results in dimerization), causing accumulation of mutagenic 8-oxodG that is not repaired efficiently. (**3a**) Repaired 8-oxodG is bypassed nonmutagenically by DNA polymerase (DNAP) during replication. (**3b**) Unrepaired 8-oxodG is also bypassed by DNAP during replication, which can establish a permanent mutation. (**4b**) Increased levels of 8-oxodG and a decreased 8-oxodG repair capacity may cause an increase in mutation frequency underlying the

association between S326C-*Ogg1* and tumorigenesis, particularly under oxidizing conditions.

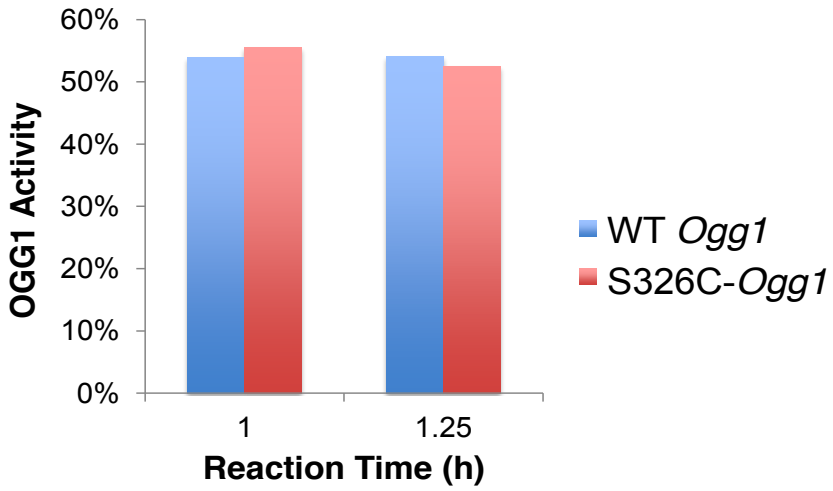

Fig. S1. *In vitro* oligonucleotide cleavage activity does not increase after 1h. *Ogg1-/-* MEFs were transfected with WT or S326C-*Ogg1,* lysed, and incubated with ^{32}P-labeled 8-oxodG-containing oligonucleotide for 1h or 1.25h. Lysates were resolved in a SDS-PAGE gel and probed for OGG1 (HA), which confirmed similar levels of expression. The oligonucleotide and its fragments were resolved in a polyacrylamide urea gel, and the percentage of cleaved oligonucleotide was quantified ("OGG1 activity"). Previous studies showed that OGG1 activity in such assays is complete after 15 min (33), and all *in vitro* cleavage reactions were carried out for 1h to ensure that they were complete. Consistent with this prediction, OGG1 activity did not increase between 1h and 1.25h for either WT or S326C-*Ogg1* cells, indicating that the oligonucleotide cleavage reaction is complete after 1h.

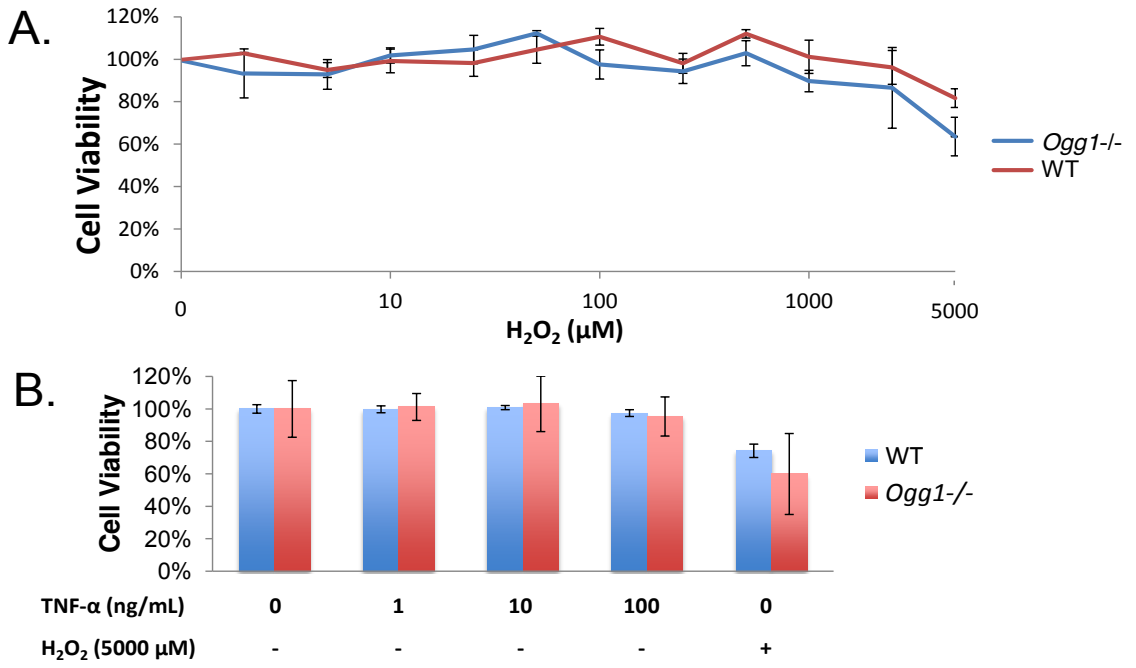

Fig. S2. Treatment with micromolar concentrations of H_2O_2 or 10 ng/mL TNF-α is not cytotoxic. **A.** To measure H_2O_2 cytotoxicity, *Ogg1*-/- and WT MEFs were seeded at 3.125 x 10^4 cells/well in a 96-well plate. After 24h, cells were treated in triplicate with varying concentrations of H_2O_2 on ice for 15 min, and 10 µL 0.5% MTT (Invitrogen) was added to cell media, then incubated at 37°C at 5% CO_2 for 5h. Media was removed, then 100 µL DMSO was added to each well, and incubated with shaking at RT in the dark until dye crystals dissolved. Survival was then measured as the difference between the average absorption at 595 nm of treated cells and untreated cells. The average survival for both *Ogg1*-/- and WT cells is 90-100% when cells are treated with 0-1000 µM H_2O_2, demonstrating that the doses used in Fig.s 1 & 2 (≤100 µM H_2O_2) are not cytotoxic. Only after treatment with 5000 µM H_2O_2 did the cells undergo a substantial degree of cytotoxicity, presumably through cytolysis, since the 15 min timeframe of treatment is

not expected to be sufficient for apoptosis. **B.** *Ogg1*-/- and WT MEFs were treated in sextuplicate with varying concentrations of TNF-α for 1h, and 10 μL 0.5% MTT (Invitrogen) was added to cell media, then incubated at 37°C at 5% CO_2 for 5h. Media was removed, then 100 μL DMSO was added to each well, and incubated with shaking at RT in the dark until dye crystals dissolved. Survival was then measured as the difference between the average absorption at 595 nm of treated cells and untreated cells. The average survival for both *Ogg1*-/- and WT cells is 90-100% when cells are treated with 0-100 ng/mL TNF-α, demonstrating that the doses used in Fig.s 2 & 3 (10 ng/mL) are not cytotoxic.

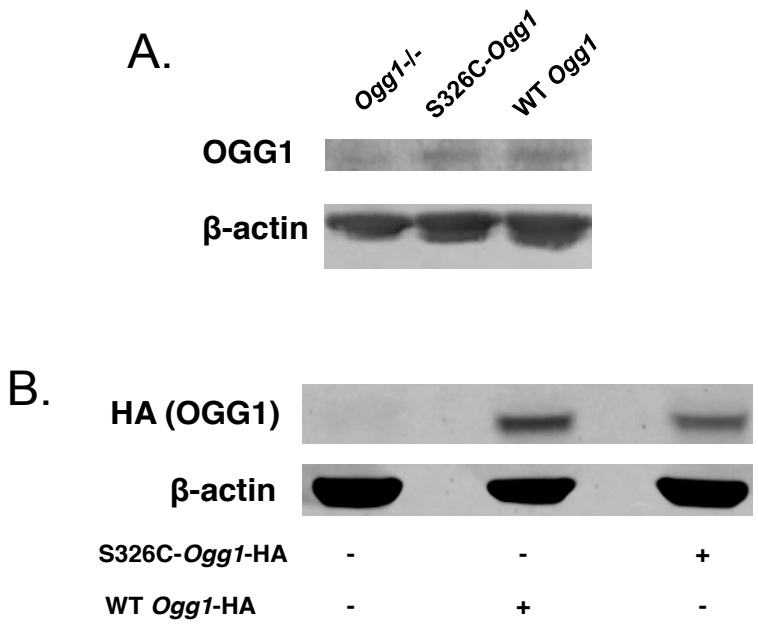

Fig. S3. OGG1 expression is similar in *Ogg1*-/- MEFs transfected with S326C-*Ogg1* and WT *Ogg1*. **A.** Untreated cell lysates from *Ogg1*-/- MEFs stably transfected with WT or S326C-*Ogg1* were resolved by SDS-PAGE and probed for OGG1 or β-actin, whose cropped bands are shown above. These cells were used in the modified comet assays in these studies, in which adjusting for transfection efficiency was impossible. Experimental samples were processed in parallel. **B.** *Ogg1*-/- MEFs were transiently transfected with WT or S326C-*Ogg1*, lysates were resolved using SDS-PAGE and probed for HA (OGG1-HA) or β-actin. These cells were used in the *in* vitro activity assays in these studies. Experimental samples were processed in parallel.

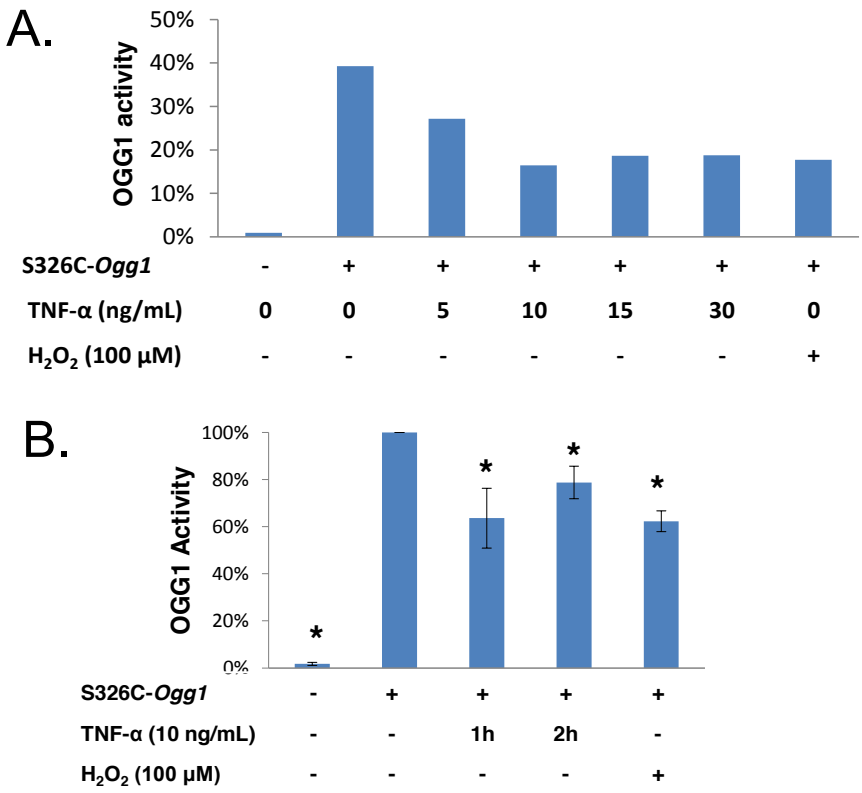

Fig. S4. S326C-OGG1 variant activity is inhibited by cellular exposure to TNF-α. **A.** TNF-α treatment causes inhibition of S326C-OGG1 activity. *Ogg1*-/- MEFs were transfected with S326C-*Ogg1*, then treated with varying concentrations of TNF-α for 1h. Cells were then lysed and incubated with ^{32}P-labeled 8-oxodG-containing oligonucleotides. Oligonucleotide fragments were resolved in a polyacrylamide urea gel, then quantified and adjusted for OGG1 expression as described in Materials & Methods. The lowest concentration at which maximal activity inhibition occurred was 10 ng/mL. **B.** The activity of S326C-OGG1 is inhibited by TNF-α treatment. *Ogg1*-/- MEFs were transfected with S326C-*Ogg1*, treated with 10 ng/mL TNF-α for 1 or 2 h or 100 µM H_2O_2 for 15 min, then subjected to the oligonucleotide cleavage assay described previously. Results shown are the average of 3 independent experiments, all ± SE. *Ogg1*-/- MEFs

were transfected with WT or S326C-*Ogg1*, treated with 10 ng/mL TNF-α for 1h ± pre-treatment with1 mM NAC for 2.5h or 100 μM H_2O_2 for 15 min, then subjected to the oligonucleotide cleavage assay described previously. All values represent the average of ≥3 independent experiments ± SE. Significant differences are represented by (*) $p <$ 0.05 according to one-way ANOVA. "N.S." represents a non-significant difference.

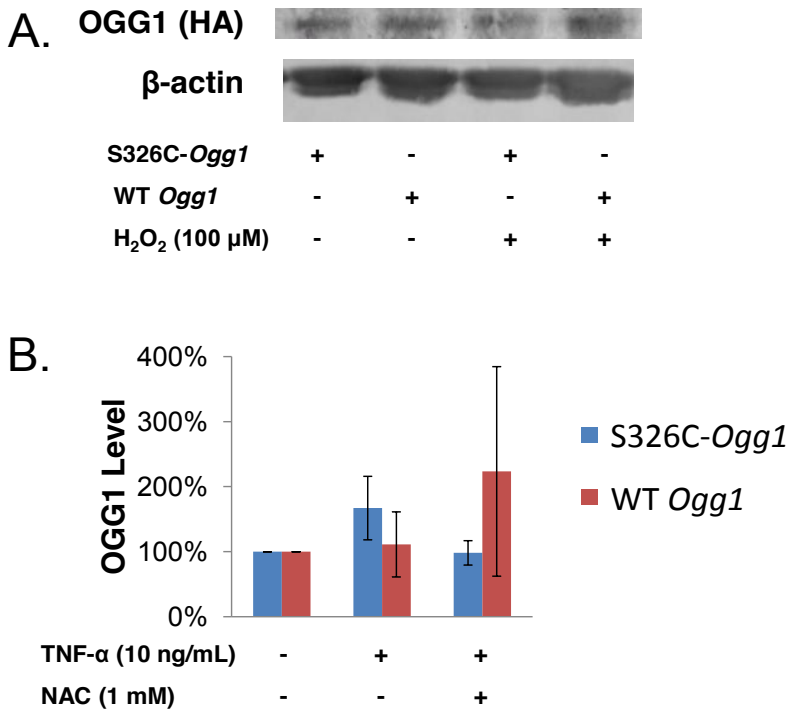

Fig. S5. OGG1 is not degraded following treatment with oxidative stressors. **A.** *Ogg1-/-* MEFs were transfected with WT *Ogg1-HA* or S326C-*Ogg1-HA*, then treated with 100 μM H_2O_2 for 15 min. The cells were then lysed, and the lysates were resolved in a SDS-PAGE gel. The proteins were transferred to a membrane probed with anti-OGG1 antibody. H_2O_2 treatment did not cause decreased levels of OGG1. **B.** The same experimental protocol as in A was undertaken with cells treated with TNF-α for 1 h, or pretreated with N-acetylcysteine (NAC) for 2.5 h. However, the membrane was probed with anti-HA (OGG1) antibody. The results show an average of ≥ 3 independent experiments ± SE. Treatment with neither TNF-α not NAC caused decreased levels of OGG1.

A.

Cysteine sequence position	Distance (AA)	Bond	Score
140 - 253	113	QDPIE**C**LFSFI-TKVAD**C**ICLMA	0.9999
241 - 326	85	AHKAL**C**ILPGV-ADLRQ**C**RHAQE	0.99977
241 - 253	12	AHKAL**C**ILPGV-TKVAD**C**ICLMA	0.99948
146 - 326	180	LFSFI**C**SSNNN-ADLRQ**C**RHAQE	0.99403
140 - 326	186	QDPIE**C**LFSFI-ADLRQ**C**RHAQE	0.81653
140 - 241	101	QDPIE**C**LFSFI-AHKAL**C**ILPGV	0.80522

B.

Cysteine sequence position	Distance (AA)	Bond	Score
140 - 253	113	QDPIE**C**LFSFI-TKVAD**C**ICLMA	0.99991
241 - 253	12	AHKAL**C**ILPGV-TKVAD**C**ICLMA	0.99934
140 - 241	101	QDPIE**C**LFSFI-AHKAL**C**ILPGV	0.87908

Fig. S6. Cys326 is one of the strongest candidates for OGG1 residues likely to undergo disulfide bond formation. **A**. The amino acid sequence of WT OGG1 (NCBI accession number NP_002533.1) was modified by replacing Ser326 with Cys326 and entered into the DiANNA software. **B**. The amino acid sequence of WT OGG1 (NCBI accession number NP_002533.1) was entered into the DiANNA software. From the disulfide bonds predicted, only those whose likelihood was calculated as significant (score > 0.8) are shown. Cys326 is likely to undergo disulfide bond formation with Cys241, Cys146, and Cys140, and is one of the cysteines most likely to form disulfide bonds.

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

Research Issues Explored in this Dissertation

The genomes of both bacteria and mammalian cells are under continuous attack from genotoxic agents (1). For example, DNA deamination occurs spontaneously, but its rate is thought to be very slow (2). However, deamination occurs at a significantly increased rate during exposure to nitrosylating agents such as nitric oxide (3), prevalent throughout the human body (4), and nitrous acid (5). Depending on their cellular genotype, different cell populations have different DNA repair capacities (6). For example, because DNA deamination in *Escherichia coli* is typically repaired by alternative excision repair (7), mutation of the *nfi* gene encoding Endonuclease V responsible for the initial cleavage at the deaminated base hypoxanthine significantly decreases the hypoxanthine repair capacity of the cell (8). However, the decreased repair capacity in *nfi* cells has effects beyond the mutagenic bypass of hypoxanthine during DNA replication. The *nfi* repair defect allows hypoxanthine to remain unrepaired, as might many other DNA repair defects found among bacteria. In nature, bacteria do not undergo continuous cell division, and thus, at most points during their existence, the ability to acquire new phenotypes depends on mechanisms other than replicative bypass of DNA damage. One such mechanism is transcriptional mutagenesis (TM), arising from transcriptional bypass of mutagenic DNA lesions on the transcribed strand, which has been demonstrated using damage-containing constructs in *E. coli* (9, 10). However, no studies to date have measured the contribution of TM to phenotypic reversion. Thus, in repair-compromised *nfi* cells, retromutagenesis is not only possible, but was demonstrated in the studies in Chapter 2.

Other studies in this dissertation highlight another phenotype arising in the context of compromised DNA repair. One of the most prevalent forms of DNA damage is oxidation. Oxidation can cause more than 90,000 DNA lesions in each mammalian cell per day (11, 12). Oxidative DNA damage is in mammalian cells is typically repaired by base excision repair (13), and arises from reactive oxygen species (ROS) that arise partly from cellular metabolism. Base excision repair (BER) is the primary means by which cells limit the toxic effects of non-strand-distorting DNA damage. Such damage, including oxidative damage, is removed efficiently in healthy cells containing a fully functional set of BER enzymes. However, there are many cellular conditions that compromise the BER capacity of a cell, including oxidative stress. Likewise, there are a number of variant BER enzymes in human populations, several of which have been associated with pathologies presumed to arise from their dysfunction. However, the mechanism by which BER enzymes dysfunction arises has been studied among few such enzymes (14). The studies described in this work address the connection between oxidative stress imposed by physiologically relevant oxidative stressors and the dysfunction of a variant BER enzyme.

Discussion of Major Findings

The studies described herein measure the responses of bacterial and mammalian cells to genotoxic stress. In chapter 2, we studied the degree to which mutations indicative of transcriptional mutagenesis contribute to Lac^+ reversion in *E. coli*. The important findings from these studies include: 1) DNA exposure to nitrous acid causes a significant increase in mutations on the non-transcribed strand of the TAG stop codon at

lacZ amber codon 17 in the absence of catabolite repression; 2) after imposing catabolite repression, nitrous acid exposure causes mutations more frequently on the transcribed strand than on the non-transcribed strand of *lacZ*(Am)50 after immediate Lac selection, regardless of leading- or lagging-strand orientation; 3) nitrous acid exposure causes similar numbers of mutations on the transcribed strand than on the non-transcribed strand of *lacZ*(Am)50 under non-selective conditions with catabolite repression, regardless of leading- or lagging-strand orientation; 4) deaminating ($NaNO_2$) treatment causes a substantial increase in TA>AT mutations under non-selective conditions; 5) because such TA>AT mutations in our reporter allele cause a Lys substitution in *lacZ* codon 17, the glutamine residue encoded by the predominant TA>CG mutations have no selective functional advantage.

In chapter 3, the ability of S326C-OGG1 to carry out repair of 8-oxodG under different cellular environments was explored. Several important findings arose: 1) S326C-OGG1 has a similar level of *in vitro* activity when compared to WT OGG1 in the absence of oxidative stress; 2) S326C-OGG1 displays a decreased level of *in vitro* activity under oxidative stress; 3) S326C-OGG1 displays a similar level of cellular repair activity compared to the WT allele; 4) oxidative stress, arising from inflammatory cytokine exposure, causes a loss in cellular repair activity in the S326C-*Ogg1* background; 5) in the absence of agents that cause cysteine oxidation, such activity loss is not observed, suggesting that Cys326 oxidation mediates loss of repair activity.

Nitrous acid causes an increase in non-transcribed strand lac reversion mutations in the absence of catabolite repression

In Chapter 2, we described experiments *E. coli* studying mutations consistent with transcriptional mutagenesis that mediated Lac^+ reversion indicative of retromutagenesis. In order to measure phenotypic reversion, we constructed strains with a premature TAG stop codon in the 17^{th} codon of *lacZ*, necessary for the metabolism of lactose. This stop codon was cloned into both the leading- and lagging-strand orientations to control for any effects of mutagenic replication. To measure strand-specific mutations, we selected an amber allele as a reporter because it contains adenines on both the transcribed and the non-transcribed strands, and thus gives rise to different mutations depending on which strand underwent damage. To induce base-specific DNA damage, we exposed cells to nitrous acid, which induces deamination of adenines into hypoxanthine and thereby induces AT>GC mutations during replication. We exposed our *lacZ* amber strains to nitrous acid for 9 min before incubating cells in rich media, then spreading a portion of cells on selective media. We then measured the frequency with which mutations arose consistent with transcriptional mutagnesis, allowing the cell to replicate and establish an analogous DNA mutation indicative of retromutagenesis.

The mutations that arose among revertants in the absence of catabolite repression were exclusively on the non-transcribed strand, consistent with the known sensitivity of this strand in a transcription bubble. Importantly, this result illustrated that reversion could occur from the mutagenic sensitivity imposed by transcription (15). When compared to subsequent results, this result validated that lac reversion at the amber allele could arise from mutations on either the transcribed- or non-transcribed strand.

Nitrous acid exposure causes no strand-specific mutation bias under non-selective conditions

The aforementioned mutagenesis scheme was modified in several ways to study mutations on both strands of the amber allele. We utilized catabolite repression to limit *lacZ* transcription (16) and allow both strands to have a similar sensitivity to mutagenesis. After nitrous acid exposure, we incubated a portion of cells immediately on selective media, and another portion in rich media prior to selection, which should not induce a mutagenic strand bias. In both the leading- and lagging-strand orientation, *lacZ* amber cells underwent at least a 19-fold increase in Lac^+ revertants after immediate selection, indicating that 80 mM $NaNO_2$ treatment for 9 min was sufficient to cause a significant induction in Lac^+ reversion. After overnight growth, as was expected, we measured up to 100-fold increases in the frequency of lac reversion, illustrating that the damage causes by $NaNO_2$ caused mutagenesis during the non-selective replication.

Intermediate incubation in rich media allowed cells to undergo replication, thereby allowing replication to bypass damaged DNA and permanently establish mutations in the DNA. Under this condition, exposure to nitrous acid induced similar numbers of mutations on the transcribed and non-transcribed strand of the amber allele among cells subjected to selection after intermediate replication. Therefore, each strand of the *lacZ* amber allele has a similar sensitivity to DNA damage and a similar ability to encode revertant ß-galactosidase enzymes, illustrating that the amber allele in our cells is suitable for studying strand-specific mutations caused by retromutagenesis.

Nitrous acid exposure causes a transcribed-strand mutation bias after immediate lac selection

After immediate selection, exposure to nitrous acid induced more mutations on the transcribed than the non-transcribed strand of the amber allele. Reversion under immediate selection requires that cells express a functional full-length *lacZ* transcript (**Figure 1**). The predominance of transcribed-strand mutations suggests that TM allowed the expression of functional ß-galactosidase protein that induced replication that bypassed hypoxanthine. This finding was upheld for both the leading- and lagging-strand amber allele strains, suggesting that transcription, not replication, gave rise to the transcribed-strand mutation bias found among immediately selected revertants.

S326C-OGG1 has a similar level of in vitro activity compared to WT OGG1

The activity of S326C-OGG1 has been the subject of controversy. We began our studies on S326C-OGG1 by measuring the activity of this enzyme *in vitro.* To begin, we transfected *Ogg1*-/- mouse embryonic fibroblasts (MEFs) with S326C-*Ogg1* or WT *Ogg1* from which we generated whole-cell lysates. These lysates were incubated with ^{32}P-labeled 8-oxodG oligonucleotides, and these reactions were resolved on a denaturing urea gel. The radioactive signal from the gel was transferred to a phosphor screen, which was scanned, and the band signals of cleaved vs. uncleaved oligonucleotide was quantified. As a control for transfection efficiency, the lysates were also probed using a standard Western blot protocol for the HA tag on the *Ogg1* constructs, as well as the β-actin signal as a loading control. The proportion of cleaved oligonucleotide signal, adjusted for OGG1 and β-actin signal, was used to measure the activity level of the WT vs. S326C-

OGG1 enzymes. In the absence of oxidative stress, we measured no statistically significant difference between the activity of the two enzymes, suggesting that the difference between their activities *in vivo* must arise under a stress condition in their cellular environments.

S326C-OGG1 has a decreased level of in vitro activity under oxidative stress

Several studies have observed that S326C-OGG1 is subject to activity loss under oxidative stress conditions. This observation has been upheld using a number of different non-physiological cellular treatments that give rise to oxidative stress conditions. Therefore, to investigate the oxidative inactivation of S326C-OGG1 activity, we exposed the MEFs transfected with S326C-*Ogg1* or WT *Ogg1* to H_2O_2 before the lysis step of the *in vitro* activity assay described in the previous section. H_2O_2 is a cellular oxidative stressor, found at low micromolar concentrations in cells in infected microenvironments. In our experimental methodology, we treated our cells with a range of H_2O_2 concentrations for 15 minutes on ice. This short treatment period helps ensure that the H_2O_2 does not undergo spontaneous dissociation. However, the brevity of this treatment may explain why only higher concentrations of H_2O_2 caused a statistically significant decrease in S326C-OGG1 activity.

S326C-OGG1 has a similar level of cellular repair activity to the WT allele

Standard methodologies for assessing *in vitro* enzymatic activity involve alteration of the native redox conditions of the enzyme, such as the alkaline elution technique (17). To investigate the repair phenotype of S326C-*Ogg1* cells, we utilized a

modified comet assay in which cellular 8-oxodG repair could be measured directly. We obtained Ser326-hOGG1, Cys326-OGG1, and KO OGG1 MEFs (Nik Hodges (School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK), stable transductant cell lines that allowed us to study the ability of S326C-OGG1 to carry out effective repair of oxidative DNA damage without resorting to the variability imposed by transfection. To study S326C-*Ogg1* repair phenotypes, we cultured the *Ogg1* cell lines above, released the cells, then resuspended them in low-melting point agarose, and transferred the cells onto a glass slide. After lysis, cellular DNA was digested by treatment with OGG1 enzyme, causing single-stranded breaks to arise at sites of 8-oxodG damage. Cellular DNA was then subjected to alkaline electrophoresis, which causes the denaturation of the DNA, and the migration of single-stranded DNA demonstrated the induction of 8-oxodG damage arising from $KBrO_3$ treatment. This migration was visualized after staining with SYBR® Green dye (Life Technologies, Inc.) and fluorescent microscopy visualization. Using software, the DNA in individual cells was quantified by the percentage that migrated beyond the nuclear area. This percentage served as a measure of the induction of 8-oxodG DNA damage in each background and cellular condition. By comparing the percentage of migrated DNA between cells before and after 1 h recovery from genotoxic treatment, we were able to measure the degree to which *Ogg1* alleles were able to effectively repair 8-oxodG. These measurements allowed us to determine that S326C-OGG1 and WT OGG1 undergo comparable levels of repair in the absence of oxidative stress. Therefore, we concluded that Cys326 oxidation may underlie the loss of activity seen in S326C-OGG1 under oxidative stress.

Inflammatory cytokine treatment causes a loss in S326C-OGG1 activity in vitro and ex vivo

S326C-OGG1 is known to be inactivated by oxidative stress. However, little is known about the degree to which physiological oxidative stressors cause the inactivation of S326C-OGG1. Therefore, we chose to study a physiological oxidative stressor, tumor necrosis factor alpha (TNF-α). TNF-α is an inflammatory cytokine implicated in every step of tumor progression. Furthermore, TNF-α causes an increase in cellular ROS over the course of several hours, which increases the likelihood that the oxidative stress it causes could have mutagenic consequences. In the aforementioned comet assay, some cells were given 1 h to recover from genotoxic damage before lysis, while another group of cells were subjected to lysis immediately. By comparing the 8-oxodG before and after recovery, we were able to measure the amount of 8-oxodG repair. After exposing cells to TNF-α for 1 h, we noted a significant decrease in OGG1 repair activity in S326C-*Ogg1* and *Ogg1-/-* lysates, but WT *Ogg1* lysates underwent effective repair. We also measured an increase in cellular H_2O_2 after exposure to TNF-α by utilizing the Amplex® Red reagent (Life Technologies, Inc.), according to the manufacturer's recommendations.

These results suggested that TNF-α may induce oxidative stress that damages DNA and inactivates S326C-OGG1. We were able to confirm this finding using a control experiment in which we exposed cells to $KBrO_3$, an oxidative stressor that causes DNA oxidation, including 8-oxodG formation, but does not cause oxidation of amino acids (18). Therefore, $KBrO_3$ treatment allowed us to study DNA damage independent of Cys326 oxidation. We carried out the aforementioned comet assay in order to measure the level of unrepaired 8-oxodG present in cells after exposure to $KBrO_3$. *Ogg1-/-* cells

were unable to repair 8-oxodG induced by $KBrO_3$, but WT and S326C-*Ogg1* cells carried out effective repair. Therefore, we concluded that Cys326C oxidation presumably underlies the inactivation of S326C-OGG1 induced by TNF-α. This inactivation may be mediated by dimerization, either inter- or intramolecularly (19), or via gross conformational changes in the spatial arrangement of functional domains (**Figure 2**). This result would be particularly interesting because, although inactivating modifications are predicted to occur inside functional domains (20), Cys326 exists outside any known functional OGG1 domains,

Biological implications for future studies

Part I

Implications for limiting resistance in antibiotic therapy

The findings in Chapter 2 illustrate that adaptive mutations may arise from retromutagenesis. Retromutagenesis may explain why bacteria develop adaptive resistance mutations to certain antibiotics. Antibiotic resistance can arise by many mechanisms, but one mechanism involves single-base mutations that confer resistance to certain antibiotics. For example, ciprofloxacin is an antibiotic that inhibits DNA synthesis, and stationary-phase cells under ciprofloxacin selection ultimately develop resistance mutations. This finding is consistent with the transcription-dependent role that retromutagenesis may play in generating antibiotic resistance. However, no resistance mutations have been observed in cells under selection with rifampin, a transcription inhibitor (21). Therefore, if antibiotic resistance can arise via retromutagenesis, the

inhibition of transcription with antibiotics like rifampin would limit resistance to antibiotics caused by single-base mutations.

Implications for studying evolutionary adaptive forces in bacteria

Adaptive mutagenesis occurs via several different mechanisms. One major mechanism of adaptive mutagenesis is amplification (22). In a population of cells under selection, mutants will arise via amplification over time (**Figure 3**), and eventually one revertant colony will tend to outgrow the others in competition for nutrients and survival. However, the same pattern of natural selection will occur in cells that have undergone retromutagenesis, allowing them to acquire mutations that render them able to divide under previously selective conditions. In this way, retromutagenesis may shape the evolution of cells under selective conditions. By expanding the scope of experiments such as those described in Chapter 2 over many generations of growth, mutation analysis could illustrate the degree to which retromutagenesis contributes to phenotypic evolution.

Implications for Measuring TNF-α Levels as a Biomarker in Humans

In Chapter 3, TNF-α was shown to cause both DNA damage and a loss in S326C-OGG1 activity, suggests that TNF-α could serve as a biomarker in human populations. TNF-α expression is already studied as a biomarker for inflammatory and immunological responses (23). Humans who are homozygotic for the *S326C-Ogg1* allele have an increased risk of cancer (24), but the degree to which this risk is affected by systemic concentrations of TNF-α is unknown. Nonetheless, serum concentrations of TNF-α have been determined in pathological pro-inflammatory states (5 ng/mL) (25), and we have

established that TNF-α concentration is implicated in both dose-dependent cytotoxicity and oxidative inactivation of S326C-OGG1. Therefore, humans who are *S326C-Ogg1* homozygotes may have a greater likelihood of developing cancer in their lifetimes, depending on their level of cellular oxidative stress. Since TNF-α causes chronic oxidative stress, it would be useful to determine the correlation between serum TNF-α concentration and lifetime cancer risk among people who are homozygotic for S326C-*Ogg1*. If such a correlation is found, people who are determined to be homozygotic for S326C-*Ogg1* could benefit from the measurement of serum TNF-α concentration by allowing individual patients to be assessed for relatively high or low cancer risk. Such an assessment could help guide patients toward managing oxidative stress via lifestyle changes, such as smoking cessation and dietary supplementation with antioxidants. Overall, the association of lifetime cancer risk with serum TNF-α concentration among S326C-*Ogg1* homozygotes could help establish a clinical range of cancer risk using TNF-α concentration as a prognostic biomarker.

Part II

Future directions

Ultimately, the studies described in Chapter 2 would best be validated by the construction of strains containing reporter alleles at alternative loci. Retromutagenesis could be subject to variation at different loci depending on damage sensitivity, damage repair, transcription, or replication. Unfortunately, the pursuit of this experimental goal was met with difficulty, presumably due to the different phenotypes encoded by the different mutations arising from damage to the two strands. However, future work could

identify loci at which reporter allele mutations arise with equal frequency on either strand, and then mutagenesis studies similar to those in Chapter 2 could be conducted to test the locus specificity of retromutagenesis.

Also, the studies in Chapter 2 could be utilized as a template for designing studies of antibiotic resistance. For example, many single-base substitutions in *gyrA* have a dramatic effect on ciprofloxacin resistance (21). Strains could be constructed that contained a reporter allele that could confer resistance by the same mutation on either strand of the allele. These strains could then be exposed to a base-specific mutagen and then subjected to antibiotic selection either immediately or following non-selective growth. DNA sequencing of the reporter locus could determine the frequency with which mutations arose predicted to arise from damage to the transcribed strand. If intermediate growth in non-selective media gave rise to similar mutation frequencies on either strand, this would demonstrate that the two strands of the reporter allele have similar mutagenic sensitivity, and that the mutations on either strand give rise to similar resistance phenotypes. If immediate growth in selective media also gave rise to a high frequency of mutations on the transcribed strand, this would suggest that retromutagenesis can contribute to antibiotic resistance.

In Chapter 3, the study of oxidative inactivation focused on a single enzyme. However, the study of other glycosylase variant enzymes associated with cancer could prove informative. For example, little is known about the mechanistic basis on which BER glycosylase NEIL1 variant S82C is presumed to be dysfunctional (26), but like S326C-OGG1, this may arise from disulfide bridges at Cys82 arising from oxidation. Another informative possibility would be to measure the repair of 8-oxodG at multiple

timepoints during the comet assay. By studying the repair of 8-oxodG over time, future studies could determine the degree to which S326C-OGG1 activity recovers after exposure to oxidizing conditions. Such a recovery could arise from reversible oxidation of Cys326 oxidation. For example, sulfinic acid is a reversible modification of cysteine (27), while cysteine sulfonic acid is an irreversible modification (28). Although both cysteine modifications could be similarly deleterious to enzymatic activity in the short term, the recovery of repair activity over time would illustrate the reversibility of this modification over time.

Alternative Experimental Approaches

Utilization of Alternative Mutation Reporter Systems

The studies in Chapter 2 measure the degree to which retromutagenesis, and thus transcriptional mutagenesis, contributes to adaptive mutations. Instead of utilizing a premature stop codon in *lacZ*, we could have instead designed a reporter system in the *trp* gene necessary for tryptophan synthesis. However, in strains containing a similar premature stop codon, *trp* reversion has been measured at high (approximately 50%) frequencies from suppressor mutations (29). Such findings suggest that premature stop codons in *trp* do not overwhelmingly limit the activity of the encoded tryptophan synthase protein, allowing residual growth under selection. Residual growth would allow inheritance of mutations via natural selection over time, rather than through the retromutagenesis under selection we measured. Likewise, as found in other adaptive mutation studies, we could have utilized a *lacZ* reporter allele containing a frameshift mutation. Although such an allele could have been designed to measure strand-specific

mutagenesis, the reversion of frameshift alleles frequently occurs by recombination or polymerase errors (30). Therefore, a reporter allele containing a frameshift mutation would have introduced a range of mutagenic events, limiting our ability to study the single-base substitutions predicted by retromutagenesis.

Instead of studying strand-specific reversion mutations, we could also have designed an experimental system to study the sequences of both RNA and DNA in small populations of revertant cells. Retromutagenesis predicts that, in a wild-type population, DNA damage will allow the creation of mutant RNA, which will ultimately contribute to analogous mutations in DNA. One way to validate this model would be by removing samples of the population under selection at different time points, then analyzing the RNA sequence (after cDNA conversion) and DNA sequence to measure the same reversion mutations arising first in RNA, then in the DNA. However, because the cellular samples would be destroyed during sequencing, there would be no way to validate that reversion arose because of the identified mutations. Likewise, the low frequency at which a given locus would contain a damaged base, compounded by the low frequency of mutagenic transcriptional bypass, would severely limit the detection power of sequencing to measure transcriptional mutagenesis.

Utilizing Alternative Selection Techniques

The studies in Chapter 2 could have been carried out using selection for resistance to killing via a bacteriophage. This method would have had the advantage of actively killing non-revertant cells, rather than allowing them to persist in a non-growth state on selective media. However, phage resistance is mediated by multiple genes, and thus

revertant colonies could contain mutations in several different loci (31). Moreover, the growth of non-revertant cells was minimized in our experiments by incubation of selective media with a carbon scavenger strain prior to growth of experimental cultures. This scavenger treatment also prevented the remnants of killed cells from providing trace carbon sources that would allow non-revertant cells to form colonies on selective media.

Direct Measurement of Cys326 Oxidation

The studies in Chapter 3 measured the degree to which TNF-α contributes to inactivation of S326C-OGG1 via Cys326 oxidation. There are several different labeling protocols that allow the measurement of alternative redox states by utilizing mass spectrometry (32). However, mass spectrometric analysis involves distinguishing mass shifts in response to labeling (33) that would have required utilizing cell lines containing a tagged *Ogg1* construct for purification of an appropriate sample size. Likewise, even labeling techniques that do not require sophisticated instrumentation such as OxyBlot (Millipore) would have necessitated generating cell lines with a tagged *Ogg1* construct in order to measure the oxidation of OGG1 specifically (34). Future studies could carry out such a technique in order to compare the measurement of cysteine oxidation between WT and S326C-*Ogg1* cells.

Concluding Remarks

Overall, the results reported in this dissertation illustrate the importance of utilizing simple model organisms to understand basic biological questions that may underpin the mechanisms of biologically important events such as the development of

antibiotic resistance. Likewise, our results also demonstrate the importance of studying inflammatory signaling in order to better understand processes involved in influencing human cancer risk.

References

1. Lindahl T. 1993. Instability and decay of the primary structure of DNA. Nature 362:709-715.
2. Frederico LA, Kunkel TA, Shaw BR. 1990. A sensitive genetic assay for the detection of cytosine deamination: determination of rate constants and the activation energy. Biochemistry 29:2532-2537.
3. Wink DA, Kasprzak KS, Maragos CM, Elespuru RK, Misra M, Dunams TM, Cebula TA, Koch WH, Andrews AW, Allen JS, et al. 1991. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254:1001-1003.
4. Kroncke KD, Fehsel K, Kolb-Bachofen V. 1997. Nitric oxide: cytotoxicity versus cytoprotection--how, why, when, and where? Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society 1:107-120.
5. Schouten KA, Weiss B. 1999. Endonuclease V protects Escherichia coli against specific mutations caused by nitrous acid. Mutat Res 435:245-254.
6. Lindahl T, Wood RD. 1999. Quality control by DNA repair. Science 286:1897-1905.
7. Kow YW. 2002. Repair of deaminated bases in DNA. Free radical biology & medicine 33:886-893.
8. Guo G, Weiss B. 1998. Endonuclease V (nfi) mutant of Escherichia coli K-12. Journal of bacteriology 180:46-51.

9. Bregeon D, Doddridge ZA, You HJ, Weiss B, Doetsch PW. 2003. Transcriptional mutagenesis induced by uracil and 8-oxoguanine in Escherichia coli. Molecular cell 12:959-970.

10. Clauson CL, Oestreich KJ, Austin JW, Doetsch PW. 2010. Abasic sites and strand breaks in DNA cause transcriptional mutagenesis in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America 107:3657-3662.

11. Beckman KB, Ames BN. 1997. Oxidative decay of DNA. The Journal of biological chemistry 272:19633-19636.

12. Fraga CG, Shigenaga MK, Park JW, Degan P, Ames BN. 1990. Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proceedings of the National Academy of Sciences of the United States of America 87:4533-4537.

13. Seeberg E, Eide L, Bjoras M. 1995. The base excision repair pathway. Trends in biochemical sciences 20:391-397.

14. Wilson DM, 3rd, Kim D, Berquist BR, Sigurdson AJ. 2011. Variation in base excision repair capacity. Mutat Res 711:100-112.

15. Davis BD. 1989. Transcriptional bias: a non-Lamarckian mechanism for substrate-induced mutations. Proceedings of the National Academy of Sciences of the United States of America 86(13):5005-5009.

16. Pastan I, Perlman RL. 1969. Repression of beta-galactosidase synthesis by glucose in phosphotransferase mutants of Escherichia coli. Repression in the

absence of glucose phosphorylation. The Journal of biological chemistry 244:5836-5842.

17. Moritz E, Pauly K, Bravard A, Hall J, Radicella JP, Epe B. 2014. hOGG1-Cys326 variant cells are hypersensitive to DNA repair inhibition by nitric oxide. Carcinogenesis.

18. Murata M, Bansho Y, Inoue S, Ito K, Ohnishi S, Midorikawa K, Kawanishi S. 2001. Requirement of glutathione and cysteine in guanine-specific oxidation of DNA by carcinogenic potassium bromate. Chem Res Toxicol. Jun;14(6):678-85.

19. Hill JW, Evans MK. 2006. Dimerization and opposite base-dependent catalytic impairment of polymorphic S326C OGG1 glycosylase. Nucleic Acids Res 34:1620-1632.

20. Mohrenweiser HW, Wilson DM, 3rd, Jones IM. 2003. Challenges and complexities in estimating both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. Mutat Res 526:93-125.

21. Riesenfeld C, Everett M, Piddock LJ, Hall BG. 1997. Adaptive mutations produce resistance to ciprofloxacin. Antimicrobial agents and chemotherapy 41:2059-2060.

22. Roth JR, Kugelberg E, Reams AB, Kofoid E, Andersson DI. 2006. Origin of mutations under selection: the adaptive mutation controversy. Annu Rev Microbiol 60:477-501.

23. Young CN, Koepke JI, Terlecky LJ, Borkin MS, Boyd Savoy L, Terlecky SR. 2008. Reactive oxygen species in tumor necrosis factor-alpha-activated primary

human keratinocytes: implications for psoriasis and inflammatory skin disease. J Invest Dermatol. Nov;128(11):2606-14.

24. Hung RJ, Hall J, Brennan P, Boffetta P. 2005. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. Am J Epidemiol 162:925-942.

25. Turner DA, Paszek P, Woodcock DJ, Nelson DE, Horton CA, Wang Y, Spiller DG, Rand DA, White MR, Harper CV. 2010. Physiological levels of TNFalpha stimulation induce stochastic dynamics of NF-kappaB responses in single living cells. J Cell Sci 123:2834-2843.

26. Prakash A, Carroll BL, Sweasy JB, Wallace SS, Doublié S. 2014. Genome and cancer single nucleotide polymorphisms of the human NEIL1 DNA glycosylase: activity, structure, and the effect of editing. DNA Repair (Amst). 2014 Feb;14:17-26.

27. Jönsson TJ, Tsang AW, Lowther WT, Furdui CM. 2008. Identification of intact protein thiosulfinate intermediate in the reduction of cysteine sulfinic acid in peroxiredoxin by human sulfiredoxin. J Biol Chem. Aug 22;283(34):22890-4.

28. Lim J.C., Choi H.I., Park Y.S., Nam H.W., Woo H.A., Kwon K.S., Kim Y.S., Rhee S.G., Kim K., Chae H.Z. Irreversible oxidation of the active-site cysteine of peroxiredoxin to cysteine sulfonic acid for enhanced molecular chaperone activity. J. Biol. Chem. 2008;283:28873–28880.

29. Ohta T, Tokishita S, Tsunoi R, Ohmae S, Yamagata H. 2002. Characterization of Trp(+) reversions in Escherichia coli strain WP2uvrA. Mutagenesis. 2002 Jul;17(4):313-6.

30. Foster, PL. 2004. Adaptive Mutation in Escherichia coli. Journal of Bacteriology. 186;15:4846–4852.

31. Labrie SJ, Samson JE, Moineau S. 2010. Bacteriophage resistance mechanisms. Nat Rev Microbiol. 2010 May;8(5):317-27.

32. Zhang J, Ye ZW, Gao P, Reyes L, Jones EE, Branham-O'Connor M, Blumer JB, Drake RR, Manevich Y, Townsend DM, Tew KD. 2014. Glutathione S-transferase P influences redox and migration pathways in bone marrow. PLoS One 9:e107478.

33. Tew KD, Manevich Y, Grek C, Xiong Y, Uys J, Townsend DM. 2011. The role of glutathione S-transferase P in signaling pathways and S-glutathionylation in cancer. Free Radic Biol Med. Jul 15;51(2):299-313.

34. Romagnoli M, Gomez-Cabrera MC, Perrelli MG, Biasi F, Pallardó FV, Sastre J, Poli G, Viña J. 2010. Xanthine oxidase-induced oxidative stress causes activation of NF-kappaB and inflammation in the liver of type I diabetic rats. Free Radic Biol Med. 2010 Jul 15;49(2):171-7.

35. Bruner SD, Norman DP, Verdine GL. 2000. Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. Nature 403:859-866.

36. Kelley LA, Sternberg MJ. 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nature protocols **4:**363-371.

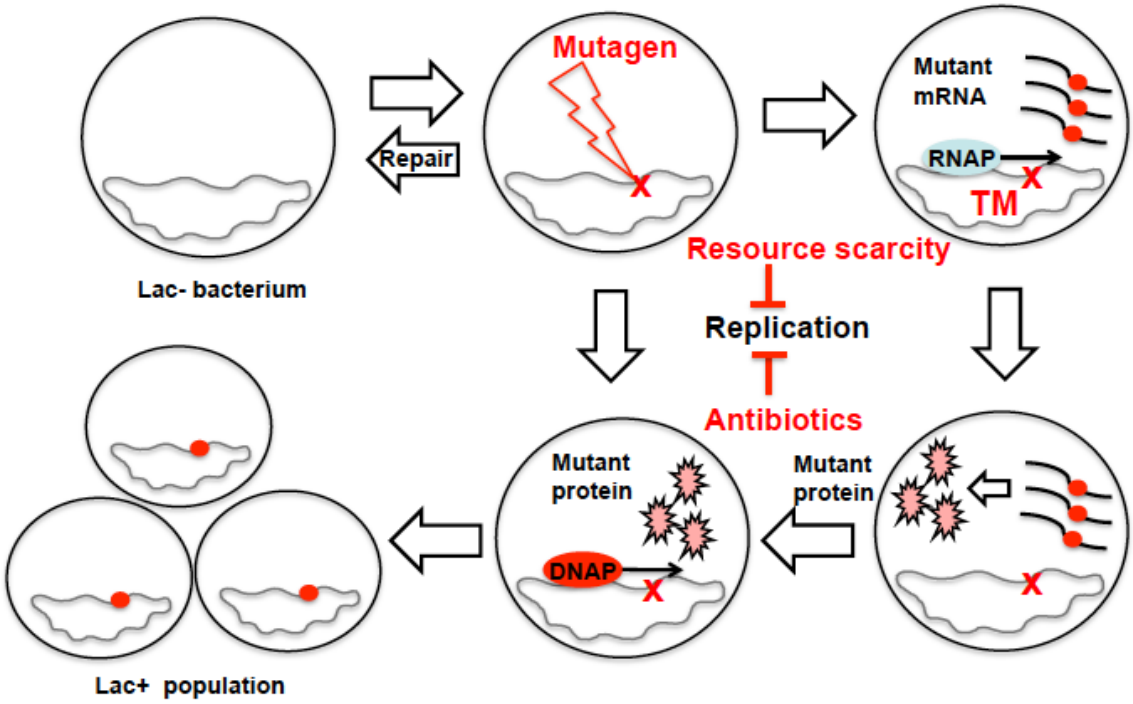

Figure 1. Retromutagenesis allows cells to undergo phenotypic reversion to a pro-growth state. Cells that are initially incapable of replicating in a selective growth environment may undergo DNA damage. This damage undergoes mutagenic bypass by RNA polymerase, generating a mutant transcript that allows the expression of a mutant protein. This mutant protein allows a cell to overcome selection and undergo DNA replication, causing DNA polymerase to bypass the lesion that caused TM and permanently establish an analogous mutation in the DNA of a dividing cell population.

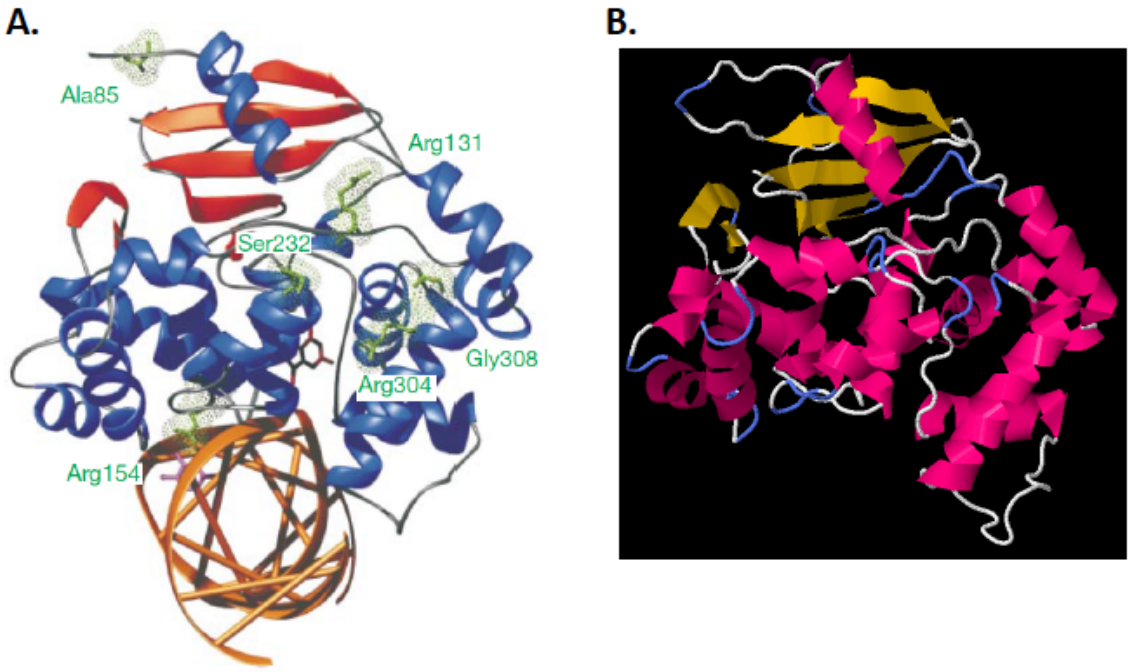

Fig. 2. S326C-OGG1 may undergo conformational rearrangement. (**A**) The structure of WT OGG1 in a complex with DNA illustrates the relative positions of the residues for which crystallographic information is available. Adapted from Bruner *et al.* (35) with permission from the publisher. (**B**) The structure of S326C-OGG1 illustrates an altered conformation around the C-terminal region matching that labeled in **A**, suggesting that Cys326 may affect native protein structure. Protein structure predicted using methods of Kelley and Sternberg (36).

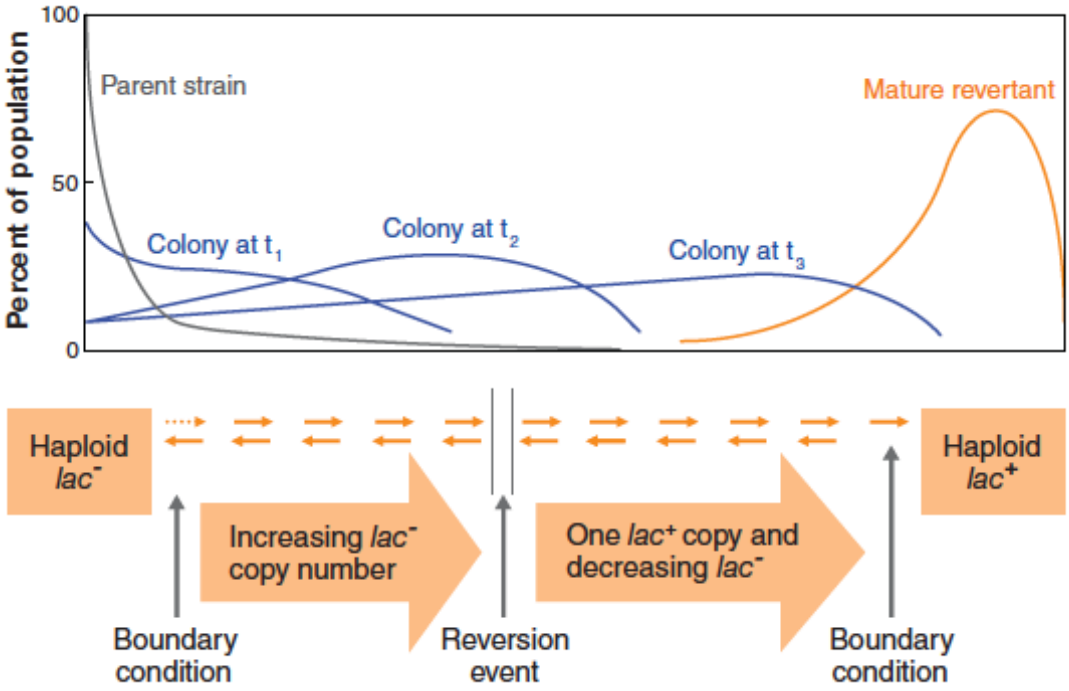

Figure 3. Adaptive mutation can occur via the competitive outgrowth of mutant colonies. Adaptive mutations may arise by several mechanisms, one of which is the amplification of genes under selection, such as *lac*. Residual activity of the protein encoded by such a gene can allow a cell to undergo replication under selective conditions. Importantly, although retromutagenesis has an important role in reversion under selection, such amplification events may occur simultaneously in populations of cells in nature. Moreover, colonies arising from retromutagenesis will be subject to a similar pattern of competitive selection for growth resources. From Roth *et al.* (22) with permission from the publisher.

CHAPTER S1

TRANSCRIPTIONAL MUTAGENESIS AND ITS POTENTIAL ROLES IN THE ETIOLOGY OF CANCER AND BACTERIAL ANTIBIOTIC RESISTANCE

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Abstract

Most cells do not undergo continuous cell division and DNA replication, yet they can still acquire novel RNA mutations that can result in the production of mutant proteins and induce a phenotypic change. All cells are frequently subjected to genotoxic insults that give rise to damaged nucleotides which, similarly to DNA replication, can undergo base mispairing during transcription. This mutagenic lesion bypass by RNA polymerase, transcriptional mutagenesis (TM), has been studied in a variety of systems and organisms, and may be involved in diverse pathogenic processes, such as tumorigenesis and the acquisition of bacterial antibiotic resistance. Tumor cells and bacteria within the human body are subject to especially high levels of oxidative stress, which can damage DNA and consequently drive TM. Mutagenesis at the level of transcription may allow cells to escape growth arrest and undergo replication that could permanently establish mutations in DNA in a process called retromutagenesis (RM). Here, we review the broad range of DNA damages which may result in TM including a variety of non-bulky lesions and some bulky lesions, which recent studies indicate may not completely block transcription, and emerging evidence supporting the RM concept in the context of tumorigenesis and antibiotic resistance.

Introduction

DNA damage from endogenous sources, such as reactive oxygen species (ROS) produced by mitochondria during respiration, and environmental sources, including ultraviolet (UV) light is ubiquitous and, poses a constant threat to genomic integrity. All cells, regardless of their proliferation state, are constantly exposed to DNA damage. DNA base damages can often have miscoding properties and be mutagenically bypassed by DNA polymerase. The well-established mutagenic potential of DNA damage during DNA replication has been linked to genomic instability and a variety of diseases, including cancer. However, largely quiescent cells may not be protected from the potentially deleterious consequences of DNA damage, as many of these lesions can also be bypassed by RNA polymerase (RNAP), giving rise to mutant RNA molecules. This process, termed transcriptional mutagenesis (TM), can alter the phenotype of non-proliferating cells (1, 2, 3). Since analogous bases are often incorporated opposite the lesion by both DNA and RNA polymerases, if a phenotype conferred by the transcriptional mutation is cell cycle entry, one of the resulting daughter cells could acquire a permanent DNA mutation, and thus permanent establishment of the phenotype. This mechanism, which can potentially allow quiescent cells to enter the cell cycle and acquire novel mutations, has been termed retromutagenesis (RM) (4).

Two highly conserved pathways, base excision repair (BER) and nucleotide excision repair (NER), repair base damage in humans, bacteria, yeast as well as other organisms. BER primarily repairs non-bulky, non-helix-distorting lesions, which are generally bypassed by RNAP and may result varying levels of TM. Bulky lesions present in non-transcribed DNA are repaired by global genome repair (GGR) sub-pathway of

NER, while RNAP stalled at lesions occurring in DNA undergoing transcription serves as the first point of recognition for the transcription-coupled repair (TCR) sub-pathway. BER and NER pathway deficiencies have been associated with an increased susceptibility to cancer, neurodegenerative disease, as well as developmental defects (5, 6) and the consequences of the interactions of unrepaired DNA damage with DNA and RNA polymerases may influence these outcomes. TM and RM may have deleterious impact on human health by contributing to the etiology of such diseases as well as giving rise to antibiotic-resistant pathogenic bacteria.

Transcriptional Mutagenesis Induced by Non-Bulky Base Damage

BER is initiated by lesion-specific glycosylases, which recognize and excise damaged bases, leaving an apurinic/apyrimidinic (AP) site, which is then further processed and replaced by an undamaged base. Such lesions arise from four primary sources of damage: alkylating agents, reactive oxygen species, reactive nitrogen species, and deaminating agents (7). RNAP bypass of a plethora of such non-bulky lesions as well as the BER pathway intermediates AP sites and single-strand breaks, that can also arise spontaneously, give rise to TM (8). Non-bulky lesions allow the bypass by both RNA and DNA polymerases, and thus mediate potential transcriptional and replicative mutagenesis, respectively. TM may be important in a variety of contexts and organisms. For example, adenine is misincorporated opposite an 8-oxoguanine (8-oxoG) lesion by mammalian RNAPII, as well as yeast, bacterial, and mitochondrial RNAP (9).

In the presence of oxidative DNA damage, BER deficiency can dramatically increase the levels of TM, as was found in mouse embryonic fibroblasts (MEFs) for 8-

oxoG in an 8-oxoguanine glycosylase null ($Ogg1^{-/-}$) background (3). Within BER-deficient backgrounds, a TM-causative lesion may persist long enough to be mutagenically bypassed by DNA polymerase, and thus permanently establish mutations in a cell's genome through retromutagenesis (10). However, although 8-oxoG can be bypassed by RNAP before repair, bypass is obstructed following interaction with Ogg1 (11).

Encounters of RNAP with Bulky DNA Lesions

Unlike BER substrates, bulky lesions distorting the double helix frequently stall RNAP, which serves as an impediment to replication and transcription and may induce apoptosis, unless repaired by TCR. However, emerging evidence indicates that the mechanisms by which RNAP stalls differ depending on the lesion and, for some damages, transcription may not be entirely prevented (12, 13, 14). When the block of transcription is not complete, and a fraction of RNAPs are able to bypass the damage, transcriptional mutagenesis may occur.

Using a novel assay allowing the quantitative estimation of transcriptional mutagenesis and lesion bypass efficiency, relative to undamaged sequence, it was recently demonstrated that this is the case for some bulky oxidative damages and thiopurine drug-induced lesions (13, 14). This Competitive Transcription and Adduct Bypass assay employs non-replicating vectors containing site-specific base modifications premixed with lesion-free competitor plasmids, from which run-off transcripts are reverse-transcribed, restriction-digested and analyzed by PAGE and LC-MS/MS (13). By employing the assay and HeLa nuclear extracts or transfection into human cells, it was

determined that 8,5-cyclo-2'-deoxyadenosine (cdA) and 8,5'-cyclo-2'-deoxyguanosine (cdG) prevent transcription by human RNAPII *in vitro* and strongly inhibit transcription in human cells. However, the limited bypass of the lesions resulted in TM (13). Moreover, a major lesion induced by thiopurine drugs, widely used for the treatment of cancers such as leukemia as well as other conditions, S6-methylthioguanine, also strongly inhibits transcription but is also transcriptionally mutagenic (14). It has been therefore proposed that thiopurine drug induced DNA damage may not only contribute to the cytotoxic effects of these agents, likely due to its transcription blocking effects, but may also play a role in the pathogenesis of thiopurine therapy-associated cancers (14).

While the structure-outcome relationship by which the above lesions inhibit transcription and result in TM are not yet known, mechanistic insight has been gained from crystallographic studies of *Saccharomyces cerevisae* RNAP in complex with two other NER substrate lesions, cyclobutane pyrimidine dimers (CPDs) (12, 15) and cisplatin lesions (16), as well as the BER substrate 8-oxoG (17). CPDs, which are covalently linked adjacent pyrimidines and are the most common DNA damages induced by UV light, can enter the RNAP active site, but translocation is disfavored following lesion-templated misincorporation of uridine (15). Nevertheless, with the CPD outside the active site, a non-templated adenine can be inserted at the first thymine of a CPD lesion, allowing the entry of the lesion into the active site and non-mutagenic elongation past the lesion (12). In contrast, bypass has not been observed for the 1,2-d(GpG) intrastrand crosslinks induced by the chemotherapeutic drug cisplatin, which do not enter the RNAP active site and block translocation (17). Also, similar to the encounter of RNAP or DNA polymerase with an abasic site or strand break (18), a non-templated

adenine is misincorporated by RNAP stalled at a cisplatin lesion, suggesting that the "A-rule" observed for DNA polymerases when they incorporate adenine as they replicate through a non-informative site may also apply to transcribing RNAP (16).

Bacterial Transcriptional Mutagenesis and Antibiotic Resistance

The first models of transcriptional mutagenesis were established *in vitro*. Experiments with plasmid vectors containing site-specific abasic sites showed efficient bypass by *E. coli* RNA polymerases, giving rise to transcripts containing an adenine at the site corresponding to the template DNA abasic site (19). Bacteriophage polymerases were also used to illustrate TM *in vitro* when bypassing dihydrouracil lesions (20), as well as uracil, O6-methylguanine, and 8-oxoguanine, each of which promoted a distinct range of RNA misinsertions (21). Finally, the mutagenic specificity of lesions was compared between RNA and DNA polymerase bypass, and found to be the same for dihydrouracil (22). Analogous RNAP and DNA polymerase misinsertion at the site of a lesion is essential to the model of retromutagenesis, as it would allow permanent establishment of the original transcriptional mutation.

In vivo models were later established for the study of TM. Uracil was cloned into a stop codon engineered into the firefly luciferase gene, allowing TM at this locus to permit the synthesis of a full-length luciferase transcript. When this construct was transformed into non-dividing cells, luciferase activity was used to measure TM arising from the transgene and found to be dependent on DNA base damage and uracil repair background (1). This experimental system was used to measure TM arising from uracil, as well as from 8-oxoguanine, which was found to be increased in a transcription-coupled

repair-deficient background (2). The same system was used to study TM arising from abasic sites and strand breaks, which, *in vivo*, gave rise to TM (18). One advancement to this system is the introduction of site-specific lesions into the *E. coli* chromosome, which could improve the physiological relevance of the bypass of such lesions (23). Interestingly, a recent study has illustrated an increased frequency of *lac* operon expression switching in cells with decreased RNAP fidelity, causing lac to undergo derepression without undergoing reversion mutations, yet also causing heritable changes in lac expression (24). This reinforces the notion that mutant transcripts can confer heritable phenotypes.

TM is a mechanism by which bacteria may escape growth restrictions imposed by their environment. TM is particularly important in conditions where bacteria divide at lower rates *in vivo*; for example, *E. coli* divides 4-6 times slower in the gut than it does in culture (25). In addition, antibiotic treatment, to which bacteria in a physiological setting are often subjected, is typically cytostatic, and can even impair the fidelity of translation (26). Under such non-dividing conditions, point mutations arise that allow phenotypic reversion over the course of several days, indicating that such mutations were not present before cells were plated on selective media, and the mechanisms by which these mutations arise could include retromutagenesis (27). The oxidative DNA lesions from which TM arises are common among bacteria, which are often subjected to oxidative stress that may alter their phenotype. Bacteria in infection microenvironments undergo oxidative stress (28), which can give rise to lesions known to contribute to stationary-phase mutagenesis (29). Strains with greater endogenous oxidative stress undergo more frequent adaptive mutations, possibly through TM (30). Oxidative damage is known to

facilitate *lacZ* reversion because mutating *MutT*, which sanitizes oxidative damage from dNTP pools, allows increased *lacZ* missense reversion under aerobic conditions (31). Antibiotics induce oxidative stress, which has been correlated with but may not be necessary for their bactericidal effects (32), yet the ROS induced by sub-lethal concentrations of antibiotics can induce mutations causing antibiotic resistance (33). TM may allow cells to acquire antibiotic resistance through the expression of a mutated target protein that allows the cell to survive antibiotic selection, and the daughter cells may acquire the same mutation through retromutagenesis.

Phenotypic Consequences in Mammalian Cells and Tumor Development

Cancer is a disease of cellular proliferation, and perhaps some of the most notorious hallmarks of cancer include aberrant proliferative signaling and evasion of signals, which suppress growth (34). Yet multiple tumor types can originate from largely quiescent cells, such as adult stem cells, following the introduction of limited number of defined genetic alterations, including single-base substitutions. For example, the G12D mutation of K-Ras leads to bronchioalveolar stem cell expansion and lung tumorigenesis (35), and the combined inactivation of p53 and Rb in stem cells of the ovarian surface epithelium results in ovarian adenocarcinoma in mice (36). Since it may allow quiescent cells to acquire new oncogenic mutations, TM may serve as one possible route to tumor initiation for cancers with stem cell origins, following retromutagenesis, but it may also play a role in cancers that originate from highly proliferative cells, such as progenitors. If a TM event initiates aberrant differentiation and proliferation of stem cells and the acquisition of one out of a set of mutations required for tumorigenesis, it might initiate

the acquisition of one trait required for a multistep tumorigenesis process, or a pre-malignancy. Such aberrantly proliferating cells may be rendered more susceptible to acquire subsequent mutations and more likely to become cancerous. In addition, loss-of-function mutations in tumor suppressors that can negatively regulate proliferation, such as p53 in the event of extensive DNA damage, may allow proliferative cells to evade harsh conditions that could otherwise lead to growth arrest, apoptosis, or necrosis.

Most human cancers possess somatic mutations with pro-mitogenic effects, such as loss-of-function mutations in negative regulators of proliferation, including p53, or gain-of-function mutations in oncogenes, such as the Ras family of GTPases, B-raf or PI3-kinases that can lead to the constitutive activation of pro-mitogenic signaling. The mutational spectra can vary between tumor types, likely due to exposure to different types of DNA damage or defects in repair (37). Many of these mutations are consistent with those occurring due to lesions known to induce TM, supporting the hypothesis that TM is one possible route to tumor development. Importantly, more direct evidence supporting this idea came from a study using a mouse embryonic fibroblast (MEF) system and a non-replicating construct containing a site-specifically inserted 8-oxoG lesion in the transcribed strand of H-Ras, designed such that mutagenic bypass of the lesion would result in the production of the constitutively active Q61K mutant form of H-Ras (3). Bypass of the lesion resulted in approximately 14% of the transcripts being mutant, which was sufficient to produce a measurable activation of the H-Ras downstream effector ERK, thus demonstrating the ability of TM to initiate oncogenic signaling.

Conclusions

While TM can drive Ras downstream oncogenic signaling, it remains unclear whether these molecular changes are sufficient to serve as the first step of the tumor-initiating process. It is likely that non-bulky BER substrate lesions which are efficiently bypassed by RNAP and are highly transcriptionally mutagenic play a more significant role in inducing such phenotypic changes. They may play important roles during tumor initiation or progression when they trigger the aberrant activation of a pro-proliferative oncogene or when they result in the loss-of-function mutation of a tumor suppressor. In contrast, NER-substrate lesions which inhibit transcription but are mutagenic when RNA and DNA polymerase bypass occurs may be more significant in the etiology of cancers when they result in the loss-of-function mutation of a tumor suppressor, since the total transcript levels following bypass by RNAP may or may not be sufficient to drive a phenotypic change that depends on a gain-of-function mutation, and some could contribute to the cytotoxic effects of DNA damaging chemotherapeutics, such as thiopurine drugs (14). However, gain-of-function mutations may be more relevant to the retromutagenesis concept, as heterozygous tumor suppressor loss may not be sufficient to drive phenotypic change. While loss of heterozygosity (LOH) by point mutation of the wild type allele cannot be excluded, that is commonly not the case. For example, tumor development in $p53^{+/-}$ mouse models of the cancer-predisposing Li-Fraumeni syndrome is associated with LOH by large-scale genetic aberrations, such as chromosomal nondisjunction (38), which is not expected to arise by a TM mechanism.

Bacteria also remain an important crucible for the phenotypic consequences of TM. Although oxidative stress contributes to antibiotic resistance and adaptive

mutagenesis, it remains to be seen whether oxidative base damage can drive antibiotic resistance through retromutagenesis. A major technical challenge to the study of the retromutagenesis concept in physiologically relevant contexts is the development of technologies for the study of TM initiated *in situ*, due to DNA damage occurring in genomic DNA. A single bacterium acquiring a transcriptional mutation conferring antibiotic resistance may give rise to a population of resistant bacteria. However, studying TM before retromutagenesis has occurred and given rise to a population of antibiotic resistant DNA mutants requires identifying such rare transcriptional mutant cells among a large population of bacteria that are not transcriptional mutants. The development of reliable technologies for the identification of DNA damage lesions and single cell transcriptomics may facilitate the study of such rare transcriptional mutants within large, highly heterogeneous populations of cells. Future high-throughput studies may also help address which lesions are most transcriptionally mutagenic and thus most likely to contribute to important phenotypes such as antibiotic resistance and tumorigenesis through TM and retromutagenesis.

References

1. Viswanathan A, You HJ, Doetsch PW. 1999b. Phenotypic change caused by transcriptional bypass of uracil in nondividing cells. Science 284(5411):159-162.

2. Bregeon D, Doddridge ZA, You HJ, Weiss B, Doetsch PW. 2003. Transcriptional mutagenesis induced by uracil and 8-oxoguanine in Escherichia coli. Mol Cell 12(4):959-970.

3. Saxowsky TT, Meadows KL, Klungland A, Doetsch PW. 2008. 8-Oxoguanine-mediated transcriptional mutagenesis causes Ras activation in mammalian cells. Proc Natl Acad Sci U S A 105(48):18877-18882.

4. Doetsch PW. 2002. Translesion synthesis by RNA polymerases: occurrence and biological implications for transcriptional mutagenesis. Mutat Res 510(1-2):131-140.

5. Cleaver JE, Lam ET, Revet I. 2009. Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity. Nat Rev Genet 10(11):756-768.

6. Fu D, Calvo JA, Samson LD. 2012. Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat Rev Cancer 12(2):104-120.

7. Friedberg EC, Aguilera A, Gellert M, Hanawalt PC, Hays JB, Lehmann AR, Lindahl T, Lowndes N, Sarasin A, Wood RD. 2006. DNA repair: from molecular mechanism to human disease. DNA Repair (Amst) 5(8):986-996.

8. Bregeon D, Doetsch PW. 2011. Transcriptional mutagenesis: causes and involvement in tumour development. Nat Rev Cancer 11(3):218-227.

9. Nakanishi N, Fukuoh A, Kang D, Iwai S, Kuraoka I. 2013. Effects of DNA lesions on the transcription reaction of mitochondrial RNA polymerase: implications for bypass RNA synthesis on oxidative DNA lesions. Mutagenesis 28(1):117-123.

10. Viswanathan A, Liu J, Doetsch PW. 1999a. E. coli RNA polymerase bypass of DNA base damage. Mutagenesis at the level of transcription. Ann N Y Acad Sci 870:386-388.

11. Kitsera N, Stathis D, Luhnsdorf B, Muller H, Carell T, Epe B, Khobta A. 2011. 8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1. Nucleic Acids Res 39(14):5926-5934.

12. Walmacq C, Cheung AC, Kireeva ML, Lubkowska L, Ye C, Gotte D, Strathern JN, Carell T, Cramer P, Kashlev M. 2012. Mechanism of translesion transcription by RNA polymerase II and its role in cellular resistance to DNA damage. Mol Cell 46(1):18-29.

13. You C, Dai X, Yuan B, Wang J, Brooks PJ, Niedernhofer LJ, Wang Y. 2012a. A quantitative assay for assessing the effects of DNA lesions on transcription. Nat Chem Biol 8(10):817-822.

14. You C, Dai X, Yuan B, Wang Y. 2012b. Effects of 6-thioguanine and s6-methylthioguanine on transcription in vitro and in human cells. J Biol Chem 287(49):40915-40923.

15. Brueckner F, Hennecke U, Carell T, Cramer P. 2007. CPD damage recognition by transcribing RNA polymerase II. Science 315(5813):859-862.

16. Damsma GE, Alt A, Brueckner F, Carell T, Cramer P. 2007. Mechanism of transcriptional stalling at cisplatin-damaged DNA. Nat Struct Mol Biol 14(12):1127-1133.

17. Damsma GE, Cramer P. 2009. Molecular basis of transcriptional mutagenesis at 8-oxoguanine. J Biol Chem 284(46):31658-31663.

18. Clauson CL, Oestreich KJ, Austin JW, Doetsch PW. 2010. Abasic sites and strand breaks in DNA cause transcriptional mutagenesis in Escherichia coli. Proc Natl Acad Sci U S A 107(8):3657-3662.

19. Zhou W, Doetsch PW. 1993. Effects of abasic sites and DNA single-strand breaks on prokaryotic RNA polymerases. Proc Natl Acad Sci U S A 90(14):6601-6605.

20. Liu J, Zhou W, Doetsch PW. 1995. RNA polymerase bypass at sites of dihydrouracil: implications for transcriptional mutagenesis. Mol Cell Biol 15(12):6729-6735.

21. Viswanathan A, Doetsch PW. 1998. Effects of nonbulky DNA base damages on Escherichia coli RNA polymerase-mediated elongation and promoter clearance. J Biol Chem 273(33):21276-21281.

22. Liu J, Doetsch PW. 1998. Escherichia coli RNA and DNA polymerase bypass of dihydrouracil: mutagenic potential via transcription and replication. Nucleic Acids Res 26(7):1707-1712.

23. Pages V, Mazon G, Naiman K, Philippin G, Fuchs RP. 2012. Monitoring bypass of single replication-blocking lesions by damage avoidance in the Escherichia coli chromosome. Nucleic Acids Res 40(18):9036-9043.

24. Gordon AJ, Halliday JA, Blankschien MD, Burns PA, Yatagai F, Herman C. 2009. Transcriptional infidelity promotes heritable phenotypic change in a bistable gene network. PLoS Biol 7(2):e44.

25. De Paepe M, Gaboriau-Routhiau V, Rainteau D, Rakotobe S, Taddei F, Cerf-Bensussan N. 2011. Trade-off between bile resistance and nutritional competence drives Escherichia coli diversification in the mouse gut. PLoS Genet 7(6):e1002107.

26. Brakier-Gingras L, Phoenix P. 1984. The control of accuracy during protein synthesis in Escherichia coli and perturbations of this control by streptomycin, neomycin, or ribosomal mutations. Can J Biochem Cell Biol 62(5):231-244.

27. Quinones-Soto S, Roth JR. 2011. Effect of growth under selection on appearance of chromosomal mutations in Salmonella enterica. Genetics 189(1):37-53.

28. Farr SB, Kogoma T. 1991. Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol Rev 55(4):561-585.

29. Vidales LE, Cardenas LC, Robleto E, Yasbin RE, Pedraza-Reyes M. 2009. Defects in the error prevention oxidized guanine system potentiate stationary-phase mutagenesis in Bacillus subtilis. J Bacteriol 191(2):506-513.

30. Aubron C, Glodt J, Matar C, Huet O, Borderie D, Dobrindt U, Duranteau J, Denamur E, Conti M, Bouvet O. 2012. Variation in endogenous oxidative stress in Escherichia coli natural isolates during growth in urine. BMC Microbiol 12:120.

31. Taddei F, Hayakawa H, Bouton M, Cirinesi A, Matic I, Sekiguchi M, Radman M. 1997. Counteraction by MutT protein of transcriptional errors caused by oxidative damage. Science 278(5335):128-130.

32. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. 2013. Killing by bactericidal antibiotics does not depend on reactive oxygen species. Science 339(6124):1213-1216.

33. Kohanski MA, DePristo MA, Collins JJ. 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell 37(3):311-320.

34. Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. Cell 144(5):646-674.

35. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. 2005. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 121(6):823-835.

36. Flesken-Nikitin A, Hwang CI, Cheng CY, Michurina TV, Enikolopov G, Nikitin AY. 2013. Ovarian surface epithelium at the junction area contains a cancer-prone stem cell niche. Nature 495(7440):241-245.

37. Greenman C, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN,

Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR. 2007. Patterns of somatic mutation in human cancer genomes. Nature 446(7132):153-158.

38. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. 1994. Tumor spectrum analysis in p53-mutant mice. Curr Biol 4(1):1-7.

39. Charlet-Berguerand N, Feuerhahn S, Kong SE, Ziserman H, Conaway JW, Conaway R, Egly JM. 2006. RNA polymerase II bypass of oxidative DNA damage is regulated by transcription elongation factors. EMBO J 25(23):5481-5491.

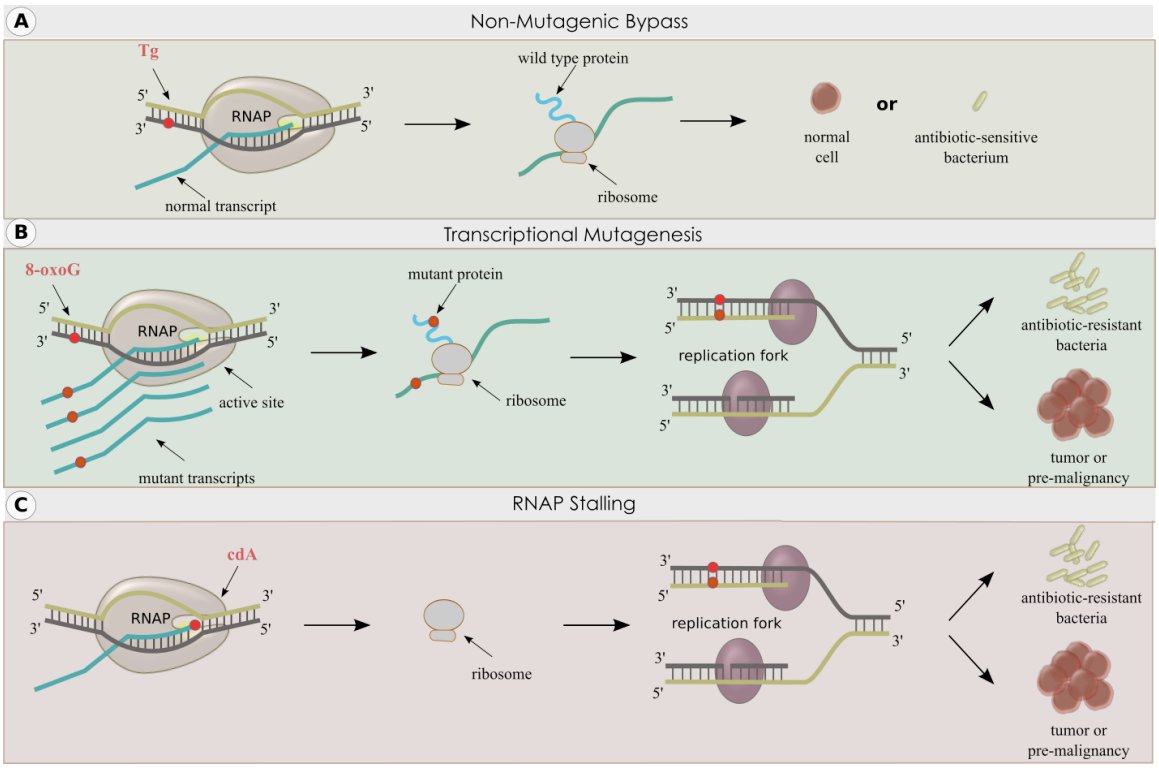

Figure 1. Potential Phenotypic Consequences of DNA Damage during Transcription

A. Some BER substrates, such as thymine glycol (Tg) (39), can be bypassed with only low levels of TM, unlikely to induce a phenotypic change. **B.** Some non-bulky BER substrates, such as 8-oxoG, can be bypassed and result in significant levels of TM. If the TM event drives a transcriptional gain-of-function mutation and aberrant activation of a proliferation-promoting oncogene, it may initiate tumorigenesis. In the case of bacteria, if the transcriptional mutation is such that it confers antibiotic resistance, it may allow the cells to evade cell death or cytostasis and give rise to a population of antibiotic resistant bacteria. **C**. Bulky lesions which partially stall RNAP but are mutagenic may result in reduced expression of a tumor suppressor and low levels of mutant transcripts, and may allow cells to evade growth-suppressive conditions or apoptosis. Bypass of such lesions in bacteria may allow cells to acquire mutations of antibiotic target proteins, which allow them to survive antibiotic selection. Reprinted with permission from the publisher.