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Dna Glycosylases Remove Oxidized Base Damages From G-Quadruplex Dna Structures

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DNA GLYCOSYLASES REMOVE OXIDIZED BASE DAMAGES FROM G-QUADRUPLEX DNA STRUCTURES

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ABSTRACT

The G-quadruplex DNA is a four-stranded DNA structure that is highly susceptible to oxidation due to its G-rich sequence and its structure. Oxidative DNA base damages can be mutagenic or lethal to cells if they are left unrepaired. The base excision repair (BER) pathway is the predominant pathway for repair of oxidized DNA bases. DNA glycosylases are the first enzymes in BER and are responsible for removing base lesions from DNA. How DNA glycosylases remove base lesions from duplex and single-stranded DNA has been intensively studied, while how they act on G-quadruplex DNA remains to be explored.

In Chapter II of this dissertation, we studied the glycosylase activity of the five mammalian DNA glycosylases (OGG1, NTH1, NEIL1, NEIL2 and mouse Neil3) on G-quadruplex DNA formed by telomere sequences that contain a single base lesion. We found that telomeric sequences that contain thymine glycol (Tg), 8-oxo-7,8-dihydroguanine (8-oxoG), guanidinohydantoin (Gh) or spiroiminodihydantoin (Sp) all formed the basket form of an antiparallel G-quadruplex DNA structure in Na^+ solution. We also showed that no glycosylase was able to remove 8-oxoG from quadruplex DNA, while its further oxidation products, Sp and Gh, were good substrates for mNeil3 and NEIL1 in quadruplex DNA. In addition, mNeil3 is the only enzyme that removes Tg from quadruplex DNA and the glycosylase strongly prefers Tg in the telomere sequence context in both single-stranded and double-stranded DNA.

In Chapter III, we extended our study to telomeric G-quadruplex DNA in K^+ solution and we also studied quadruplex DNA formed by promoter sequences. We found that 8-oxoG, Gh and Sp reduce the thermostability and alter the folding of telomeric quadruplex DNA in a location-dependent manner. Also, the NEIL1 and NEIL3 DNA glycosylases are able to remove hydantoin lesions but none of the glycosylases, including OGG1, are able to remove 8-oxoG from telomeric quadruplex DNA in K^+ solution. Interestingly, NEIL1 or NEIL3 do not efficiently remove hydantoin lesions at the site that is most prone to oxidation in quadruplex DNA. However, hydantoin lesions at the same site in quadruplex DNA are removed much more rapidly by NEIL1, NEIL2 and NEIL3, when an extra telomere TTAGGG repeat is added to the commonly studied four-repeat quadruplex DNA to make it a five-repeat telomere quadruplex DNA. We also show that APE1 cleaves furan in selected positions in Na^+-coordinated telomeric quadruplex DNA structures. We use promoter sequences of the *VEGF* and *c-MYC* genes as models to study promoter G-quadruplex DNA structures, and show that the NEIL glycosylases primarily remove Gh from Na^+-coordinated antiparallel quadruplex DNA but not from K^+-coordinated parallel quadruplex DNA containing *VEGF* or *c-MYC* promoter sequences.

Taken together, our data show that the NEIL DNA glycosylases may be involved in both telomere maintenance and gene regulation.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1. DNA Damages

DNA is the carrier of genetic information and is susceptible to damage from multiple sources. First of all, biological molecules are inevitably subject to spontaneous chemical reactions, mostly hydrolysis (Gates, 2009). For example, the glycosidic bonds that connect the nucleobases to the DNA backbone are weak points of the DNA, and spontaneous hydrolysis of glycosidic bonds happens at a significant rate in cells. It was calculated that hydrolytic cleavage of the glycosidic bonds of purines (or commonly referred as "depurination") happens at a rate of 10,000 per cell per day, which results in the abasic site being one of the most popular DNA damages in cells (Lindahl and Nyberg, 1972). Hydrolytic deamination of DNA bases is another source of DNA damage. For instance, cytosine residues can undergo hydrolytic deamination to yield uracil residues (Lindahl and Nyberg, 1974). DNA damage also occurs during normal biological processes such as DNA replication. Base mismatches occur when DNA polymerases fail the proofreading process. A common example is the incorporation of uracil instead of thymine opposite adenine (Gadsden et al., 1993; Goulian et al., 1980).

Cells are constantly exposed to DNA damaging agents produced by endogenous and exogenous insults. Ionizing radiation such as gamma-rays and X-rays directly generate DNA strand breaks, while ultraviolet (UV) light causes pyrimidine dimers (i.e. thymidine dimers). High-energy ionizing radiation also generates a variety of reactive oxygen

species (ROS) (Ward, 1988), which produces over one hundred different oxidative DNA lesions, such as base modifications, single- and double-strand breaks, and DNA-protein cross-links (Cadet et al., 1997). DNA damages caused by ROS seem to be inevitable, because ROS are by-products of normal cellular processes such as cellular respiration and they play important physiological roles in healthy cells (Sena and Chandel, 2012).

DNA damages can be deleterious to cells and need to be repaired. The focus of this dissertation is on oxidized DNA base damages and how these damages are repaired by the base excision repair pathway, which will be discussed in detail below.

1.2. DNA Repair Pathways

The high frequency of DNA lesions places a genetic burden on cells and cells have evolved several DNA repair pathways to ensure the faithful passage of genetic information. There are four major DNA repair mechanisms that repair DNA damages, base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER) and double-strand break repair (Dexheimer, 2013; Helleday et al., 2014).

Base excision repair, as the name implies, is the predominant pathway responsible for the repair of damaged DNA bases, including oxidized bases, alkylated bases, as well as base loss (Wallace, 2014). BER is also essential for the repair of single strand breaks (Zharkov, 2008). This pathway is accomplished by a sequence of steps, including damage

recognition, damage removal, DNA backbone incision, end processing at the gap, gap filling and nick ligation (Zharkov, 2008).

The mismatch repair pathway corrects mismatched bases like those that have been misincorporated by DNA polymerases and escaped their proofreading activity. In addition, MMR also corrects insertion and deletion loops that results from polymerase slippage during replication of repetitive DNA (Li, 2008). The MMR pathway can be also divided into multiple steps: a recognition step where mispaired bases are recognized by MutS proteins, an excision step where the error-containing strand is degraded by exonuclease I in the MutS-MutL-DNA ternary complex and a repair synthesis step where the gap is filled by the replicative DNA synthesis machinery (Fukui, 2010).

Nucleotide excision repair is responsible for the removal of bulky lesions (Costa et al., 2003). These include cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts produced by UV light. NER also takes care of bulky lesions caused by chemicals, which include N2-guanine adducts caused by benzo[a]pyrene diol epoxide (Hess et al., 1997) and intra-strand crosslinks generated by cisplatin (Furuta et al., 2002; Zamble et al., 1996). The principle of NER is similar to BER and includes damage recognition, DNA excision, DNA resynthesis and ligation. However, NER is much more complicated, and requires some thirty different proteins in humans (Shuck et al., 2008).

Lastly, double-strand break repair takes care of one of the most deleterious types of DNA damages, the DNA double-strand breaks. There are two main mechanisms for double-strand break repair. Homologous recombination (HR) is largely an error-free

mechanism where the lost sequence is copied from its sister chromatid (Li and Heyer, 2008), while non-homologous end-joining (NHEJ) is normally error-prone with information lost at the broken DNA ends (Lieber, 2010).

Figure 1.1 summarizes the DNA damaging sources, DNA damages, and functions of the four DNA repair pathways. BER is the subject of this dissertation and will be discussed in more detail below.

1.3. Oxidative DNA Base Damages and Consequences of Base Oxidation

1.3.1. Oxidative base damages

It has been estimated that 30,000 DNA damages per cell per day are produced due to endogenous metabolic processes, of which over one third are oxidative damages (Lindahl and Barnes, 2000). ROS are the primary sources of oxidative DNA damages. ROS, including superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$), are highly reactive species that constantly attack DNA. DNA damages from ROS are inevitable, because they are generated constantly as byproducts of normal cellular metabolic processes and biochemical reactions (Sena and Chandel, 2012). DNA bases are vulnerable targets for oxidation, which result in a variety of oxidative base adducts.

7,8-Dihydro-8-oxoadenine (8-oxoA) (Van Hemmen and Bleichrodt, 1971), 2-hydroxyadenine (2-OHA) (Olinski et al., 1992), 4,6-diamino-5-formamidopyrimidine (Fapy-A) (Dizdaroglu et al., 1991; Olinski et al., 1992) are three examples of free radical-

damaged adenine bases. The eighth position on the imidazole ring of guanine is very reactive and readily oxidized (Boiteux et al., 1992), leading to the formation of highly mutagenic 7,8-dihydro-8-oxoguanine (8-oxoG) (Grollman and Moriya, 1993). The ring fragmentation product of guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G), is also a common lesion caused by ionizing radiation (Boiteux et al., 1992). Moreover, 5,6-dihydro-5,6-dihydroxythymine (thymine glycol) is the most common thymine lesion found after treatment by oxidizing agents such as hydrogen peroxide and ionizing radiation, and is also the most commonly studied thymine lesion (Breimer and Lindahl, 1985; Frenkel et al., 1981). Lastly, cytosine glycol is the major product of hydroxyl radical attack on cytosine (Tremblay et al., 1999). Cytosine glycol is highly unstable and spontaneously deaminates to form uracil glycol or dehydrates to form 5-hydroxycytosine (5-OHC). Uracil glycol can also dehydrate to form 5-hydroxyuracil (5-OHU) (Douki et al., 1996; Wagner et al., 1992). Uracil glycol, 5-OHC and 5-OHU are three stable forms of cytosine oxidation products in cells.

Several dozen kinds of oxidative base lesions have been identified, including damages to all four bases (Cooke et al., 2003). Figure 1.2 shows the structures of some common oxidatively damaged bases that are investigated in this dissertation (Cooke et al., 2003).

1.3.2. Biological consequences of base oxidation

Numerous studies have shown that damaged bases cause biological consequences, some more severe than others (Wallace, 2002). Base lesions can be mutagenic or lethal.

For example, thymine glycol disturbs the base alignment at its 5' and blocks DNA polymerases (Greenberg and Matray, 1997; Ide et al., 1985). Fapy-A and Fapy-G can also block DNA polymerases (Graziewicz et al., 2000; O'Connor et al., 1988). Since stopping progression of a replicative DNA polymerase can be catastrophic to a cell, these lesions are potentially lethal. DNA polymerases do bypass some base lesions, and depending on whether the cognate base is incorporated, the base lesion can be either benign or mutagenic. For instance, the cytosine lesions uracil glycol, 5-OHC and 5-OHU are readily bypassed by DNA polymerases. However, *in vitro* polymerase incorporation studies revealed that 5-OHC pairs correctly with guanine as well as incorrectly with adenine, while uracil glycol and 5-OHU always pair with adenine, thus these three lesions are potentially premutagenic (Purmal et al., 1994; Purmal et al., 1998). These *in vitro* observations predicted the results *in vivo*. It was later shown that 5-hydroxyuracil and uracil glycol induce an extremely high frequency of C to T transition mutations while the frequency of C to T transitions for 5-OHC was much lower but significant (Kreutzer and Essigmann, 1998).

1.3.3. 8-oxoG and its further oxidation products Gh and Sp

8-oxoG is the certainly the most well-studied oxidized base lesion if not the most abundant lesion in cells. In fact, 8-oxoG is used as a biomarker to evaluate cellular oxidative stress. It is estimated that the steady-state level of endogenous 8-oxoG is around one lesion per 10^5–10^6 DNA bases (Escodd, 2002), and the level increases under conditions of oxidative stress (Malins and Gunselman, 1994). One of the reasons why 8-

oxoG is among the most abundant base lesion in cells is that guanine has the lowest redox potential of the four natural bases. 8-oxoG can mispair with adenine, and is thus is a premutagenic lesion (Grollman and Moriya, 1993). Chemical oxidation of 8-oxoG *in intro* results in the formation of multiple products including guanidinohydantoin (Gh) that can be further oxidized to spiroiminodihydantoin (Sp) diastereomers (*R* and *S*) (Luo et al., 2001; Luo et al., 2000). The hydantoins (Gh and Sp) have been shown to mispair with adenine and guanine (Duarte et al., 1999; Kornyushyna et al., 2002), and they are also capable of blocking DNA polymerases (Aller et al., 2010). Whether Gh and Sp exist in cells is still under debate. However, when guanine is oxidized to 8-oxoG, the later has even lower redox potential than guanine, thus further oxidation of 8-oxoG seems to be an inevitable consequence.

1.4. Base Excision Repair (BER)

1.4.1. Base excision repair overview

BER is the predominant pathway to repair DNA single-stranded breaks and base damages, including oxidative base damage, alkylation, deamination and sites of base loss. This repair mechanism is well conserved throughout evolution from bacteria to humans and I will focus on the human enzymes. BER is accomplished by series of consecutive enzymatic reactions (Figure 1.3). The first enzyme in BER is a DNA glycosylase, which searches for, recognizes and removes damaged bases by catalyzing the hydrolysis of the N-glycosidic (also known as glycosylic, hence glycosylase) bond of the damaged

deoxyribonucleoside. This action of glycosylases converts the damaged base to an abasic site (also known as apurinic/aprymidinic site or AP site), which is a substrate for AP endonucleases such as APE1. AP endonucleases hydrolyze the phosphodiester bond immediately 5' to the AP site and create a strand break. Some DNA glycosylases (bifunctional DNA glycosylases) also possess a lyase activity that cleaves the abasic site generated by the glycosylase activity.

Although both AP endonucleases and the lyase activity of bifunctional glycosylases cleave AP sites, they generate different products at the 5' and 3' ends of the gap that need to be processed by different enzymatic activities before DNA synthesis and ligation can happen. First, AP endonucleases produce 3' hydroxyl termini that are substrates for DNA polymerases but the 5' ends harbor the fragment of the deoxyribonucleotide that must be trimmed before repair can be completed (Doetsch and Cunningham, 1990). The hanging 5' dRP (2-deoxyribo-5'-phosphate) is excised primarily by the dRP lyase activity of DNA polymerase β to recover the ligation-suitable 5'-end (Allinson et al., 2001; Matsumoto and Kim, 1995; Prasad et al., 1998). On the other hand, the lyase activity of bifunctional glycosylases generates unmodified 5'-ends and polymerase blocking 3'-ends. Some glycosylases catalyze beta-elimination that produces 3'-phospho-α,β-unsaturated aldehyde (3'-PUA), which is efficiently removed by AP endonuclease APE1 (Izumi et al., 2000; Suh et al., 1997). Other glycosylases (such as NEIL2 and NEIL3) perform beta and delta elimination steps that generate a 3'-phosphate, the removal of which is independent of APE1 but involves the 3' phosphatase activity of polynucleotide kinase (PNK) (Das et al., 2006; Wiederhold et al., 2004).

After cleaning either the 5'- or the 3'-end, the one nucleotide cap is filled by a DNA polymerase. BER branches to short-patch BER (SP-BER) and long-patch BER (LP-BER), depending on the size of newly synthesized DNA, and the two branches use different sets of enzymes. In short-patch BER, a single nucleotide is incorporated by DNA polymerase β, and the nick is sealed by a DNA LigaseIIIα/XRCCI (XRCC1, X-ray repair complementing defective repair in Chinese hamster cells 1). The long-patch BER utilizes either Pol β or the replicative DNA polymerases (polymerase δ and ε) and their accessory proteins (PCNA, RFC, RPA, *etc.*) to accomplish DNA strand displacement synthesis (Robertson et al., 2009). The processivity factor PCNA is required for DNA synthesis in LP-BER (Frosina et al., 1996; Matsumoto et al., 1994) presumably to stimulate the activities of polymerases and FEN1 (Klungland and Lindahl, 1997). Although the strand displacement synthesis activity of Pol β is weak, it can be stimulated by other BER proteins (Harrigan et al., 2003; Klungland and Lindahl, 1997; Prasad et al., 2001). The newly synthesized DNA displaces a stretch of the parental DNA (2–20 nucleotides (Sung and Mosbaugh, 2003)) into a flap DNA structure, which is then processed by a flap endonuclease FEN1 (Klungland and Lindahl, 1997). FEN1 is absolutely required for successful LP-BER but not SP-BER (Kim et al., 1998). Although it is still controversial, the nick-sealing step is catalyzed by DNA ligase I in LP-BER and by LigIIIα/XRCC1 in SP-BER (Robertson et al., 2009).

1.4.2. Base excision repair complex

While each enzyme in BER catalyzes an individual step, BER reconstitution experiments *in vitro* suggest that repair occurs by a handoff mechanism (Prasad et al., 2011). Some glycosylases such as OGG1 remain bound to the generated AP site product until displaced by APE1 (Sidorenko et al., 2007, 2008). Results from functional protein-protein interaction studies suggest that BER likely functions as a repair complex (or BERosome) in cells (Das et al., 2006; Hegde et al., 2008a; Parlanti et al., 2007; Vidal et al., 2001). XRCC1 plays a coordinating role in BER by its interaction with glycosylases, APE1, Polβ, and ligases (Das et al., 2006; Dianova et al., 2004; Marsin et al., 2003; Sano et al., 2004; Vidal et al., 2001). In fact, immunoprecipitates of XRCC1 alone are able to completely repair AP sites (Hanssen-Bauer et al., 2011). However, as many of these interactions are possibly transient and damage search by a BERosome is unlikely, these repair complexes may only occur transiently during certain circumstances.

1.5. Biological Function of BER

Besides the role of DNA repair in cancer prevention, non-conventional roles of BER have emerged. These include the functions of BER in telomere homeostasis, gene regulation, active demethylation, the immune system, aging, and neurodegenerative diseases (reviewed in (Wallace, 2014)). Studies in this dissertation are focused on the functions of BER in telomere homeostasis and gene regulation, which are discussed in the next sections.

1.5.1. BER in telomere homeostasis

Telomeres are DNA-protein complexes at the ends of chromosomes. By capping the chromosome ends, telomeres prevent chromosome degradation, undesired fusion of these ends and also improper activation of DNA damage response (de Lange, 2005, 2009; Hackett et al., 2001). Due to the inability of the replication machinery to replicate the lagging strand DNA at the very distal end (the 'end replication problem'), telomeres shorten as cells progress through each cell cycle (Webb et al., 2013). Cells counteract telomere shortening by a special reverse transcriptase called telomerase, which adds DNA repeats to the 3' end using its self-contained RNA template. Telomerase was first identified in *Tetrahymena* extracts (Greider and Blackburn, 1985), and it was later discovered that human cells use similar mechanism (Hiyama et al., 1996). Since somatic cells normally lack telomerase activity, they become senescent after a certain number of passages when their telomeres pass a critical length limit. On the other hand, telomeres in immortal cells (i.e. stem cells and cancer cells) are maintained by telomerase, which allows these cells to continue to proliferate (Kim et al., 1994; Mocellin et al., 2013).

Protection of telomeres is accomplished by the shelterin complex, a protein complex that allows cells to distinguish chromosome ends from sites of DNA double strand breaks (de Lange, 2002, 2010). Three shelterin proteins, TRF1, TRF2 and POT1, directly recognize TTAGGG telomere repeats and they are interconnected by three additional shelterin proteins, TIN2, TPP1, and RAP1 (de Lange, 2005). All of the shelterin proteins have been shown to function in telomere protection and/or maintenance (de Lange, 2010;

Griffith et al., 1998; Kibe et al., 2010; Loayza and De Lange, 2003; Sfeir and de Lange, 2012; van Steensel et al., 1998; Ye and de Lange, 2004; Ye et al., 2004). TRF1 and TRF2 are scaffolding proteins that recruit a number of other proteins, including POT1, TPP1, TIN2, RAP1, NHEJ protein Ku (Chen et al., 2008; de Lange, 2002) and BER enzymes polymerase β and FEN1 (Fotiadou et al., 2004; Muftuoglu et al., 2006). Both TRF1 and TRF2 bind to double-stranded telomere repeat DNA with exquisite specificity (Broccoli et al., 1997), while POT1 specifically binds to single-stranded telomere DNA (Baumann and Cech, 2001; Christiansen et al., 1973). While human telomere DNA can form four-stranded G-quadruplex DNA structures (discussed in detail below), the POT1 protein unfolds quadruplex DNA structures (Wang et al., 2011). Together with TPP1, POT regulates the structural dynamics of the telomere 3'tail (Hwang et al., 2012).

Telomere DNA consists of kilobases of 5'-TTAGGG-3' repeats, most of which are double-stranded DNA, with about 150 bp of a single-stranded overhang at the distal 3' end of chromosomes (McElligott and Wellinger, 1997). The G-rich telomere DNA sequence is more susceptible to oxidation than random non-telomeric DNA sequences *in vitro* by UVA radiation (Oikawa et al., 2001) and hydrogen peroxide (Oikawa and Kawanishi, 1999), and the telomere regions harbor more base damages than other regions of the genome upon oxidative treatment to cells (O'Callaghan et al., 2011; Vallabhaneni et al., 2013; von Zglinicki, 2002; Wang et al., 2010). Oxidizing and alkylating agents also specifically induce single-strand breaks in telomeric DNA (Petersen et al., 1998) and provoke erosion of the telomeric 3' single-stranded tail (Stewart et al., 2003). Although the exact mechanism of oxidative stress-induced telomere erosion is unknown, these data

indicate that telomeres may require extra care from base excision repair. Base damages in telomeres have to be repaired to ensure the proper function of telomeres, because they may hinder telomerase activity and disrupt the shelterin complex. 8-oxoG present at selected positions in the telomere sequence has been shown to block telomerase activity (Szalai et al., 2002), while 8-oxoG and abasic sites in telomere DNA disrupt the binding of TRF1 and TRF2 (Opresko et al., 2005).

Both the length and integrity of telomeres have to be carefully maintained. Shortening and loss of function of telomeres lead to telomere-mediated cell senescence and premature aging syndromes in humans (Armanios, 2009; Baird et al., 2003). On the other hand, telomere lengthening due to overactive telomerase has been linked to cancer (Artandi et al., 2002; Kim et al., 1994). Also, the exact TTAGGG telomeric sequence is critical for proper formation and function of telomeres, presumably because of the stringent sequence specificity of the TRF proteins (Hanish et al., 1994). Thus repair of mutagenic lesions, such as 8-oxoG, before they are fixed and accumulate in telomere DNA is necessary. In addition, base damages such as 8-oxoG and abasic sites in telomere DNA may reduce the binding of TRF1 and TRF2 (Opresko et al., 2005).

An increasing number of studies have suggested that BER may be actively involved in telomere homeostasis through repair. DNA polymerase β interacts with TRF2 and induces telomere dysfunction when ectopically expressed in a murine mammary cell line (Fotiadou et al., 2004). TRF2 also interacts with FEN1, and TRF2 stimulates the activities of both Pol β and FEN1 (Muftuoglu et al., 2006). More recently, TRF1, TRF2

and POT1 were found to stimulate the individual steps of LP-BER, including APE1, FEN1, Ligase I as well as the complete LP-BER (Miller et al., 2012). In addition, NEIL1 and the mouse Neil3 DNA glycosylases were found to remove oxidative damages such as Tg and Gh from quadruplex DNA structures formed by human telomere DNA, while OGG1 and NTH1 are not able to remove their preferred lesions from quadruplex DNA (Zhou et al., 2013). Interestingly, the Neil3 glycosylase shows a marked preference for removing damages from the telomere sequence compared to a random sequence (Zhou et al., 2013).

Studies in cells deficient in BER enzymes suggest that BER is required for maintenance of healthy telomeres. MEFs from Nthl1 nullizygous mice were found to harbor a higher level of lesions recognized by Nthl1 in telomeres compared to a non-telomeric locus, and the repair of oxidative damages in telomeres in these cells was slow (Vallabhaneni et al., 2013). Nthl1$^{-/-}$ bone marrow cells also suffer telomere attrition and increased recombination, DNA damage foci and fragile telomere sites when cultured in the present of 20% oxygen. Interestingly, telomerase deficiency exacerbates telomere shortening in Nthl1 deficient mouse cells, which suggests that base excision repair cooperates with telomerase to maintain telomere integrity (Vallabhaneni et al., 2013). In Ogg1-deficient mouse cells, both telomere lengthening and shortening were observed, depending on the oxygen tension provided during cell culture (Wang et al., 2010). In addition, Ogg1 deletion in yeast cells leads to telomere lengthening which may be a result of attenuated binding of telomere proteins to telomere DNA (Lu and Liu, 2010). More recently, Ung deficiency in primary mouse hematopoietic cells led to increased uracil

levels that resulted in abnormal telomere lengthening (Vallabhaneni et al., 2015). The frequency of sister chromatid exchanges and fragile telomeres in these cells was significantly increased in the absence of telomerase as well. The telomere lengthening appears to be a result of better access of telomerase to the uracil-containing telomere 3' tail, since binding of POT1 to the uracil-containing single-stranded telomere DNA was attenuated but the extension activity of telomerase was not affected (Vallabhaneni et al., 2015). Besides glycosylases, APE1 deletion causes telomere dysfunction and segregation defects in immortalized cells, which concurs with dissociation of TRF2 from the telomeres and these effects are independent of the telomerase activity (Madlener et al., 2013). The dissociation of TRF2 in APE1-deficient cells agrees with a previous *in vitro* study showing that abasic sites inhibit binding of TRF1 and TRF2 to telomere DNA (Opresko et al., 2005).

1.5.2. BER in gene regulation

In recent years, BER enzymes have been implicated in the regulation of gene transcription (reviewed in (Li et al., 2013) and (Wallace, 2014)). ROS have been proposed to act as second messengers in signal transduction during hypoxia, a stimulus for angiogenesis and vascular remodeling (Chandel et al., 1998). The Gillespie group found hypoxia-induced clusters of oxidized bases in a *VEGF* promoter sequence that harbored the AP-1 and HIF-1 response elements (HIF-1, hypoxia induced factor 1) (Grishko et al., 2001). Later, they found that oxidative modifications of the hypoxia response elements (HREs) coincided with the onset of mRNA accumulation of hypoxia-

inducible genes, including the *VEGF* gene (Gillespie et al., 2010). Interestingly, when the oxidation-targeted guanine was replaced with an abasic site, they observed increased incorporation of HIF-1 and the BER enzyme/transcriptional coactivator, APE1/Ref-1 (Ziel et al., 2005). These data suggested a ROS-dependent, nucleotide modification-specific mechanism of gene regulation. Moreover, Clark *et al.* showed that G4-forming sequences are targets of oxidation in hypoxia-induced signaling, after which the BER enzymes, OGG1 and APE1, are recruited to these damaged G4 sequences presumably to remove damages and create DNA strand breaks (Clark et al., 2012). Based on these observations, they hypothesized a model of controlled DNA damage and repair to regulate gene expression, by altering transcription factor binding and increasing DNA/chromatin flexibility (Gillespie et al., 2010; Ziel et al., 2005).

Besides APE1, OGG1 glycosylase has been shown to function in gene transcription regulation by altering the secondary structure of local DNA and chromosomal arrangement. Perillo *et al.* showed that OGG1 was recruited to *bcl2* promoter and enhancer regions during estrogen-induced expression of the *bcl2* gene. It turns out that the H_2O_2 generated from the reaction of LSD1-mediated H3K9me2 demethylation oxidizes the nearby guanine bases to 8-oxoG (LSD1, lysine-specific demethylase 1) (Perillo et al., 2008). The subsequent recruitment of OGG1 to the 8-oxoG sites, together with TOPO IIβ, generates nicks in DNA and chromosomal changes that lead to induced expression of the *bcl2* gene (Perillo et al., 2008). Similarly, a BER-coupled LSD1-dependent mechanism has been shown in MYC-induced expression of the *Ncl* gene,

where OGG1 and APE1 (instead of TopIIIα) are required (Amente et al., 2010a; Amente et al., 2010b).

Taken together, BER may function in gene regulation through repair of the targeted DNA base oxidation, either by or a DNA sequence-specific mechanism or by the LSD1-dependent mechanism. Nevertheless, more studies have to be done to show that the BER-coupled gene regulation is a general mechanism that cells utilize.

1.6. DNA Glycosylases

As the first enzyme in base excision repair pathway, a DNA glycosylase plays a critical role in the successful repair of damaged DNA bases. DNA glycosylases are highly conserved from bacteria to human, indicating their importance in cell survival. Early research identified uracil DNA glycosylase (Udg), endonuclease III (Nth) and formamidopyrimidine DNA glycosylase (Fpg) glycosylase in *E. coli*. Eukaryotic orthologs were subsequently characterized, including human UDG, NTH1 (or NTHL1), NEIL1, NEIL2 and NEIL3. Based on their substrate specificity, tertiary structure, active site characteristics and the AP lyase reaction they perform, these glycosylases can be categorized into three families and named after the Udg, Nth and the Fpg glycosylases (McCullough et al., 1999; Zharkov, 2008). There is another group of glycosylases that specifically remove alkylated DNA bases, which includes human AAG (alkyladenine glycosylase, also known as N-methylpurine-DNA glycosylase or MPG), *E.coli* AlkA (3-methyladenine DNA II) and Tag (3-methyladenine DNA glycosylase I).

1.6.1. The Udg family

The *Homosapiens* UDG super family includes UDG, thymine DNA glycosylase (TDG), and Single-Strand-Selective Monofunctional Uracil-DNA Glycosylase (SMUG1). Since they lack of lyase activity, all three enzymes are monofunctional glycosylases. Uracil is mutagenic yet abundant in the human genome due to cytosine deamination and misincorporation of dUMP by DNA polymerases. Uracil DNA glycosylase was the first DNA glycosylase discovered in *E. coli* after searching for an enzymatic activity that would recognize uracil in a G-U base pair (Lindahl, 1974). Subsequently, similar UDG enzymes were identified in other bacteria, yeast, plants, and mammalian cells (Krokan et al., 2002). The enzyme is highly specific for uracil in single-stranded and double-stranded DNA due to its tightly fit active site for uracil, which allows UDG to distinguish U from the structurally similar normal pyrimidine bases in DNA such as C or T (Slupphaug et al., 1996). Crystal structures and enzymatic studies of UDG have illustrated a 'flipped-out' mechanism for how glycosylases remove damages from double helical DNA (Mol et al., 1995a; Mol et al., 1995b; Slupphaug et al., 1996), which set a milestone for research of base recognition and excision.

The human genome encodes three additional glycosylases (TDG, SMUG1 and MBD4) that are able to remove uracil from DNA (Krokan et al., 2001). SMUG1 was initially described as being specific for uracil in single-stranded DNA, as implied by its name (Haushalter et al., 1999). However, it was later found that double-stranded substrates were the preferred substrate of SMUG1 providing that APE endonuclease was present to

stimulate the enzyme's turnover (Nilsen et al., 2001). SMUG1 also excises 5-hydroxyuracil, 5-hydroxymethyluracil (5-hmU), and 5-formyluracil (Masaoka et al., 2003; Wibley et al., 2003), which suggests that the enzyme may function in active demethylation when coupled to active deamination and oxidation of 5-methyl-cytosine. TDG excises thymine and uracil from T:G and U:G mismatch (Hardeland et al., 2003). Since TDG is also able to remove oxidation and deamination derivatives of 5-methylcytosine (e.g. 5-hydroxymethylcytosine and 5-hydroxymethyluracil), a role for TDG in active demethylation has been proposed (Wu and Zhang, 2010). Recent studies show that indeed TDG is directly involved in active demethylation in an AID/Gadd45a-dependent (Cortellino et al., 2011) or TET-dependent mechanism (Hu et al., 2014; Maiti and Drohat, 2011; Zhang et al., 2012).

1.6.2. The Nth family

The Nth family of glycosylases includes Nth (NTHL1), MutY (MUTYH), OGG1 *E.coli* AlkA, and MBD4 in humans (Zharkov, 2008). While all glycosylases of the Udg family are monofunctional, the NTHL1 and OGG1 of the Nth family are bifunctional (Cadet et al., 2000). NTHL1 and OGG1 utilize an internal Lys residue to catalyze the β-elimination reaction at the AP site. Crystal structures of the family members reveal that these enzymes contain two well defined domains. One domain is the HhH/GPD domain, a helix–hairpin–helix motif followed by a Gly and Pro rich loop with an absolutely conserved Asp. The other domain is the Iron-Sulfur (Fe-S) cluster. Some of the family members have lost the Fe-S cluster but they always keep the HhH/GPD domain (Denver

et al., 2003). The HhH/GPD domain is a versatile structure that allows glycosylases of the Nth superfamily to cover nearly the full spectrum of lesions (discussed below).

1.6.3. The Fpg/Nei family

The Fpg/Nei family includes Fpg (MutM in human) and Nei (Nei like glycosylases NEIL1, NEIL2, and NEIL3 in human) (Hegde et al., 2008a; Prakash et al., 2012). This family of glycosylases utilizes an N-terminal Pro as the nucleophile to initiate base excision and perform a β,δ elimination (McCullough et al., 1999; Wiederhold et al., 2004). There is one exception. NEIL3 utilizes a N-terminal valine instead of proline as a nucleophile and primarily catalyzes a β elimination reaction (Liu et al., 2010). The crystal structures of the Fpg/Nei family glycosylases reveal that they consist of two domains connected by a flexible linker (Zharkov et al., 2003). The N-terminal domain contains mostly beta sheets and the C-terminal domain includes two DNA-binding motifs: a helix-two-turn-helix (H2TH) motif and a zinc finger motif (Prakash et al., 2012). While NEIL2 and NEIL3 have all the conserved domains (Liu et al., 2013b; Prakash et al., 2013), NEIL1 lacks the cysteine residues that coordinate Zn^{2+} in the zinc finger. Instead, the NEIL1 presents a "zinc-less finger", a motif that is structurally indistinguishable from a zinc finger but does not contain the Zn^{2+} (Doublie et al., 2004). Moreover, NEIL3 contains an atypical long, disordered C-terminus that is about the same size of the N-terminus glycosylase domain. Neither the structure nor the function of this C-terminal domain is known (Liu et al., 2010).

1.6.4. AAG glycosylase

Human alkyladenine glycosylase AAG and *E. coli* 3-methyladenine glycosylase AlkA recognize and excise a variety of alkylated bases from DNA. The two enzymes remove N-alkylpurines, including 3-methylA, 3-methylG, 7-methylA and 7-methylG (Asaeda et al., 2000; Sedgwick et al., 2007; Wyatt et al., 1999). AAG also excises 1,N^6-ethenoadenine (Saparbaev et al., 1995) and deamination products of A and G (hypoxanthine and xanthine, respectively) (Saparbaev and Laval, 1994). The *E. coli* genome also encodes the Tag glycosylase that specifically removes 3-methylA (Metz et al., 2007; Sakumi et al., 1986) and much less efficiently 3-methylG (Bjelland et al., 1993).

The alkylated base-specific glycosylases are monofunctional enzymes (Metz et al., 2007; Sedgwick et al., 2007). Structural studies have revealed that 3-methylA glycosylases flip the alkylated base out of the DNA helix into the active site cleft and utilize an activated water molecule to initiate the cleavage of the glycosylic bond (Labahn et al., 1996; Yamagata et al., 1996). In addition, the three-dimensional structures of the AlkA reveal that it contains a Helix-hairpin-Helix motif, which puts AlkA in the Nth/HhH superfamily. However, the overall structure of human AAG is very different from *E.coli* AlkA, and lacks the HhH motif (Wyatt et al., 1999).

1.6.5. Substrate specificity of oxidative damage-specific DNA glycosylases

In this dissertation, the five DNA glycosylases that are specific for removing oxidized DNA bases in human cells, namely OGG1, NTH1, NEIL1, NEIL2, and NEIL3 (De Bont and van Larebeke, 2004; Fromme and Verdine, 2004; Grin and Zharkov, 2011; Hegde et

al., 2008a) were studied. Although glycosylases have overlapping substrate specificities, each glycosylase has its own substrate spectrum, narrow or wide. OGG1 and NTH1 primarily remove oxidized purines and pyrimidines from duplex DNA, respectively (Asagoshi et al., 2000; Zharkov et al., 2000). OGG1 is a very specific enzyme that removes 8-oxoG and Fapy-G opposite C in duplex DNA (Bjoras et al., 1997; David et al., 2007; van der Kemp et al., 1996). NTH1 has a broader lesion specificity that includes Tg, DHU, 5-OHC, 5OHU, FapyG and hydantoins, and it also only acts on duplex DNA (Aspinwall et al., 1997; Dizdaroglu et al., 1999; Hazra et al., 2001). Both NTH1 and OGG1 are bifunctional enzymes, although the lyase activity of OGG1 is weak (Aspinwall et al., 1997; Girard et al., 1997; van der Kemp et al., 1996).

The NEIL glycosylases have broad substrate specificities, in terms of both lesion type and DNA conformation. NEIL1 removes pyrimidine lesions such as Tg, 5-OHU, and 5-OHC (Bandaru et al., 2002), and the enzyme also excises purine lesions such as FapyA, FapyG, Sp and Gh (Krishnamurthy et al., 2008). Although NEIL1 prefers damages in duplex DNA, it also removes lesions from single-stranded and bubble DNA structures (Dou et al., 2003). Both NEIL2 and NEIL3 prefer oxidized pyrimidines and some purine damages in single-stranded DNA (Dou et al., 2003; Liu et al., 2010); however, they do have weaker activity on duplex DNA. The lesions that NEIL2 and NEIL3 remove are similar to those of NEIL1. We have previously shown that mouse Neil3 and human NEIL1 remove lesions from G-quadruplex DNA (Zhou et al., 2013). Although, 8-oxoG is not a substrate for any of the NEIL glycosylases, its further oxidation products, Gh and Sp, are the best substrates for all three enzymes (Hailer et al., 2005; Krishnamurthy et al.,

2008; Liu et al., 2010; Liu et al., 2013a). The three NEIL glycosylases are all bifunctional glycosylases, although the AP lyase of NEIL3 is very weak (Krokeide et al., 2013; Liu et al., 2010).

1.6.6. Cellular function of DNA glycosylases that are specific for oxidative damages

OGG1 and NTH1 are both housekeeping enzymes, the expression of which is ubiquitous (Karahalil et al., 2002; Winter et al., 2003). The *ogg1* knockout mice are healthy until at least 18 months of age, but deletion of this gene leads to accumulation of 8-oxoG in the genome and spontaneous development of lung adenoma/carcinomas (Sakumi et al., 2003). These results suggest that MutY may have prevented tumor development by correcting the mismatched adenine opposite 8-oxoG in the *ogg*$^{-/-}$ mice. Indeed, the *ogg1 mutY* double knockout mice exhibit increased spontaneous tumorigenesis (particularly in lung and ovary) and lymphoma (Xie et al., 2004), while the *mutY* single knockout mice do not develop tumors (Xie et al., 2004) or only develop intestinal tumors in more aged mice and when treated with an oxidizing agent (Sakamoto et al., 2007). In addition, the *nth1* knockout mice do not have overt abnormalities due to the presence of the back-up glycosylase activities of the NEIL glycosylases (Takao et al., 2002a; Takao et al., 2002b). Nevertheless, a recent study of a human NTH1 variant D239Y, which occurs in ~6.2% of the global population, shows that this single nucleotide polymorphism induces genomic instability and cellular transformation (Galick et al., 2013). Moreover, the *Neil1* knockout mice only display obesity and fatty liver phenotypes in some but not all males, which is a result of unrepaired oxidative damages

in mitochondrial DNA (Vartanian et al., 2006). However, the *nth1 neil1* double knockout mice develop pulmonary and hepatocellular tumors in a much higher incidence than either of the single knockouts (Chan et al., 2009). Remarkably, the $nth^{-/-}$ $neil1^{-/-}$ mice contain exclusively the activating GGT→GAT transition mutations in codon 12 of *K-ras* gene (Chan et al., 2009), while the $Ogg1^{-/-}$ $MutY^{-/-}$ mice harbors activating GGT→GTT transversion mutations (Xie et al., 2004). These data suggest that deficiency in Ogg1 or Nth1 alone may not cause cancer predisposition, but a coupled deficiency may do so. OGG1 and NTH1 have also been shown to function in the maintenance of healthy telomeres, as described above (Lu and Liu, 2010; Vallabhaneni et al., 2013; Wang et al., 2010).

The NEIL1 glycosylase has been shown to be expressed at highest levels during S phase (Hazra et al., 2002a), although controversial results were observed (Neurauter et al., 2012), suggesting that the expression of NEIL1 may be cell type dependent. Nevertheless, NEIL1 physically interacts with the replication machinery, including PCNA, RPA and FEN1 (Dou et al., 2008; Hegde et al., 2008b; Theriot et al., 2010). These protein-protein interactions suggest that NEIL1 may be involved in replication-associated DNA repair. A recent study shows that NEIL1 presents at the replication folk and prevents folk collapse by removing the base lesions ahead of the replication folk (Hegde et al., 2013). Since the NEIL1 interacts with the replicative polymerase as well as FEN1, it can also initiate long patch BER. On the other hand, the expression of NEIL2 is not cell cycle regulated (Hazra et al., 2002b; Neurauter et al., 2012). However, NEIL2 prefers lesions in the single-stranded regions of bubble DNA structures (Dou et al., 2003). Also, NEIL2 interacts with

RNA polymerase II and the transcriptional regulator heterogeneous nuclear ribonucleoprotein-U (hnRNP-U), and the actively transcribed regions harbor fewer base damages in cells in a NEIL2 dependent manner (Banerjee et al., 2011). These data suggest NEIL2 may function in concert with the transcription machinery (transcription-coupled BER).

NEIL3 is an atypical glycosylase with a large C-terminal domain, which contains a RanBP-like zinc finger motif, a putative nuclear localization signal, and two tandem GRF zinc fingers (Bandaru et al., 2002; Liu et al., 2010; Morland et al., 2002; Torisu et al., 2005). The NEIL3 protein has been proven to be difficult to study due to its large, disordered C-terminus that appears to be unstable during purification (Krokeide et al., 2009; Takao et al., 2009). It was only a few years ago that the enzymatic properties of the N-terminal glycosylase domain were clearly characterized (Krokeide et al., 2013; Liu et al., 2010; Liu et al., 2012). However, purification of the full length human NEIL3 is still challenging, although the mouse full length enzyme appears to be easier to study (Liu et al., 2010; Liu et al., 2012).

The cellular function of NEIL3 has been difficult to study and remains elusive due to the fact that cells are not viable either during NEIL3 knockdown or overexpression (Reis and Hermanson, 2012; Rolseth et al., 2013). NEIL3 is highly expressed during S phase through G2/M and the expression is controlled by the DREAM (DP1, RB p130, E2F4 and MuvB core) complex (Neurauter et al., 2012). The expression of NEIL3 is also tissue/cell type-specific. In mice, NEIL3 is only expressed in highly proliferating cells,

including stem cells, neuron progenitor cells in the brain, and hematopoietic cells (Regnell et al., 2012; Reis and Hermanson, 2012; Rolseth et al., 2013; Sejersted et al., 2011; Torisu et al., 2005). In adult humans, NEIL3 is only expressed in thymus and testes (Morland et al., 2002; Torisu et al., 2005). NEIL3 is also expressed in cancer cells (Hildrestrand et al., 2009; Kauffmann et al., 2008). These observations indicate that NEIL3 plays an important role in highly proliferating cell types.

1.7. G-quadruplex DNA

1.7.1. Quadruplex DNA structures

G-quadruplex DNA (G4) is a four-stranded DNA structure formed by G-rich sequences. The G4 structures consist of two or more layers of four-guanine structures called G-quartets (or G-tetrads), in which the four Gs are Hoogsteen base paired to each other (reviewed in (Burge et al., 2006)). The classic model contains three layers of G-quartets with two monovalent cations sandwiched in between, which coordinate the layers and stabilize the entire structure (Hardin et al., 1991). An intramolecular quadruplex is a quadruplex formed by one DNA strand. The DNA sequences that form intramolelucar quadruplex DNA require four G stretches (or G-runs) separated by at least one base, or $(G_{n\geq 2}N_{\geq 1})_4$ (Huppert and Balasubramanian, 2005; Todd et al., 2005). There are a variety of G-quadruplex DNA topologies, and different structures can form even with very similar DNA sequences, depending on the solutions they are folded in and 5'- and 3'end capping (Burge et al., 2006; Olsen et al., 2009). The quadruplex topologies can

be categorized in bulk into three groups based on their strand directionality and loop arrangement. In parallel quadruplex DNA, all four "strands" point in the same direction. The four G stretches in an intramolecular parallel quadruplex DNA are usually connected by double-chain-reversal loops, and thus are called a propeller-like quadruplex DNA. In antiparallel quadruplexes, the neighboring "strands" have opposite directionality that are connected by lateral (edge-wise) and diagonal loops (Burge et al., 2006). The hybrid quadruplex DNA, as the name implies, has mixed strand directionality and all the connecting loop types may be used in these structures (Ambrus et al., 2006). An intermolecular quadruplex is formed by two or more DNA strands (usually two or four). The ciliate *Tetrahymena* telomere consists of $_{d}$(T2G4) repeats, which forms both intermolecular (Sundquist and Klug, 1989) and intramolecular (Wang and Patel, 1994) quadruplex DNA *in vitro*.

It has been more than three decades since the discovery of quadruplex DNA. Structural studies have revealed that the telomere DNA can form parallel, antiparallel, and hybrid structures (reviewed in (Burge et al., 2006)). In a NMR solution structure, Wang *et al.* show that the telomere DNA forms an antiparallel basket structure in sodium solutions (Wang and Patel, 1993). The quadruplex structures formed in potassium solutions are more complicated. The telomere DNA can form both parallel and hybrid quadruplex DNA, depending on the 5' and 3' capping sequences. The crystal structure of Parkinson *et al.* shows that the A(GGGTTA)$_3$GGG sequence forms a propeller-like all parallel quadruplex DNA structure (Parkinson et al., 2002). Ambrus *et al.* show that the AAA(GGGTTA)$_3$GGGAA sequence (Tel26) forms a hybrid-type of quadruplex DNA in

K^+ solution. This NMR structure was assigned Hybrid-1 quadruplex DNA, with a double-chain-reversal loop followed by two lateral loops (Ambrus et al., 2006). Later, the same group resolved another NMR structure with the $TTA(GGGTTA)_3GGGTT$ sequence (wtTel26), which adopts another hybrid-type quadruplex DNA (Hybrid-2) in K^+ solution (Dai et al., 2007). Analysis of the structures reveals that that the 3'-capping sequence is essential for the formation of hybrid quadruplex DNA. In particular, the T8:A9:T25 triplet capping structure stabilizes the Hybrid-2 structure while the A25:T14 base pair stabilizes the Hybrid-1 structure (Ambrus et al., 2006; Dai et al., 2007).

1.7.2. Methods for studying quadruplex DNA structures

Over the decades, native gel electrophoresis and CD spectrometry have been used as routine techniques to analyze the formation of quadruplex DNA (Karsisiotis et al., 2011). The native gel electrophoresis separates single-stranded DNA and quadruplexes by their size. Because of their more compact size and globular shape, the quadruplex DNA migrates faster in a native gel than the single-stranded DNA with the same molecular weight (Oganesian et al., 2006; Zhou et al., 2013). It is important to notice that the native gel electrophoresis does not have the capability to determine if a band with higher mobility is quadruplex. Instead, it only shows researchers that a "more compact" structure is formed.

CD spectrometry is another routinely used technique in quadruplex research. The technique allows researchers to quickly get information about the DNA secondary structures, which is the strand orientation in the case of quadruplex DNA. Numerous CD

studies have been done on quadruplex DNA structures, which allow the researchers to distinguish different folding patterns of the quadruplex DNA structures based on their CD spectra. The CD spectrum of a parallel quadruplex features a 265 nm maximum and a 240 nm minimum, while the CD spectrum of the basket antiparallel quadruplex shows a 295 nm maximum and a 265 nm minimum (Balagurumoorthy and Brahmachari, 1994; Balagurumoorthy et al., 1992). Although a CD spectrum can provide information about quadruplex folding, it does not have the resolution to visualize the structure at a single-nucleotide level. For example, CD spectra are not able to distinguish the type 1 and type 2 hybrid quadruplex DNA structures, which both present a 295 nm maximum, a 270 nm shoulder and a 235 nm minimum (Karsisiotis et al., 2011).

Thermostability studies of quadruplex DNA can also be done by CD spectrometry. Melting temperature (T_m) is an indicator of the stability of quadruplex DNA, which can be determined by monitoring the signature peaks of a quadruplex DNA structure. T_m values can also provide some structural information (Bochman et al., 2012). For example, the T_m value of quadruplex DNA is usually high (above 50^oC) and some quadruplex DNAs can have a T_m value of over 80^oC (Phan et al., 2004; Tran et al., 2011). A high T_m may indicate the formation of a highly-organized structure, as the faster moving band in native gel electrophoresis does. In addition, for the same sequence, the propeller-like parallel quadruplex in K^+ solution has greater T_m values (more stable) than the antiparallel quadruplex DNA in Na^+ solution (Tran et al., 2011). Also, base damage in a G-quartet leads to a significant drop in the T_m of quadruplex DNA, which can be used to determine if the base guanine is involved in the formation of G-quartet.

While X-ray crystallography and NMR are powerful techniques to reveal high-resolution structural details of quadruplex DNA, such structures are difficult to obtain. Moreover, if a structure is not very stable, it may be impossible to grow crystals or difficult to collect NMR data. Thus, native gel electrophoresis and CD spectra are very helpful techniques to quickly obtain information about DNA secondary structures such as quadruplex DNA folding. In this dissertation, native gel electrophoresis, CD spectroscopy, and T_m measurements have all been used to study quadruplex DNA structures.

1.8. Biological Function of Quadruplex DNA *in vivo*.

Bioinformatics studies have revealed that the human genome is rich in potential quadruplex forming sequences, with about 375,000 in the human genome (Huppert and Balasubramanian, 2005; Todd et al., 2005). Since the discovery of G-quadruplex DNA, several biological roles have been proposed based on the results from biochemical assays and studies in cells, including the function of telomeric quadruplex DNA in telomere length control and promoter quadruplexes in gene transcription regulation (Lipps and Rhodes, 2009; Reis and Hermanson, 2012). G-quadruplex DNA structures formed by the telomere DNA sequence and promoter sequences are the topics of these dissertation and will be discussed in detail below. Other functions of G-quadruplex structures including blocking DNA lagging strand replication and pathogen control have been discovered (Harris and Merrick, 2015).

1.8.1. Function of telomeric quadruplex DNA

The telomere TTAGGG repeats form stable quadruplex DNA structures *in vitro*. Also, the telomeric quadruplex DNA has been shown to inhibit telomerase activity. These results led to the proposed functions of quadruplex DNA in telomere-length control and cancer suppression. Since cancer cells are usually telomerase positive in order to maintain the length of telomere and keep its pluripotency, inhibiting telomerase by stabilizing the quadruplex DNA structure at telomeres is a plausible way to inhibit cancer cell growth. Indeed, quadruplex stabilizing agents such as TMPyP4 and telomestatin do inhibit cancer cell growth as well as induce telomere defects. In fact, some quadruplex-stabilizing compounds are under clinical trials as anti-cancer drugs (Neidle and Parkinson, 2002; Rezler et al., 2002).

1.8.2. Function of quadruplex DNA formed by promoter sequences

Intriguingly, promoter regions are also enriched in quadruplex-forming sequences. It is estimated more than 40% of the genes in the human genome contain at least one quadruplex-forming sequence in their promoter regions (Huppert and Balasubramanian, 2007). The formation of quadruplex DNA at a promoter inhibits the transcription machinery such as transcription factor binding to the promoter sequence, and thus suppresses the transcriptional activity of the promoter. Quadruplex forming sequences at promoters have been shown to regulate a number of genes that are associated with cancers, including *VEGF, c-MYC, Hif1α, Kit-2, and bcl-2,* and as a result, targeting G-quadruplexes in gene promoters is emerging as a novel anticancer strategy (reviewed in

(Balasubramanian et al., 2011)). The quadruplex-forming NHEs (nuclease hypersensitive elements) of *VEGF* and c-MYC promoter sequences have been intensively studied and revealed important insights of how quadruplex DNA participates in the regulation of gene transcription.

VEGF promoter quadruplex DNA

The human vascular endothelial growth factor (VEGF) is a key regulator of new blood vessel formation (angiogenesis) and plays an important role in tumor survival, growth and metastasis (Ferrara, 2000; Folkman, 2002; Hicklin and Ellis, 2005). The cytokine VEGF binds to its receptor and promotes angiogenesis that provides oxygen and nutrition for tumor cells (Folkman, 2002).

The -85 to -50 bp G-rich sequence of the human *VEGF* promoter was shown to be critical for both basal and inducible expression. Deletion of this sequence decreases at least 90% of the basal promotor activity in multiple cell lines (Finkenzeller et al., 1997; Shi et al., 2001). The 36bp G-rich sequence contains five guanine runs and harbors multiple transcription factor binding sites, including three Sp1 and Sp3 binding sites (Finkenzeller et al., 1997). The sequence was later shown to form quadruplex DNA using CD, DMS footprinting, DNase I/S1 nuclease footprinting and DNA polymerase stoppage techniques (Guo et al., 2008; Sun et al., 2011). Interestingly, quadruplexes formed in this region involve only the 5' four successive G-runs in K^+ solution (Guo et al., 2008; Sun et al., 2011). An NMR structure of the quadruplex DNA formed by the 5' four G-runs of the G-rich sequence (VEGF-Pu22) was recently solved, which presents an all parallel,

propeller-type G-quadruplex DNA structure (Agrawal et al., 2013). The quadruplex stabilizing agents enhance the quadruplex DNA structure formed by the *VEGF* promoter sequence (Sun et al., 2005), and at the same time, they also suppress the expression of VEGF (Sun et al., 2008). These results support the idea that G-quadruplex DNA at promoter regions of genes can suppress gene transcription by attenuating binding of transcription factors. Because VEGF plays important roles in tumor growth and metastasis, G-quadruplex stabilizing agents that target the -85 to -50 bp region of *VEGF* promoter may be developed as anti-cancer drug (Chen and Yang, 2012).

c-MYC promoter quadruplex DNA

c-MYC is a multifunctional transcription factor that plays an important role in a broad range of cellular processes, including gene transcription, cell cycle progression, cell growth, differentiation, transformation, angiogenesis, and apoptosis (see reviews (Dang, 1999; Eilers and Eisenman, 2008; Oster et al., 2002)). Involved in so many games, the transcription of *c-MYC* has to be tightly controlled. Aberrant expression of this proto-oncogene is one of the hallmarks of many human cancers, including breast, colon, cervix, small-cell lung cancers, glioblastomas, and myeloid leukemias (Gabay et al., 2014; Nesbit et al., 1999; Slamon et al., 1984). It is estimated that one in seven of all the deaths caused by cancer are associated with alteration in the *c-MYC* gene or its expression (Dang, 1995).

The expression of *c-MYC* is controlled by multiple promoters (P0, P1, P2, and P3) (Gonzalez and Hurley, 2010a). The *c-MYC* promoter also contains seven nuclease

hypersensitive elements (NHEs) (Gonzalez and Hurley, 2010a), one of which, the NHEIII1 located at -142 to -115 bp upstream of the P1 promoter, controls up to 90% of the total *c-MYC* transcription (Berberich and Postel, 1995; Davis et al., 1989). It has been shown that the NHEIII1 region forms non-B form DNA structures, especially under negative superhelical stress (Ashley and Lee, 2000; Liu and Wang, 1987). It was later demonstrated that the NHEIII1 sequence forms two conformations of G-quadruplex DNA structures, but only the more stable chair form is biologically relevant (Siddiqui-Jain et al., 2002). Formation of this chair form quadruplex negatively regulates the basal transcription of the *c-MYC* gene, and G-quadruplex stabilizing agent TMPyP4 is able to further suppress the *c-MYC* transcriptional activation (Siddiqui-Jain et al., 2002). Interestingly, when treated exogenously, the Pu27 oligodeoxynucleotide selectively kills leukemia cells by suppressing the expression of *c-MYC*, which is probably a result of secondary structure formation (i.e. quadruplex) at the *c-MYC* promoter between the exogenous Pu27 oligo and the genomic NHEIII1 sequence (Sedoris et al., 2012).

The NHEIII1 sequence (Pu27) of the *c-MYC* promoter also contains five G stretches (Myc-12345). Structural studies show that the Pu27 can adopt multiple forms of quadruplex DNA (Phan et al., 2004; Siddiqui-Jain et al., 2002). Two forms of antiparallel quadruplex DNA structures determined by DMS footprinting were proposed, one of which utilizes the 1^{st}, 2^{nd}, 4^{th}, and 5^{th} G-stretches (Myc1245) and forms basket form quadruplex DNA while the other utilizes the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} G-stretches (Myc-2345) and forms chair form quadruplex DNA (Siddiqui-Jain et al., 2002). Somewhat controversial, the NMR structure analysis shows that both the Myc-1245 and the Myc-

2345 sequences form intramolecular, propeller-type, parallel-stranded G-quadruplexes (Phan et al., 2004). However, it is consistent that quadruplex DNA formed by the Myc-2345 sequence is more thermodynamically stable due to the smaller size of its central loop. Moreover, DNA base damages can modulate the structure of the G-quadruplex formed by the NHEIII1 sequence. Beckett *et al.* found that the Pu27 sequence favored the formation of quadruplex DNA over duplex DNA when an abasic site or an 8-oxoG was introduced into the sequence. They also found that the quadruplex structure formed was dependent on the location of the lesion placed, which follows the general rule that the lesion is extruded to a loop region to favor the thermodynamic stability of the quadruplex (Beckett et al., 2012).

1.9. Quadruplex Oxidation

Due to its G-rich nature, the quadruplex-forming sequences are prone to oxidation. It has been shown that the telomere sequence (TTAGGG)n is more susceptible to oxidation in *vitro* (Oikawa and Kawanishi, 1999; Oikawa et al., 2001) and in *vivo* (O'Callaghan et al., 2011; Vallabhaneni et al., 2013; von Zglinicki, 2002; Wang et al., 2010). The quadruplex DNA formed by these G-rich regions is not an exception. In fact, it has been shown that the telomeric quadruplex DNA is about 2-fold more prone to oxidation than the double-stranded telomere DNA (Szalai et al., 2002). Formation of quadruplex DNA also alters the reactivity of guanine and the products of guanine oxidation compared to duplex DNA. The one-electron oxidants damage the 5'-G in G-quadruplexes leading to

Sp and 2,2,4-triamino-2H-oxazol-5-one (Z) as major products as well as 8-oxoG and Gh as minor products, while oxidation of the duplex DNA produces Gh as the major product at 5'- and middle-Gs of GGG runs (Fleming and Burrows, 2013).

Guanine oxidation in quadruplexes may lead to structural changes of the quadruplex folding. Since 8-oxoG is one of the most abundant lesions in cells, the structure and thermodynamic impacts of substitution of 8-oxoG for guanine have been investigated. Szalai *et al.* show that replacement of G by 8-oxoG in telomeric DNA may affect the formation of intramolecular G quadruplexes, depending on the position of substitution. When guanine at the 5'-position of a GGG triplet was substituted with 8-oxoG, G quadruplex formation was observed, while substitution of 8-oxoG in the middle of a GGG triplet produced multiple structures, indicating a compromised quadruplex DNA structure (Szalai et al., 2002). Similarly, Vorlickova *et al.* show that substitution of guanines by 8-oxoguanine at any position did not hinder the formation of intramolecular, antiparallel quadruplexes in NaCl solution. However, the thermostability of all modified quadruplexes was reduced, with the middle G substitution being the most dramatic (Vorlickova et al., 2012). Similar results were observed when the guanines in telomeric quadruplexes were individually substituted with abasic sites, where all abasic site-substituted telomere DNA formed quadruplexes with reduced stability, particularly when abasic sites were substituted for the guanosines involved in the formation of the central G-tetrad (middle G of a GGG triplet) (Virgilio et al., 2012). While substitution of the bases in the loop region does not affect the formation of intramolecular quadruplex DNA in NaCl solution, a recent study showed that substitution of adenines for abasic sites in a

loop promotes the transition of a hybrid quadruplex to a parallel quadruplex in K^+ solution, and the substitution also stabilizes the parallel quadruplex DNA structure (Babinsky et al., 2014). These studies lead to a notable trend: it is not the type of the lesion but the position of the modification that determines the impact of base oxidation on the conformation and stability of the quadruplex DNA.

1.10. Overview and Significance of the Studies Presented in This Dissertation

While the activity of DNA glycosylases on duplex and single-stranded DNA is wildly studied and well understood, how glycosylases remove lesions from G-quadruplex DNA remains unknown. Results from this dissertation fill this gap.

In Chapter II, telomere G-quadruplex DNA structures formed in Na^+ solution and containing damaged DNA bases were studied as well as the removal of damages from these structures by DNA glycosylases. We show, for the first time, that DNA glycosylases are able to remove lesions from quadruplex DNA. These results extended our understanding both of quadruplex DNA and of DNA glycosylases by showing that quadruplex DNA can be a substrate for DNA glycosylases.

Telomere quadruplex DNA structures and the G-rich telomere sequence itself are prone to oxidation. However, position effects on damaged base removal by DNA glycosylases in the quadruplex structures and telomere sequence context was unclear. Studies in Chapter II show that Neil3 DNA glycosylase removes Tg from quadruplex

DNA and also has a marked preference for Tg in the telomere sequence context in single-stranded, double-stranded and quadruplex DNA. Because base damages cause telomere dysfunction, repair of oxidative damages in telomeres is critical to prevent genome instability and cancer. Our data indicate Neil3 may play a role in telomere repair and prevent genome instability.

DNA base damages have been shown to reduce the thermostability of quadruplex DNA. However, how hydantoin lesions affect the stability and folding has not been studied. In Chapter III, folding and thermostability of telomeric quadruplex DNA that contains hydantoin lesions were examined in K^+ solution. These studies show that hydantoin lesions not only reduce the thermostability but also alter the folding of telomere quadruplex DNA in a location-dependent manner. In Chapter III, we also extended the enzymatic studies of DNA glycosylases to quadruplex DNA formed in K^+ solution. Hydantoins (Gh and Sp) are efficiently removed by NEIL1 and NEIL3, but neither enzyme is able to efficiently remove hydantoins at the most oxidation-prone position (the 5' of GGG). However, adding an extra TTAGGG repeat solved the dilemma by extruding the damage-containing repeat to a loop. These findings contribute to our current knowledge of DNA repair in telomeres.

Quadruplex DNA at the promoter regions can inhibit the transcription of downstream genes. Oxidation of quadruplex-forming sequences and recruitment of BER enzymes have been implicated in the regulation of gene transcription. However, the mechanism of how oxidation of quadruplex DNA controls gene transcription at the molecular level is

unknown. In Chapter III, structures of lesion-containing quadruplex DNA formed by promoter sequences and removal of the lesion from these structures were studied. Promoter sequences of the *c-MYC* and *VEGF* genes were used as models because of their biological importance. We demonstrate that the NEIL glycosylases are able to remove Gh from Na^+-coordinated promoter quadruplex DNA, especially those that adopt an antiparallel strand orientation. These data provide biochemical explanations for how glycosylases might be involved in gene regulation by utilizing damage-containing quadruplex DNA structures at promoters. We also proposed a model for this mechanism in Chapter III.

Quadruplex DNA-forming sequences are over-represented in promoter regions of genes, including oncogenes and genes that play critical roles in cancer. Ligands that stabilize promoter quadruplex DNA have been shown to suppress expression of oncogenes in many studies. Thus, understanding the structural dynamics of the promoter quadruplex DNA will provide new insights to transcription-related oncology. The results of this promoter quadruplex study also shine light on oxidative stress-induced gene transcription.

1.11. Figures

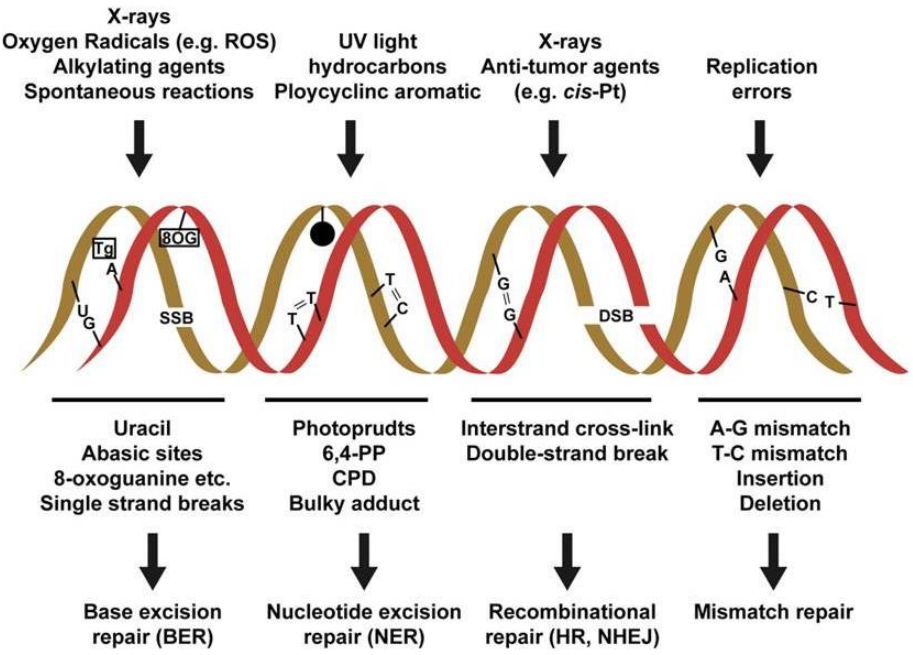

Figure 1-1. DNA repair pathways.

The diagram illustrates common DNA damaging agents, examples of common DNA lesions caused by these agents, and the responsible pathways for their repair. This figure is adapted from Boland *et al. (Boland et al., 2005).*

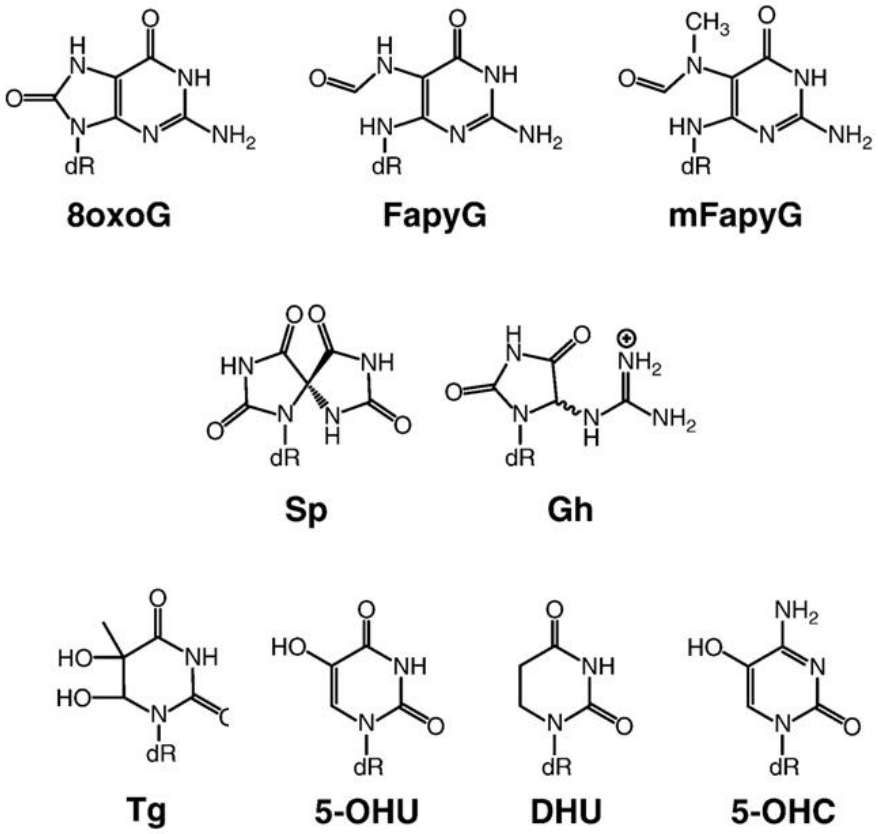

Figure 1-2. Samples of common DNA base damages that are studied in this dissertation.

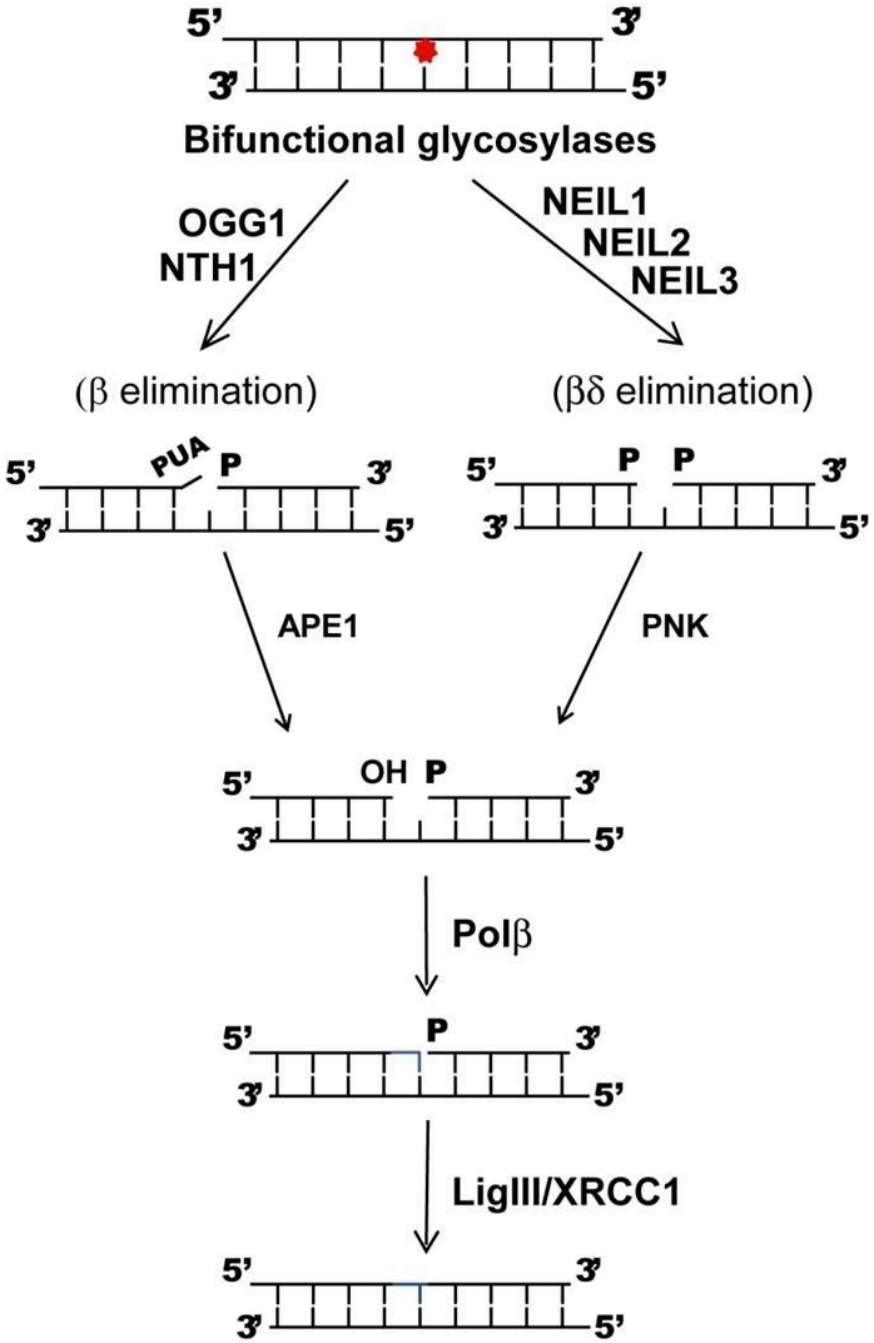

Figure 1-3. Base excision repair pathway.

This diagram illustrates the individual steps in short patch base excision repair. The base lesion is shown in red. Enzymes and the chemistry they catalyze are shown. Figure adapted from Wallace *et al.* (Wallace et al., 2012)

CHAPTER II: Neil3 AND NEIL1 DNA GLYCOSYLASES REMOVE OXIDATIVE DAMAGES FROM QUADRUPLEX DNA AND EXHIBIT PREFERENCES FOR LESIONS IN THE TELOMERIC SEQUENCE CONTEXT

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Running title: Neil3 and NEIL1 remove oxidative lesions from quadruplex DNA

Key words: Quadruplex, telomere, sequence context, base excision repair, DNA glycosylases, Neil3, NEIL1, DNA damage, DNA repair, DNA structure

Background: BER of quadruplex structures and telomere DNA has not been well studied.

Results: Neil3 and NEIL1 remove damages from quadruplex DNA and show telomeric sequence context effects.

Conclusion: Neil3 and NEIL1 may be involved in repair of damages in quadruplex DNA.

Significance: This is the first evidence of glycosylase activity on quadruplex DNA and suggests new roles for these enzymes.

2.1. SUMMARY

The telomeric DNA of vertebrates consists of d(TTAGGG)$_n$ tandem repeats, which can form quadruplex DNA structures *in vitro* and likely *in vivo*. Despite the fact that the G-rich telomeric DNA is susceptible to oxidation, few biochemical studies of base excision repair (BER) in telomeric DNA and quadruplex structures have been done. Here we show that telomeric DNA containing thymine glycol (Tg), 8-oxo-7,8-dihydroguanine (8-oxoG), guanidinohydantoin (Gh) or spiroiminodihydantoin (Sp) can form quadruplex DNA structures *in vitro*. We have tested the base excision activities of five mammalian DNA glycosylases (NEIL1, NEIL2, mNeil3, NTH1 and OGG1) on these lesion-containing quadruplex substrates and found that only mNeil3 had excision activity on Tg in quadruplex DNA and that the glycosylase exhibited a strong preference for Tg in the telomeric sequence context. While Sp and Gh in quadruplex DNA were good substrates for mNeil3 and NEIL1, none of the glycosylases had activity on quadruplex DNA containing 8-oxoG. In addition, NEIL1 but not mNeil3 showed enhanced glycosylase activity on Gh in the telomeric sequence context. These data suggest that one role for Neil3 and NEIL1 is to repair DNA base damages in telomeres *in vivo* and that Neil3 and Neil1 may function in quadruplex-mediated cellular events, such as gene regulation via removal of damaged bases from quadruplex DNA.

2.2. INTRODUCTION

Cells are continuously exposed to endogenous and environmental insults, such as reactive oxygen species (ROS), genotoxic agents, and ionizing radiation, which cause

DNA damages. ROS are by-products of cellular respiration and they play important physiological roles in healthy cells (Sena and Chandel, 2012). Unfortunately, DNA bases are particularly susceptible to ROS and oxidative base damage is almost an inevitable consequence (Duclos et al., 2012; Wallace, 2002). DNA base damages may be mutagenic and, if left unrepaired, may lead to base mispairing, blockage of DNA polymerases, and eventually result in genomic instability (Duclos et al., 2012; Wallace, 2002). For example, thymine glycol (Tg), the major oxidation product of DNA thymine, is an efficient block to DNA polymerases and thus a lethal lesion in cells (Ide et al., 1985). 8-oxo-7,8-dihydroguanine (8-oxoG), a major oxidation product of DNA guanine, and 5-hydroxyuracil (5-OHU), an oxidation product of DNA cytosine, are important premutagenic lesions both mispairing with adenine (Cheng et al., 1992; Kreutzer and Essigmann, 1998). The further oxidation products of 8-oxoG, guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp), can also mispair with adenine as well as block DNA some polymerases (Duarte et al., 1999; Henderson et al., 2003).

Base excision repair (BER) is the predominant pathway for repairing oxidative DNA base damages. DNA glycosylases are the first enzymes in BER that recognize damaged bases, excise the lesions and provide substrates for later enzymes in the pathway (Hegde et al., 2008a). Five DNA glycosylases that are specific for oxidative DNA base damages have been identified in human cells, namely OGG1, NTH1, NEIL1, NEIL2, and NEIL3 (Duclos et al., 2012; Wallace, 2002). Although there is much redundancy in substrate specificity, each glycosylase has a unique signature in terms of lesion type and DNA conformation. For example, the mammalian glycosylases OGG1 and NTH1 specifically

remove oxidized purines and pyrimidines, respectively, from duplex DNA (McCullough et al., 1999). NEIL1, NEIL2 and NEIL3 prefer oxidized pyrimidine and some purine damages (Liu et al., 2013a; Prakash et al., 2012) with NEIL2 and NEIL3 preferring lesions in single-stranded DNA (Hazra et al., 2002b; Liu et al., 2010; Wallace et al., 2003).

Telomeres are DNA-protein complexes at the end of chromosomes. By capping the chromosome ends, telomeres prevent degradation and undesired fusion of these ends (Hackett et al., 2001). Telomeres also suppress improper activation of DNA damage response pathways (de Lange, 2009). Both the length and integrity of this deoxyribonucleoprotein complex has to be carefully maintained. Shortening and loss of function of telomeres lead to telomere-mediated cell senescence and premature aging syndromes in humans (Armanios, 2009; Baird et al., 2003). On the other hand, telomere lengthening due to overactive telomerase has been linked to cancer (Artandi et al., 2002).

Under physiological salt conditions, human telomere repeats $(TTAGGG)_n$ can form quadruplex (G4) structures (Smith and Feigon, 1992; Williamson et al., 1989), which are four-stranded DNA topologies mediated by guanine-rich sequences. Several quadruplex structures containing the telomere sequence have been solved in both Na^+ (Wang and Patel, 1993) and K^+ (Parkinson et al., 2002) solutions. The basic building blocks of G-quadruplexes are layers of guanine tetrads, which consist of four guanines that Hoogsteen base pair to each other. A quadruplex consists of two or more layers of guanine tetrads,

one stacking onto the other with a monovalent cation (K^+ or Na^+) sandwiched in-between (Figure 2-1 D).

G4-forming sequences are prevalent throughout the human genome (Huppert and Balasubramanian, 2005; Todd et al., 2005). Although most structural studies of quadruplexes were done *in vitro*, there is increasing evidence supporting the existence of quadruplex structures in cells (Biffi et al., 2013; Yang et al., 2009). Quadruplex structures have been proposed to play inhibitory roles during DNA replication, gene transcription, and mRNA translation (Lipps and Rhodes, 2009). Most interestingly, quadruplex structures inhibit telomerase activity which, together with the inhibitory role of quadruplexes on oncogenes (Siddiqui-Jain et al., 2002), has led to research on quadruplex DNA as a target for cancer treatment (Neidle and Parkinson, 2002).

Guanine has the lowest redox potential among the four bases and thus is more susceptible to oxidation. Because of their G-rich nature, G4-forming sequences are vulnerable targets for oxidation through DNA charge transport (Genereux et al., 2010; Merino et al., 2008). For example, telomeric DNA $(TTAGGG)_n$ is more sensitive to UVA radiation (Oikawa et al., 2001) and hydrogen peroxide (Oikawa and Kawanishi, 1999) than non-telomeric DNA with the same nucleotide composition. Also, cellular oxidative stress induces 8-oxoG production in telomeres (Wang et al., 2010) and the production is higher than in other regions of the genome (O'Callaghan et al., 2011). DNA base damages in telomeres have to be repaired in order to preserve telomere integrity since

accumulation of base damages in telomeres may hinder telomerase activity (Szalai et al., 2002) and/or disrupt the telomere-guarding shelterin complex (Opresko et al., 2005).

Little biochemical work has been published on how glycosylases remove base lesions from quadruplex DNA or from the telomeric sequence context. Here, we tested the glycosylase activity of five mammalian oxidative DNA glycosylases, OGG1, NTH1, NEIL1, NEIL 2, and mNeil3, on quadruplex DNA containing base damages. We also investigated the impact of the telomeric sequence context on the activity of these glycosylases. Our results suggest that Neil3 and Neil1 may function in repair of damages harbored in quadruplex DNA and may play a role in base excision repair in telomeres.

2.3. EXPERIMENTAL PROCEDURES

Substrates - Sequences of oligodeoxyribonucleotides used in this study and a brief description of each are listed in Table 1. The telomeric DNA sequence (Tel) used was $_{d}$(AGGGTTAGGGTTAGGGTTAGGG). A random sequence (R) with the same nucleotide composition as the telomeric sequence was used as a control. The random sequence containing the telomeric-sequence-context (RSC) has the same backbone sequence as R, except that there is a "telomeric sequence patch" surrounding the damage. Oligodeoxyribonucleotides with Tg, 8-oxoG or 5-hydroxyuracil (5-OHU) as well as all of the non-lesion-containing oligodeoxyribonucleotides were purchased from Midland Certified Reagent Co. (Midland, TX). Gh- and Sp-containing substrates were synthesized as previously described (Krishnamurthy et al., 2008). The Tel-Sp

oligodeoxyribonucleotides used here were a mixture of Sp1 and Sp2 due to the inability to resolve these diastereomers under the purification method utilized.

All of the oligodeoxyribonucleotides were gel purified and quantified by NanoDrop spectrophotometry. The damage-containing oligodeoxyribonucleotides were ^{32}P labeled and ethanol precipitated using our standard protocol as previous described (Robey-Bond et al., 2008). The telomeric quadruplex DNA substrates were prepared as previously described (Szalai et al., 2002; Williamson et al., 1989) with minor modifications. Briefly, oligodeoxyribonucleotides were prepared in 10 mM sodium phosphate (pH 7.0) plus 100 mM NaCl in Milli-Q water. Potassium phosphate buffer (pH 7.0) and KCl of the same concentrations were used when preparing K^+-containing quadruplexes. The mixture was boiled for 5 minutes and gradually cooled down to room temperature in a water bath for 2 hours. The same procedure was used for preparing the other oligodeoxyribonucleotide substrates. In some experiments, a 1.2-fold excess of the complementary strand was added to ensure that all the lesion-containing strands were annealed into duplexes.

Enzymes - Details of the cloning, expression, and purification of the glycosylase domain of the *Mus musculus* Neil3 (MmuNeil3) were previously described (Liu et al., 2012). Unless it was specifically pointed out, the mNeil3 enzyme used in this study was the glycosylase domain of MmuNeil3 (MmuNeil3Δ324). NEIL1, NEIL2, NTH1 and OGG1 enzymes were from our laboratory stocks and were purified as previously described (Bandaru et al., 2006; Bandaru et al., 2002). Protein concentrations were determined by the BCA Protein Assay Kit (Thermo Scientific Pierce). The percentage of

active DNA glycosylase was determined by Schiff base assay or by the molecular accessibility method (Blaisdell and Wallace, 2007), and all protein concentrations used in this study were corrected to active enzyme fraction.

Native gel electrophoresis and circular dichroism (CD) - DNA substrates were prepared as described above. Samples were loaded with 5% glycerol on to a 16% acrylamide (29:1) gel with 0.5x TBE and 100 mM NaCl and the gel was run at 60 volts overnight at 4 ℃. The native gel was then dried and exposed to a phosphorimager screen for detection.

CD spectra were recorded by a JASCO-810 spectropolarimeter, using 1 mm path length cuvette in a volume of 300 µl at 37 ℃. The DNA oligodeoxyribonucleotides (20 µM) were prepared by the same procedure as described in the *Substrates* section above. For each sample, four scans were taken at wavelengths from 190 to 350 nm. An average value was then calculated from the four scans and corrected for the spectrum of the buffer control. The spectrum was then zero-corrected at 330 nm, and finally normalized to the concentration of the oligodeoxyribonucleotides.

Glycosylase activity assay - Enzymes and substrates were incubated at 37 ℃ in various glycosylase assay buffers as previously described (Liu et al., 2010). To measure the glycosylase activity only, reactions were terminated by adding NaOH to a final concentration of 0.33 N and samples were immediately put on ice. The quenched reactions were then heated at 95 ℃ for 4 minutes. An equal volume of formamide with dyes (bromophenol blue and xylene cyanol) was added to the reactions before loading on

to a 12% urea gel for separation. The gel was dried and exposed to a phosphoimage screen. Finally, bands from the screen were scanned by Molecular Imager FX (Bio-Rad Laboratories) and quantified by Quantity One software (Bio-Rad Laboratories).

Single turnover kinetics - For single-turnover kinetics analysis of mNeil3, the concentration of substrates was set at 10 nM and the concentration of enzyme was 4 μM or 6 μM, under which conditions the reaction rate reached maximum (data not shown). The glycosylase activity assay was performed as described above. The intensity of bands was quantified by Quantity One (Bio-Rad Laboratories) and analyzed by Prism 5 software. The catalytic rate (k_{obs}) was determined by fitting the data points to either a one phase or a two phase association model, depending on which was preferred.

2.4. RESULTS

The human telomeric sequence with a Tg or 8-oxoG lesion forms an antiparallel quadruplex structure in the presence of Na^+ - We first asked the question: do damage-containing oligodeoxyribonucleotides form quadruplex structures? We addressed this question using both native gel electrophoresis and circular dichroism. Because of its more compact structure, intramolecular quadruplex DNA runs faster in a non-denaturing gel than single-stranded DNA with the same nucleotide composition. In our experiments, we used the random sequence (R) as a control for the single-stranded DNA structure. As expected, the telomere sequence (Tel) had a higher mobility than R (Figure 2-1 A) and this compact structure in Na^+ solution was likely to be an intramolecular quadruplex

structure as previously demonstrated (Li et al., 2002). Similar to Tel, telomeric DNA containing a Tg lesion (Tel-Tg, Figure 2-1 A) also ran faster on a native gel than the random sequence containing Tg (R-Tg and RSC-Tg, Figure 2-1 A). This pattern indicates that Tel-Tg is able to form a compact, quadruplex-like structure in Na^+ solution. We did the same experiment using the Tel-OG oligodeoxyribonucleotide which has an 8-oxoG substitution at the 5' of the third GGG triad, and a very similar running pattern was obtained (Figure 2-1 B). This is consistent with the observation of Szalai *et.al.* (32), who showed that substitution of the 5' G of the GGG triad with 8-oxoG did not disrupt the formation of the intramolecular quadruplex structure although a significant drop in thermal melting temperature has been reported (Vorlickova et al., 2012).

Considering that the formation of a quadruplex structure is critical for our later experiments, we confirmed quadruplex formation by examining the structural details of our substrates using circular dichroism. The CD spectrum of Tel-Tg displayed a positive band at 295 nm and a negative band at 265 nm (Figure 2-1C), a characteristic CD pattern of an antiparallel quadruplex structure in NaCl solution (Li et al., 2002). The spectrum of Tel-OG was very similar to that of Tel and Tel-Tg, indicating that Tel-OG also formed an antiparallel quadruplex structure (Figure 2-1C), similar to reported spectra (Vorlickova et al., 2012). As a control, the random sequence R did not show the two-banded spectroscopic pattern (Figure 2-1C). Therefore, the CD spectra confirmed that Tel-Tg and Tel-OG fold into antiparallel quadruplex structures. These data agree with those of others that telomeric DNA forms an antiparallel structure in Na^+ solution, and that substitution of a single base does not hinder its formation (Li et al., 2002; Szalai et al., 2002;

Vorlickova et al., 2012). Based on the antiparallel quadruplex structures solved in Na^+ (Wang and Patel, 1993), we conclude that the Tg of qTel-Tg is located in the diagonal TTA loop and the 8-oxoG of qTel-OG is in an outer G-tetrad (Figure 2-1D).

mNeil3 glycosylase efficiently excises a Tg lesion from quadruplex DNA- Despite the fact that the G-rich quadruplex-forming sequences are susceptible to oxidation, there have been no biochemical studies to look at repair of base damages in quadruplex DNA structures. To examine this question, we used the five mammalian DNA glycosylases that recognize oxidative damages, NEIL1, NEIL2, mNeil3, NTH1 and OGG1 and measured their activity on Tg-containing quadruplex DNA (Figures 2-2A and data not shown). In these experiments, NaOH was added to the reactions after 15 minutes and samples were heated in order to measure glycosylase activity only. Of the five glycosylases, only mNeil3 showed significant activity on the Tg-containing quadruplex (qTel-Tg). NEIL1 and NEIL2 showed very weak glycosylase activity on the qTel-Tg substrate; there was only a trace amount of product after incubating qTel-Tg with a 10-fold excess of NEIL1 or NEIL2 for 15 minutes. NTH1 had no glycosylase activity on qTel-Tg (Figure 2-2A), although Tg itself is a good substrate for NTH1. The activity of mNeil3 was not a result of the quadruplex structure being disrupted, since the quadruplex structure of undamaged Tel was still intact after incubation with the mNeil3 enzyme. As can be seen in Figure 2-2C, the qTel substrate still ran as a unique band ahead of the single-stranded control (ssR) after incubation with mNeil3. In order to compare the activities of the glycosylases on qTel-Tg, the percentage of cleaved substrate by each enzyme was calculated (Figure 2-2B). Clearly, mNeil3 was the most active glycosylase for the Tg-containing quadruplex.

mNeil3, but not NEIL1, prefers thymine glycol in the telomeric sequence context- Next, we asked how the quadruplex substrate, qTel-Tg, compared to single-stranded or duplex DNA as a substrate for mNeil3. Single-stranded substrate (ssR-Tg) and two double-stranded substrates (dsR-Tg and dsTel-Tg) were used as controls. ssR-Tg, qTel-Tg, dsR-Tg or dsTel-Tg were incubated with mNeil3 at four different substrate-to-enzyme ratios (10:1, 1:1, 1:10, 1:50) for 15 minutes, and the glycosylase assay was carried out (Figure 2-3A and 2-3B). Surprisingly, mNeil3 showed more robust cleavage of the Tg lesion in quadruplex DNA than in single-stranded DNA (qTel versus ssR).

We also observed that dsTel-Tg was a better substrate than dsR-Tg (Figure 2-3), suggesting that sequence context might be playing a role. To examine the effect of telomeric sequence context on mNeil3 activity, we designed another random sequence RSC-Tg, which was similar to R-Tg except for the three bases on each side of the Tg lesion. In the RSC-Tg sequence, the Tg was in the same surrounding sequence context as that of telomeric DNA Tel-Tg (GGT-Tg-AGG) (Table1). If the telomeric sequence context makes dsTel-Tg a better substrate compared to dsR-Tg, then RSC-Tg, which mimics the sequence context of Tel-Tg, should be a comparable substrate to Tel-Tg. This turned out to be the case. Like dsTel-Tg, the dsRSC-Tg was cleaved much more rapidly by mNeil3 than dsR-Tg (Figure 2-3A). For example, the reaction was close to completion for dsRSC-Tg when it was incubated with a 10-fold excess of mNeil3 for 15 minutes, while the dsR-Tg substrate was barely cleaved. Similarly, ssRSC-Tg was a much better substrate than ssR-Tg (Figure 2-3A). When the experiments were quantified, it can be clearly seen that the telomeric sequence context makes the duplex DNA containing Tg

(dsRSC-Tg and dsTel-Tg) remarkably better substrates for mNeil3 (Figure 2-3B). Similar results were obtained for single-stranded substrates (ssRSC-Tg versus ssR-Tg, Figure 2-3B). Statistical analysis using Fisher's combined probability test shows that there are significant differences in the following comparisons: ssR vs. qTel, ssR vs. ssRSC, dsR vs. dsTel, and dsR vs. dsRSC, and the results are summarized in Table S1. Although the difference between ssR and qTel was significant, the difference between the means was much smaller than that of other comparisons, as can be seen in Figure 2-3B by visual inspection and by the statistical results in Table S1.

We then measured the catalytic rates of mNeil3 on the six substrates (Table 2). The rates (k_{obs}) were measured under single turnover conditions, where the enzyme concentration was much higher than the substrate concentration in order to measure the chemistry step only. Surprisingly, we found that ssRSC-Tg had a catalytic rate (k_{fast}) of 6.66 min^{-1}, which was over 25-fold faster than ssR-Tg (0.25 min^{-1}). Similar to ssRSC-Tg , the telomeric quadruplex substrate qTel-Tg had a k_{fast} of 4.95 min^{-1}, which was close to ssRSC-Tg and nearly 20 times greater than that of ssR-Tg. More strikingly, when we compared the duplex substrate with or without the telomeric sequence context, we found the k_{obs} of dsRSC-Tg (7.49 min^{-1}) to be over 80 times greater than that of dsR-Tg (0.09 min^{-1}). In fact, the k_{fast} of dsRSC-Tg (7.49 min^{-1}) was in the same range as its corresponding single-stranded DNA ssRSC-Tg (6.66 min^{-1}). It is important to note that the only difference between the two single-stranded substrates was the sequence context surrounding the Tg-lesion. The RSC-Tg has a telomeric sequence context surrounding Tg (GGT-Tg-AGG) while the R-Tg does not (GTG-Tg-GAG) (Table 1). The other duplex

substrate dsTel-Tg (1.36 min^{-1}) was also a much better substrate than dsR-Tg (0.09 min^{-1}) (Table 2).

The k_{obs} values were calculated by Prism 5 using its 'one-phase association' or 'two-phase association' model, depending on which one fit the data better. Most of the reactions with the Tg substrates turned out to be double-exponentials, giving a k_{fast} and a k_{slow} when fitted to a two-phase association model. The double exponential curves were most likely due to the presence of two isomers of Tg, which may be processed differently by different glycosylases. As observed by us (Guo et al., 2010) and others (Katafuchi et al., 2004; Miller et al., 2004) both bacterial and mammalian Nth and Nei glycosylases exhibit strong stereospecificity towards the two isomers of thymine glycol. In contrast, the ssR-Tg and dsR-Tg were best fit as a single exponential (Table 2). It is likely that the activity of mNeil3 was so slow on one of the two Tg isomers in these two substrates that we were not able to detect the second phase (k_{slow}) during the time frame of our assay.

We then tested if mNeil3 preferentially removed 5-OHU in the telomeric sequence context. The 5-OHU is a common oxidative damage of cytosine, which can occur on the opposite strand of $(TTAGGG)_n$. We found that mNeil3 also preferred 5-OHU in duplex DNA containing the telomeric sequence, although the difference in k_{obs} was less striking with 5-OHU in the telomeric sequence context being only about twice that in the non-telomeric sequence (Table 2, dsTel-5OHU versus dsR-5OHU).

We also asked if the effect of telomere sequence was unique to mNeil3 or whether it was shared by other glycosylases. Accordingly, similar experiments were done with

NEIL1, NTH1 and OGG1 glycosylases. Figure 2-4A clearly shows that the activity of NEIL1 on Tg was not affected by the presence of the telomere sequence (also see Table S1 for statistical results). OGG1 was also not sensitive to the sequence surrounding 8-oxoG in duplex DNA (Figure 2-4B), which is consistent with a previous telomeric sequence context study of OGG1 (Rhee et al., 2011). Moreover NTH1 glycosylase preferred the random sequence over the telomere sequence (Figure 2-4C). Thus we concluded that the strong preference towards thymine glycol in the telomeric sequence context was unique to Neil3.

mNeil3 and NEIL1 remove Sp and Gh from quadruplex DNA - Considering that guanine has the lowest redox potential of the four bases and is thus the most susceptible base to oxidation, we tested the activity of the glycosylases on quadruplex DNA containing oxidized guanine lesions. We started with an 8-oxoG substitution in one of the two outer G-tetrads. Since the 5'-G of the GGG triad is the major target for oxidation (Saito et al., 1995; Sugiyama and Saito, 1996), we substituted a 5' G with 8-oxoG (Tel-OG, Table 1). The oligodeoxyribonucleotide folded into an antiparallel quadruplex structure as discussed above (Figure 2-1 B and 1C). Glycosylase assays were performed using qTel-OG substrate and the five mammalian glycosylases (OGG1, NEIl1, NEIL2, mNeil3, NTH1). None of the five enzymes showed glycosylase activity on the 8-oxoG-containing quadruplex substrate (qTel-OG) (Figure 2-5A and data not shown), despite the fact that 8-oxoG is the major substrate for OGG1.

Sp and Gh are further oxidation products of 8-oxoG. Our recent data show that Sp and Gh are two major oxidation products formed after treating telomeric G-quadruplex DNA with several oxidant systems *in vitro* (Fleming and Burrows, 2013). Moreover, Sp and Gh are the two best substrates for mNeil3 (Liu et al., 2010) and NEIL1 (Krishnamurthy et al., 2008); NEIL2 (Hailer et al., 2005) and Nth (Hazra et al., 2001) also excise the two hydantoin lesions. We thus tested the activity of the glycosylases on Sp-containing and Gh-containing quadruplex substrates (qTel-Sp and qTel-Gh). Folding of the Tel-Sp and Tel-Gh to quadruplexes was confirmed by native gel electrophoresis (Figure 2-5B). Glycosylase activity assays with the five enzymes were then performed using qTel-Sp and qTel-Gh as substrates. Both mNeil3 and NEIL1 very efficiently removed Sp (Figure 2-5C) and Gh (Figure 2-5D) from quadruplex DNA. However, we did not see any activity of NEIL2, NTH1 or OGG1 on either qTel-Sp (Figure 2-5C) or qTel-Gh (Figure 2-5D).

NEIL1, but not mNeil3, prefers Gh in the telomere sequence context – In order to determine whether Gh is a preferred substrate in the telomere sequence, the glycosylase activity of mNeil3 and NEIL1 was performed on ssR-Gh, qTel-Gh, dsR-Gh and dsTel-Gh. When comparing dsR-Gh to dsTel-Gh (Figure 2-6A), mNeil3 clearly does not prefer Gh in the telomere sequence. In addition, when measured under single turnover conditions, the k_{obs} of dsR-Gh and dsTel-Gh were 5.01 ± 0.30 min^{-1} and 0.26 ± 0.01 min^{-1}, respectively. mNeil3 also does not prefer the Sp lesion in the duplex telomere sequence (data not shown). However, NEIL1 showed slightly enhanced activity on dsTel-Gh

(Figure 2-6B), as evidenced by comparing dsR and dsTel at 10 nM of NEIL1. The results of our statistical analysis are summarized in Table S1C and D.

2.5. DISCUSSION

Despite the susceptibility of the guanine rich quadruplex-forming sequence to oxidation and the biological importance of quadruplex DNA, no study has addressed the question of how damaged bases in quadruplex DNA are repaired. In this study, we show that telomeric DNA containing Tg, 8-oxoG, Sp or Gh can form quadruplex structures in the presence of physiological salts *in vitro*. We further demonstrate that the mouse ortholog of human NEIL3 can efficiently remove Tg, Sp and Gh lesions from quadruplex DNA; NEIL1 is also able to efficiently excise Sp and Gh lesions from quadruplex DNA. Interestingly, the mNeil3 glycosylase shows a marked preference towards thymine glycol (but not Sp or Gh) in telomeric DNA sequences, while NEIL1 appears to have a complementary role, with a preference for Gh in the telomeric sequence.

The structures of quadruplexes have been intensely studied for the past 20 years, but the impact of base damages on the stability of quadruplexes and how these base damages are repaired in quadruplexes remain to be investigated. The fact that Tg, 8-oxoG, Sp and Gh can be folded into quadruplex DNA structures implies that an oxidative base lesion may stably exist in quadruplex DNA *in vivo*.

The excision activities of mNeil3 and NEIL1 on lesion-containing quadruplexes led us to speculate on their biological function in cells. Quadruplex DNA has been proposed to exist during important biological functions such as DNA replication (Lipps and Rhodes, 2009), during which time the two strands of the duplex DNA molecule become separated. Interestingly, both NEIL3 (Neurauter et al., 2012) and NEIL1(Dou et al., 2008) have been linked to DNA replication. A further speculation is that genes might be regulated by glycosylases via modulation of quadruplex structures at promoter regions. Quadruplex-forming sequences are prevalent in the human genome and are enriched in promoters (Huppert and Balasubramanian, 2005; Todd et al., 2005). These guanine-rich sequences will be inevitably oxidized to create damage-containing quadruplex DNA (Clark et al., 2012). Glycosylases may function in gene regulation by removing DNA base damages from the quadruplex and creating transient strand breaks. This action may result in collapse of the quadruplex structures in promoters, which would relieve the inhibitory effect of the quadruplex on transcription. A similar mechanism has been proposed by Gillespie *et al.* (Gillespie et al., 2010) who found that the BER enzymes OGG1 and APE1 were recruited to G4 sequences in promoters of those genes exhibiting altered expression after hypoxia treatment. The recruitment of OGG1 and APE1 to the promoter regions coincides with increased 8-oxoG production and DNA strand breaks in these G4 sites (Clark et al., 2012). Interestingly, we did not find any glycosylase activity of OGG1 on 8-oxoG-containing quadruplex DNA (Figure 2-5A).

Another interesting observation is the strong sequence context effect governing the activity of mNeil3 on thymine glycol. The k_{obs} (Table 2) implies that a faster chemistry

step is significantly contributing to the preference for the telomere sequence, since the k_{obs} only reflects the catalytic step but not binding. According to the transition state theory (Berg et al., 2007), a lower activation barrier (Gibbs free energy of activation, $\Delta G‡$) due to the difference in the surrounding DNA sequence may explain the increased rates. Moreover, while the data shown in Figure 2-3B do not support a formal estimation of binding affinities and catalytic rates for the Tg-containing substrates, they suggest qualitative conclusions. The general impression is that mNeil3 binding to all these substrates is weak, a conclusion consistent with numerous EMSA experiments where we have been unable to show Neil3 to bind to Tg-containing DNA. However, the increasing extent of cleavage as enzyme: substrate increases from 1:1 to 10:1 and 50:1 implies that more binding occurs with higher protein concentrations and therefore the differences in the extent of cleavage on the same substrate do reflect differences in binding. This raises the possibility that differences between observations on different substrates also reflect differences in binding. However, inspection of the non-quadruplex substrates in Figure 2-3B also shows that both strandedness and context play a role. First, the extents of cleavage are greater for each single-stranded representative by comparison with its double-stranded mate. This is consistent with the possibility that availability of Tg for insertion into the active site pocket is greater in the single-stranded substrates due to the absence of the opposite strand and is in keeping with our prior biochemical studies of Neil3 showing its preference for lesions in single=stranded substrates (Liu et al., 2010) and with its structure (Liu et al., 2013b) showing why. Similarly, there is a strong effect of context, that is, the extents of cleavage are greater for each RSC context by

comparison with its R mate. This is consistent with both biochemical and crystallographic studies from our laboratory with DNA polymerases that have shown that the methyl and hydroxyl groups present on the 5' position of thymine glycol severely alter stacking of the 5' base and in the case of guanine, a hydrogen bond is formed with Tg. Moreover, there is an additional interaction with a 3' purine via a water molecule (Aller et al., 2007). Thus in the case of the random sequence we used, 5'G Tg G3', the Tg would be less able to be extruded into the Neil3 substrate binding pocket than in the telomere sequence context 5'T Tg G3'. So even though the enzyme is bound to the DNA, poorer extrusion of Tg into the active site pocket would explain the slower chemistry step (Table 2) observed with the random sequence compared to the telomere sequence. The preference of mNeil3 for the telomeric sequence appears to be restricted to the oxidized pyrimidines, especially thymine glycol, since there is no sequence context effect with Sp and Gh.

So why doesn't NEIL1 exhibit the same preference for Tg in the telomere sequence? It turns out that NEIL1, like most members of the Fpg/Nei family, contains three void-filling residues that help extrude the lesion from the DNA into the active site pocket while two of them are missing in mNeil3 (Liu et al., 2013b). Although NEIL1 has no preference for Tg in the telomere sequence context, it did show a slight preference for Gh (Figure 2-6B). This complementary telomere sequence context effect of mNeil3 and NEIL1 suggests that they may play special roles in telomere damage repair.

Base damages have been shown to be deleterious to telomeres. For example, 8-oxoG attenuates the binding of telomeric proteins such as TRF1 and TRF2, loss of which results in telomere dysfunction and fusions (Opresko et al., 2005). In addition, Tg, Sp and Gh are efficient blocks to some DNA polymerases (Duarte et al., 1999; Henderson et al., 2003; Ide et al., 1985). A mechanism for oxidative stress-induced telomere shortening has been proposed which argues that blocking of DNA polymerases by unrepaired bases or nucleotides is a major contributor to this shortening (von Zglinicki, 2002).

In summary, the data presented here show that Neil3 and NEIL1 may play important roles in telomere maintenance by preventing accumulation of base damages in telomeres thus protecting their integrity. However, we were unable to colocalize NEIL3 to telomere markers (TRF1, TRF2, and POT1) in HeLa or HEK293 cells or demonstrate any physical interaction between NEIL3 and TRF1 or TRF2 by co-immunoprecipitation. Since Neil3 appears to function in a cell-cycle dependent and tissue-specific manner (Neurauter et al., 2012) we suspect it may do the same for repair in telomeres. Future work is needed to address these questions.

2.6. REFERENCES

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2.7. FOOTNOTES

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[4]The abbreviations used are: Tel, telomere sequence; R, random sequence; RSC, random sequence with telomeric sequence context; q, quadruplex; ss, single-stranded; ds, double-

stranded; G4, G-quadruplex; BER, base excision repair; 8-oxoG, 8-oxo-7,8-dihydroguanine; OGG1, 8-oxoguanine DNA glycosylase; TRF1, telomere repeat-binding factor 1; TRF2, telomere repeat-binding factor 2; me-Fapy-G, 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine; Fpg, formamidopyrimidine DNA glycosylase; APE1, apurinic endonuclease 1; NEIL1, human endonuclease VIII-like glycosylase 1; NEIL2, human endonuclease VIII-like glycosylase 2; mNeil3, mouse endonuclease VIII-like glycosylase 3; NTH1, human endonuclease III glycosylase 1; Sp, spiroiminodihydantoin; Gh, guanidinohydantoin; CD, circular dichroism; RPA, replication protein A. PCNA, proliferating cell nuclear antigen.

2.8. FIGURE LEGENDS

Figure 2-1. Telomeric DNA sequences containing Tg or 8-oxoG form quadruplex DNA. All samples were prepared in 10 mM sodium phosphate (pH 7.0) and 100 mM Na+. Samples were separated using native gel electrophoresis or measured by circular dichroism as described in Experimental Procedures. (A) Tg-containing telomeric DNA forms a quadruplex. q, quadruplex DNA; ss, single-stranded DNA; ds, double-stranded DNA. (B) 8-oxoG-containing telomeric DNA forms a quadruplex. (C) CD spectrum confirms formation of an antiparallel quadruplex structure. Tel-Tg and Tel-OG show two peaks at 265 nm and 295 nm. (D) Scheme of an antiparallel quadruplex structure. Arrows indicate positions of lesion substitution.

Figure 2-2. mNeil3 has significant glycosylase activity on quadruplex DNA containing a Tg lesion. (A) Glycosylase activity of NEIL1, NEIL2, mNeil3, and NTH1 on the qTel-Tg substrate. qTel-Tg (10 nM) was incubated with buffer only or with increasing concentrations of each enzyme (1, 10, and 100 nM) for 15 minutes. Reactions were terminated by NaOH and heating to measure glycosylase activity only. (B) Quantification of substrate (qTel-Tg) cleavage. Mean and standard deviations calculated from three replicates are shown. (C) Native gel electrophoresis showing the intact quadruplex structure of qTel (without a lesion) after incubation with mNeil3.

Figure 2-3. mNeil3 prefers the Tg lesion in the telomeric sequence context. (A) Six Tg-containing substrates (10 nM) were incubated with buffer only or with increasing concentrations of mNeil3 (1, 10, 100, 500 nM) for 15 minutes. Reactions were terminated

by NaOH and heating to reveal glycosylase activity only. (B) Quantification of substrate cleavage by mNeil3. Mean and standard deviations from three replicates are shown. Fisher's combined probability test was performed between telomeric and non-telomeric substrate pairs to reveal the effect of sequence context. The stars indicate the combined *p* value of the four *t*-tests performed for the four individual concentrations. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$.

Figure 2-4. NEIL1, OGG1 and NTH1 do not prefer lesions in telomere sequence context. (A) Four Tg-containing substrates (10 nM) were incubated with buffer only or with increasing concentrations of NEIL1 (1, 10, 100 nM) for 15 minutes. Reactions were terminated by NaOH and heating to reveal glycosylase activity only. The same statistical analysis was performed as in Figure 2- 3B. There is no significant difference between ssR and qTel or between dsR and dsTel. See Table S1 for more detailed analysis. The same experimental conditions and analysis as in (A) were performed for OGG1 on 8-oxoG-containing substrates (B) and NTH1 on Tg-containing substrates (C).

Figure 2-5. mNeil3 and NEIL1 recognize Sp and Gh in telomeric quadruplex DNA. (A) OGG1 does not remove 8-oxoG from quadruplex DNA. qTel-OG and dsTel-OG (10 nM) were incubated with buffer only or increasing concentrations of OGG1 (1, 10, 100 nM) for 15 minutes. (B) Native gel electrophoresis shows that Tel-Sp and Tel-Gh sequences form quadruplexes. (C) mNeil3 and NEIL1 remove Sp from quadruplex DNA. qTel-Sp or dsTel-Sp substrate (10 nM) was incubated with each enzyme (10 nM) at 37 ℃ for 15 minutes. (D) mNeil3 and NEIL1 remove Gh from quadruplex DNA.

Figure 2-6. mNeil3 does not prefer Gh in the telomeric sequence context. (A) Quantification of substrate cleavage by mNeil3. Gh-containing substrates (10 nM) were incubated with increasing concentrations of mNeil3 (1, 10, 100 nM) for 5 minutes. Reactions were terminated by NaOH and heating to reveal glycosylase activity only. The same experiments were done using NEIL1 and results are shown in (B). Mean and standard deviations from four replicates are shown. Fisher's combined probability test was performed between dsR and dsTel to reveal the effect of sequence context. The stars indicate the combined *p* value of the four *t*-tests performed for the four individual concentrations. **, $p < 0.005$; ***, $p < 0.0001$. See Table S1 for more detailed analysis.

2.9. TABLES AND FIGURES

Table 2-1. Oligodeoxynucleotide sequences used in this study.

Name	Sequence	Description
Tel	AGGGTTAGGGTTAGGGTTAGGG	Telomeric sequence without lesion
Tel-Tg	AGGGTTAGGGT(Tg)AGGGTTAGGG	Telomeric sequence with Tg
Tel-OG	AGGGTTAGGGTTA(8OG)GGTTAGGG	8-oxoG at 5' of GGG tetrad
R	GGTGTGAGTGTGAGTGTGAGAG	Random sequence without lesion
R-Tg	GGTGTGAGTG(Tg)GAGTGTGAGAG	Random sequence with Tg
RSC-Tg	GGTGTGAGTGGT(Tg)AGGTGAGAG	Random sequence with telomeric sequence flanking Tg
Tel-Sp	TGTTAGGGTTAGGGTTA(Sp)GGTTAGGGCCAT	Telomeric sequence with Sp
Tel-Gh	TGTTAGGGTTAGGGTTA(Gh)GGTTAGGGCCAT	Telomeric sequence with Gh
Tel-5OHU	TGTCAATCCCTAA(5OHU)CCTAACCCTAACCCTGAGTCT	Telomeric sequence with 5-OHU
R-5OHU	TGTCAATAGCAAG(5OHU)GGAGAAGTCAATCGTGAGTCT	Random sequence with 5-OHU

Table 2-2. Single turnover kinetics of mNeil3 on lesion-containing substrates with or without the telomeric sequence context.

Substrates	k_{fast}, min^{-1}	k_{slow}, min^{-1}	Fast phase (%)
ssR-Tg	0.25 ± 0.01	NA, one phase	NA, one phase
ssRSC-Tg	6.7 ± 1.0	0.50 ± 0.06	50 ± 3
qTel-Tg	5.0 ± 0.7	0.62 ± 0.05	39 ± 4
dsR-Tg	0.09 ± 0.01	NA, one phase	NA, one phase
dsRSC-Tg	7.5 ± 1.4	0.19 ± 0.01	24 ± 2
dsTel-Tg	1.4 ± 0.2	0.15 ± 0.02	44 ± 5
dsR-5OHU	0.58 ± 0.20	0.04 ± 0.02	58 ± 9
dsTel-5OHU	1.7 ± 0.8	0.10 ± 0.05	53 ± 12

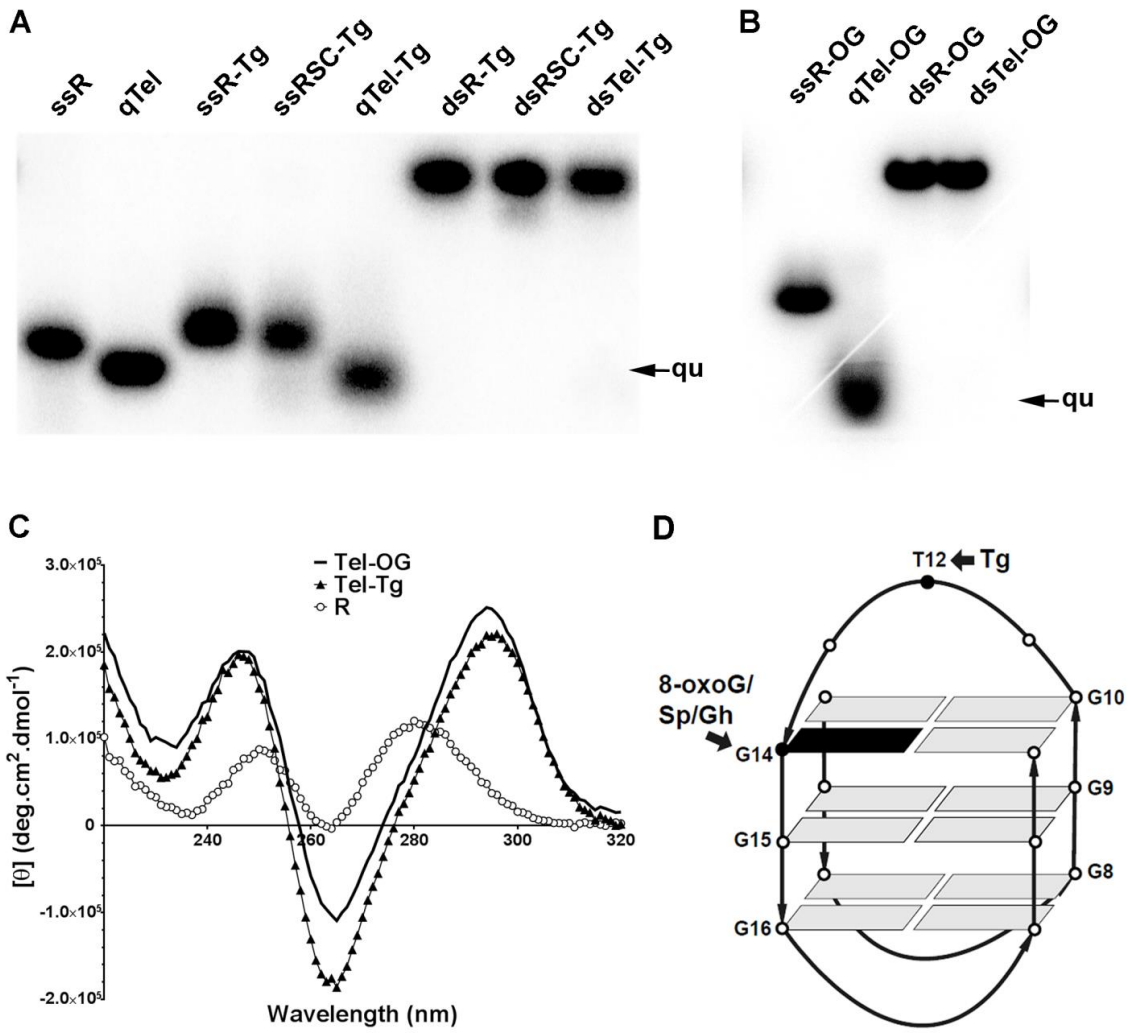

Figure 2-1. Telomeric DNA sequences containing Tg or 8-oxoG form quadruplex DNA.

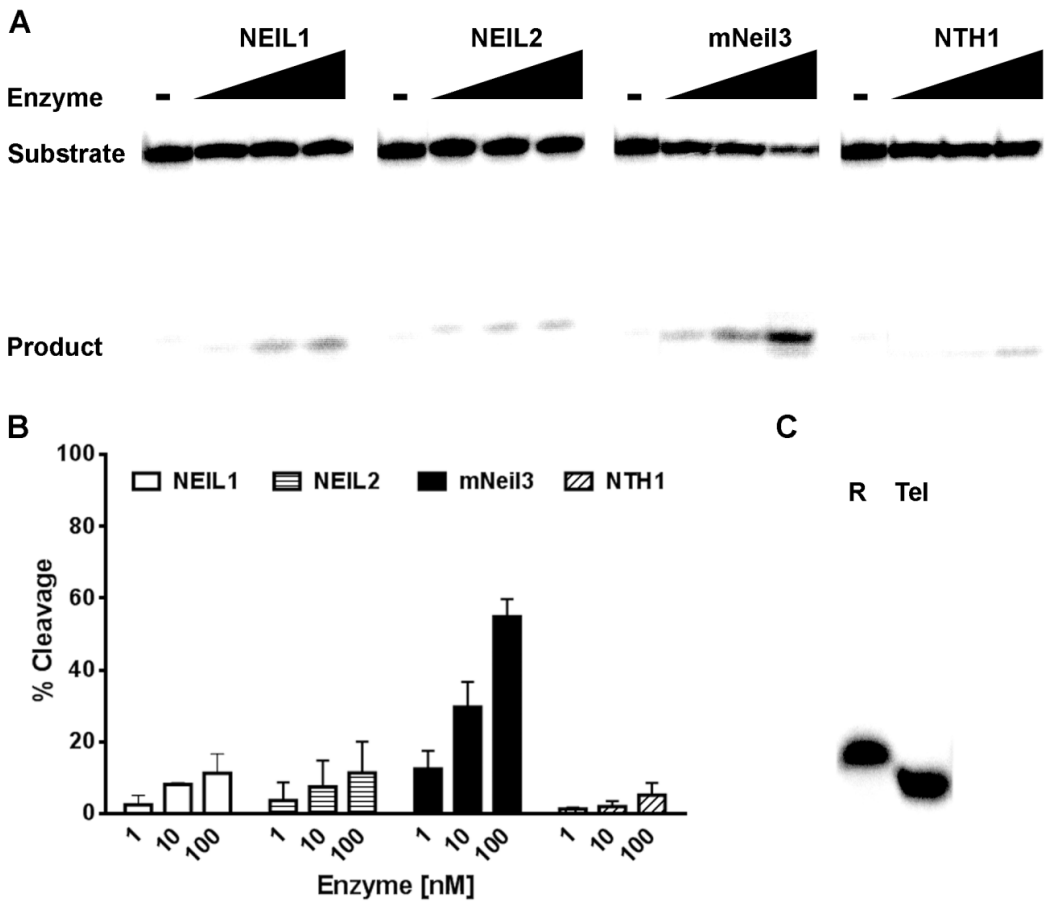

Figure 2-2. mNeil3 has significant glycosylase activity on quadruplex DNA containing a Tg lesion.

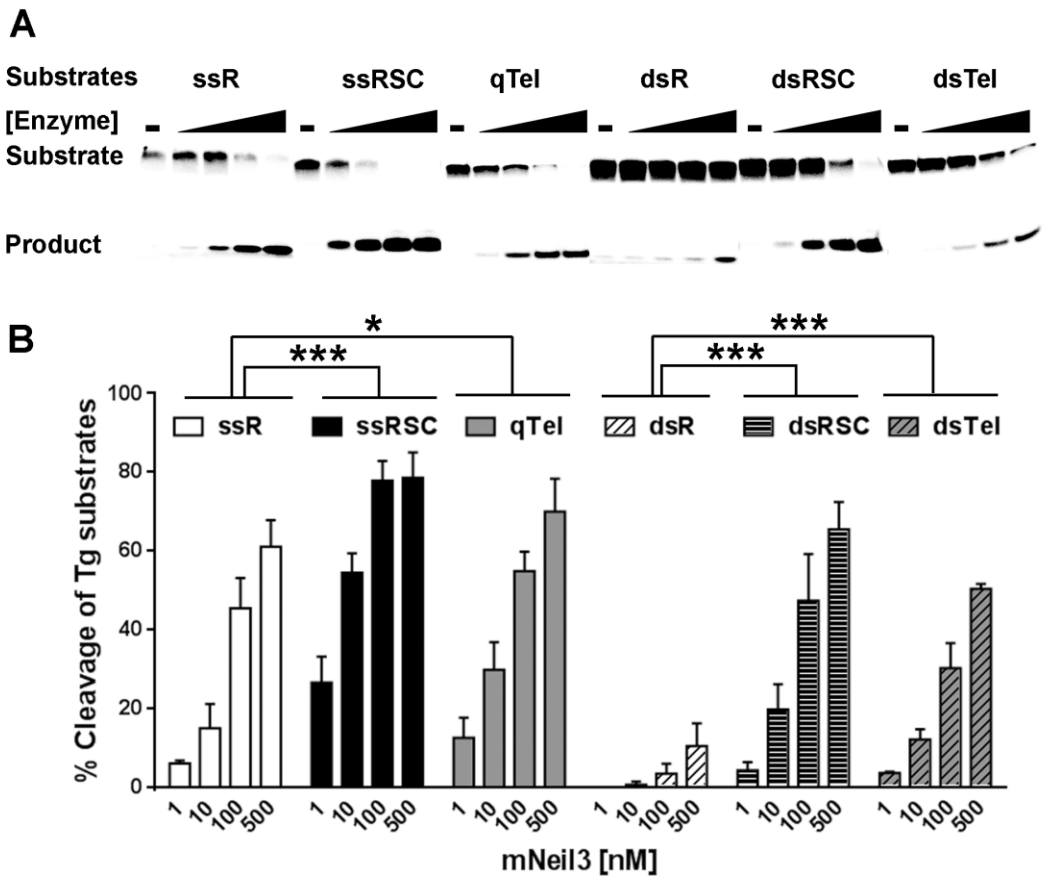

Figure 2-3. mNeil3 prefers the Tg lesion in the telomeric sequence context.

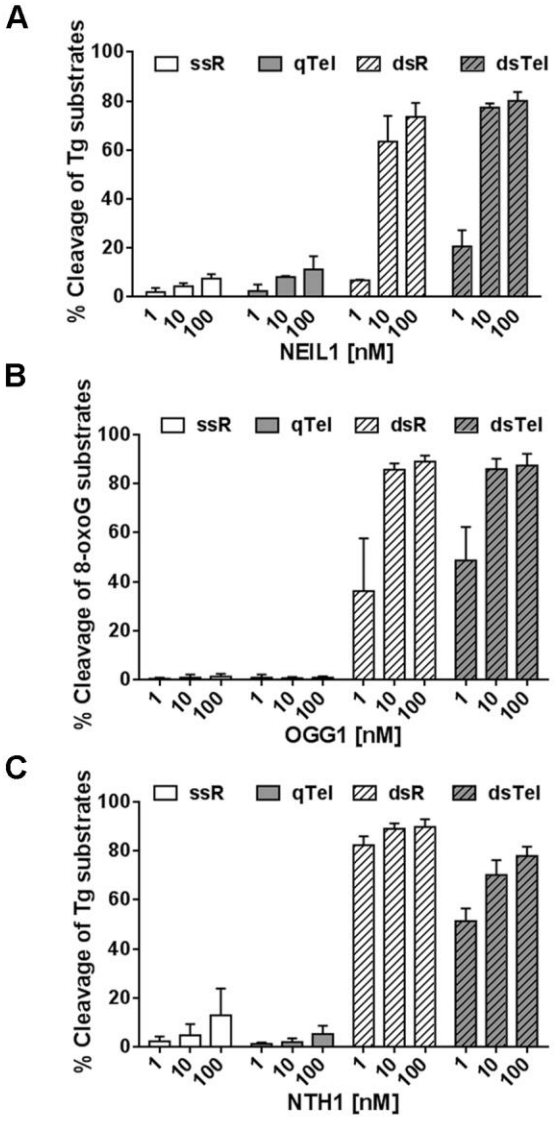

Figure 2-4. NEIL1, OGG1 and NTH1 do not prefer lesions in telomere sequence

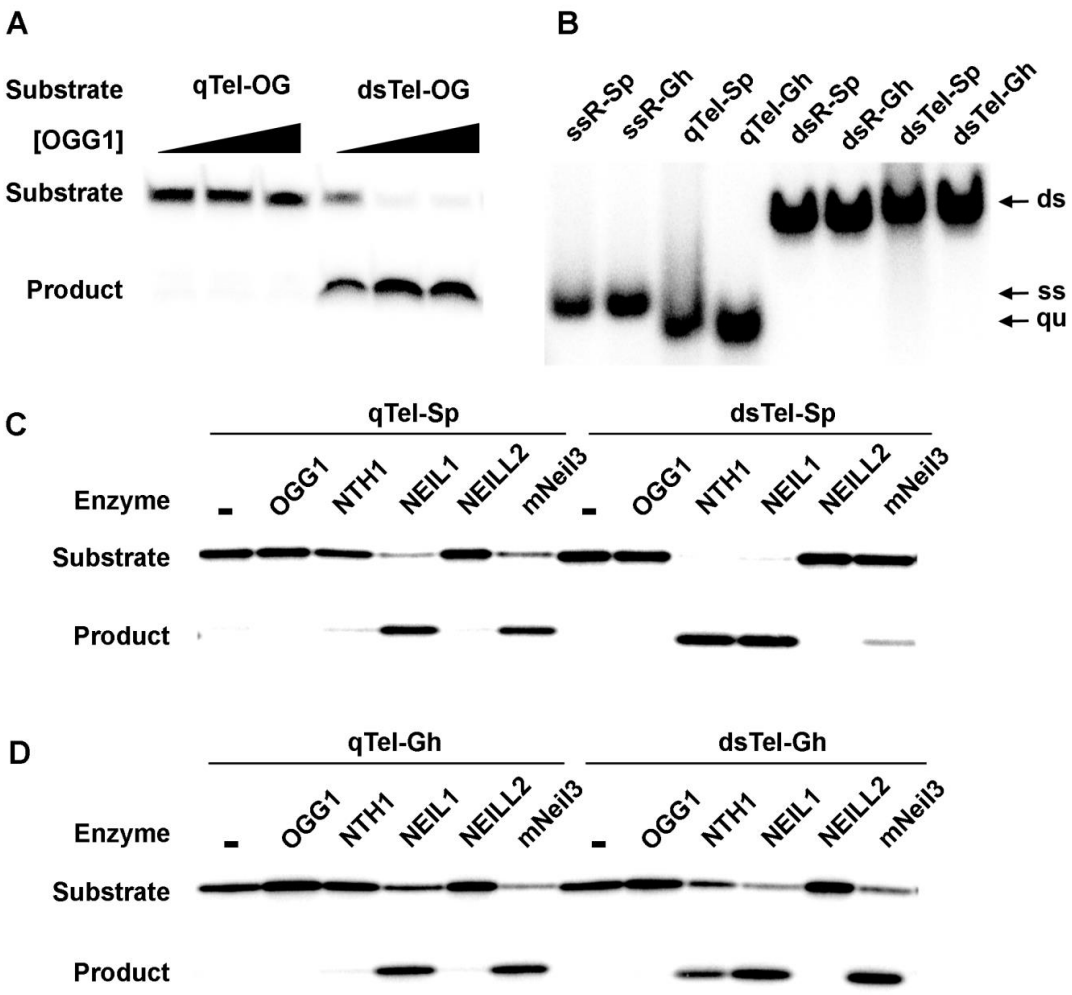

Figure 2-5. mNeil3 and NEIL1 recognize Sp and Gh in telomeric quadruplex DNA.

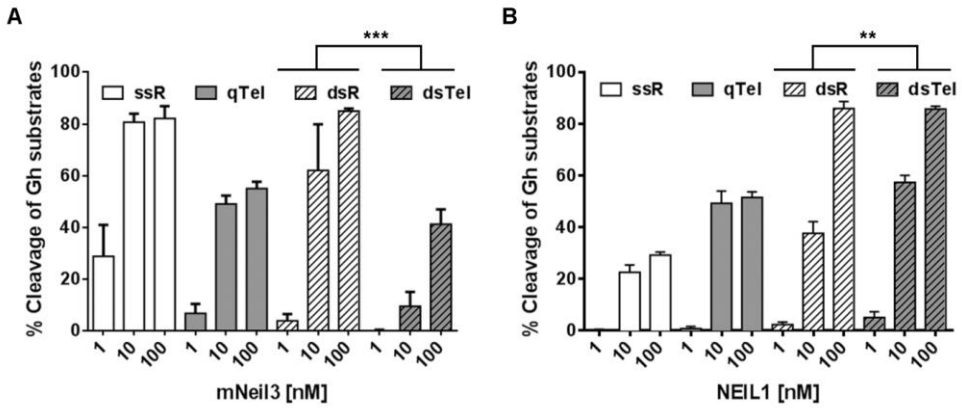

Figure 2-6. mNeil3 does not prefer Gh in the telomeric sequence context.

2.10. SUPPLEMENTAL DATA

Table 2-S1. Statistical analysis of the data from Figure 3 and Figure 5. In each table, student's *t*-test was performed for each pair at each enzyme concentration and the *p*-values are shown. Fisher's method was then used to calculate the combined probability for each pair. Briefly, Chi-square (X^2) was determined by applying the formula: $\boldsymbol{X^2 = -2\sum_{i=1}^{k}\log_e(pi)}$, where *pi* is the *p*-value for the i^{th} test and k is the number of testes. The combined *p*-values were determined from the X^2 values, using 2*k* degrees of freedom. The difference in the mean is shown if the comparison is significantly different ($p<0.05$).

Table 2-S1. Statistical analysis of the data from Figure 2-3 and Figure 2-5.

A

mNeil3 on Tg substrates in Figure 3B					
	[mNeil3]	qTel vs ssR	ssRSC vs ssR	dsTel vs dsR	dsRSC vs dsR
p values from *t*-test	1	0.154	0.031	0.003	0.075
	10	0.053	0.001	0.010	0.034
	100	0.159	0.006	0.010	0.010
	500	0.225	0.031	0.005	0.001
p value from Fisher's combined		0.0386*	<0.0001***	<0.0001***	<0.0001***
Differences in mean	1	6.557	20.580	3.500	4.140
	10	14.840	39.560	11.587	19.150
	100	9.447	32.460	26.870	44.013
	500	8.970	17.587	39.940	55.023

B

NEIL1 on Tg substrates in Figure 3C			
	[NEIL1]	qTel vs ssR	dsTel vs dsR
p values from t-test	1	0.805	0.068
	10	0.029	0.146
	100	0.354	0.181
p value from Fisher's combined		0.14	0.05
		No significant difference	

C

mNeil3 on Gh substrates in Figure 5A		
	[mNeil3]	dsTel vs dsR
p values from t-test	1	0.064
	10	0.007
	100	0.001
p values from Fishers combined		<0.0001***
Differences in mean	1	-3.753
	10	-52.530
	100	-43.720

D

NEIL1 on Gh substrates in Figure 5B		
	[NEIL1]	dsTel vs dsR
p values from *t*-test	1	0.104
	10	0.001
	100	0.929
p value from Fisher's combined		0.0038**
Differences in mean	1	2.618
	10	19.670
	100	-0.140

CHAPTER III: THE NEIL GLYCOSYLASES REMOVE OXIDIZED GUANINE LESIONS FROM TELOMERIC AND PROMOTER QUADRUPLEX DNA STRUCTURES

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3.1. ABSTRACT

G-quadruplex is a four-stranded G-rich DNA structure that is highly susceptible to oxidation. Despite the important roles that G-quadruplexes play in telomere biology and gene transcription, neither the impact of guanine lesions on the stability of quadruplexes nor their repair are well understood. Here, we show that the oxidized guanine lesions 8-oxo-7,8-dihydroguanine (8-oxoG), guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) reduce the thermostability and alter the folding of telomeric quadruplexes in a location-dependent manner. Also, the NEIL1 and NEIL3 DNA glycosylases can remove hydantoin lesions but none of the glycosylases, including OGG1, are able to remove 8-oxoG from telomeric quadruplexes. Interestingly, a hydantoin lesion at the site most prone to oxidation in quadruplex DNA is not efficiently removed by NEIL1 or NEIL3. However, NEIL1, NEIL2 and NEIL3 remove hydantoins from telomeric quadruplexes formed by five TTAGGG repeats much more rapidly than the commonly studied four-repeat quadruplex structures. We also show that APE1 cleaves furan in selected positions in Na^+-coordinated telomeric quadruplexes. In promoter G-quadruplex DNA, the NEIL glycosylases primarily remove Gh from Na^+-coordinated antiparallel quadruplexes but not K^+-coordinated parallel quadruplexes containing *VEGF* or *c-MYC* promoter sequences. Thus, the NEIL DNA glycosylases may be involved in both telomere maintenance and in gene regulation.

3.2. INTRODUCTION

Our cells are continuously exposed to endogenous reactive oxygen species (ROS), as well as ROS from environmental insults such as ionizing radiation. ROS damage to DNA results in strand breaks, sites of base loss as well as oxidized DNA bases (De Bont and van Larebeke, 2004). Both strand breaks and abasic sites can cause replication fork collapse; however, when bypass of abasic sites occurs, an adenine is preferentially inserted and can result in mutations (Loeb and Preston, 1986). The oxidized DNA bases, if left unrepaired, may also result in mutations because of base mispairing (Duclos et al., 2012; Wallace, 2002). For example, the guanine oxidation product 8-oxo-7,8-dihydroguanine (8-oxoG) can mispair with adenine, causing G to T transversion mutations (Cheng et al., 1992). 8-oxoG is susceptible to further oxidation to guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) (Figure 3-1C) (Luo et al., 2001; Luo et al., 2000) that are capable of mispairing with adenine and guanine (Duarte et al., 1999; Kornyushyna et al., 2002). Furthermore, DNA base damages such as thymine glycol (Tg), as well as Gh and Sp, efficiently block DNA polymerases (Aller et al., 2010; Ide et al., 1985).

Base excision repair (BER) is the predominant pathway that repairs oxidative DNA base damages (Barnes and Lindahl, 2004; Duclos et al., 2012; Fromme and Verdine, 2004; Izumi et al., 2003; Krokan and Bjoras, 2013; Mitra et al., 2002). The first enzyme in this pathway is a DNA glycosylase that recognizes and excises the damaged bases and provides substrates for the next step in the pathway. Bifunctional glycosylases also

possess a lyase activity that cleaves the abasic site generated by the glycosylase activity. OGG1, NTH1, NEIL1, NEIL2, and NEIL3 are the five DNA glycosylases that are specific for removing oxidized DNA bases in human cells (De Bont and van Larebeke, 2004; Fromme and Verdine, 2004; Grin and Zharkov, 2011; Hegde et al., 2008a). OGG1 and NTH1 are housekeeping glycosylases that primarily remove oxidized purines and pyrimidines, respectively, from duplex DNA (Asagoshi et al., 2000; Zharkov et al., 2000). The DNA endonuclease eight-like (NEIL) glycosylases have broader substrate specificity, and are associated with particular DNA transactions. NEIL1 acts upon pyrimidine lesions such as Tg and 5-hydroxyuracil (5-OHU) in duplex DNA (Bandaru et al., 2002), although it also removes lesions from single-stranded and bubble DNA structures (Dou et al., 2003). Both NEIL2 and NEIL3 prefer oxidized pyrimidine and some purine damages in single-stranded DNA (Dou et al., 2003; Liu et al., 2010), and we have previously shown that mouse Neil3 removes lesions from quadruplex DNA (Zhou et al., 2013). Although, 8-oxoG is not a substrate for any of the NEIL glycosylases, its further oxidation products, Gh and Sp, are the best substrates for all three enzymes (Hailer et al., 2005; Krishnamurthy et al., 2008; Liu et al., 2010; Liu et al., 2013a). In terms of cellular function, NEIL1 acts in concert with the replication fork removing lesions before they are encountered by the replicative DNA polymerases (Hegde et al., 2013) while NEIL2 appears to function during transcription-coupled repair (Banerjee et al., 2011; Dou et al., 2003). The cellular function of NEIL3 remains elusive. However, expression of Neil3 is restricted to highly proliferating cells, including embryonic stem cells, pluripotent cells in

brain and hematopoietic cells in mice (Hildrestrand et al., 2009; Regnell et al., 2012) and cancer cells in human (Hildrestrand et al., 2009; Kauffmann et al., 2008).

G-quadruplex (G4) is a four-stranded DNA structure containing two or more layers of guanine quartets, each of which consists of four guanines that Hoogsteen base pair to each other. Monovalent cations (i.e. K^+ and Na^+) stabilize G4 DNA by coordinating layers of guanine quartets. Quadruplex structures have been proposed to play regulatory roles during lagging strand replication, gene transcription, mRNA translation and telomeric DNA elongation (Lipps and Rhodes, 2009). Bioinformatics studies revealed that G4-forming sequences are prevalent throughout the human genome. Interestingly, the distribution of these sequences is not random and telomere regions and promoter regions of genes are the two places enriched for potential G4-forming sequences (Huppert and Balasubramanian, 2005; Todd et al., 2005).

Telomeres are DNA-protein complexes at the ends of chromosomes that prevent degradation, undesired fusion, and improper activation of DNA damage response pathways (de Lange, 2009; Hackett et al., 2001). Human telomeres consist of 2-20 kb of 5'-TTAGGG-3' repeats, the bulk of which is double-stranded with an approximately 150 bp 3' single-stranded overhang at the very end (Greider, 1996; McElligott and Wellinger, 1997). Telomeric DNA sequences can form G4 structures under physiological salt conditions *in vitro* (Smith and Feigon, 1992; Williamson et al., 1989). A number of quadruplex structures containing the human telomeric sequence have been solved in both Na^+ (Wang and Patel, 1993) and K^+ (Parkinson et al., 2002; Phan et al., 2007) solutions

using NMR and X-ray crystallography. Circular dichroism (CD) has been used as a routine method to study G-quadruplex DNA folding. There are three common quadruplex topologies based on the directionality of neighboring strands, namely parallel, antiparallel, and hybrid (type 1 and type 2), each having a characteristic CD spectrum (Figure 3-1 A and B). Although most structural studies of quadruplexes have been done *in vitro*, there is increasing evidence supporting the existence of quadruplex structures in cells (Biffi et al., 2013; Yang et al., 2009).

Because guanine has the lowest redox potential among the four bases, telomere DNA provides a vulnerable target for oxidation both *in vitro* (Fleming and Burrows, 2013; Oikawa and Kawanishi, 1999) and in cells (Wang et al., 2010). Accumulation of base damages such as 8-oxoG in telomeres may hinder telomerase activity (Szalai et al., 2002) and disrupt the telomere-guarding shelterin complex (Opresko et al., 2005). Thus, base damages in telomeres have to be repaired in order to preserve telomere integrity. Shortening and loss of function of telomeres can also lead to cell senescence and premature aging syndromes in humans (Armanios, 2009). On the other hand, telomere lengthening due to overactive telomerase has been linked to cancer (Artandi et al., 2002).

Two groups have shown that promoter regions of many genes are rich in G4-forming sequences (Huppert and Balasubramanian, 2005; Todd et al., 2005). Over 40% of human genes have at least one potential G4-forming sequence near their promoter regions (Huppert and Balasubramanian, 2007). Quadruplex DNA structures present at promoter sites have been linked to the transcription of downstream genes. For example, the

nuclease hypersensitive element III1 (NHE III1) upstream of the P1 promoter of the human proto-oncogene *c-MYC*, controls up to 90% of the total *c-MYC* transcription (Berberich and Postel, 1995; Davis et al., 1989), which was later demonstrated to involve quadruplex formation at this site (Siddiqui-Jain et al., 2002). The human vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis and plays an important role in tumor survival, growth and metastasis (Ferrara, 1996; Weidner et al., 1992). The -88 to -50 bp region relative to the transcription initiation site of the *VEGF* gene, which contains multiple transcription factor binding sites (i.e., Sp1 binding sites (Finkenzeller et al., 1997)), forms a quadruplex structure and has been shown to function in *VEGF* transcription (Guo et al., 2008; Sun et al., 2011). Furthermore, quadruplex-interactive agents have been shown to repress both *VEGF* and *c-MYC* expression in human tumor cells (Ou et al., 2007; Sun et al., 2008). G-quadruplexes in promoter regions of genes have emerged as therapeutic targets in oncology, and stabilization of such structures has potential as a novel anticancer strategy (Balasubramanian et al., 2011).

We have previously shown that mouse Neil3 and human NEIL1 glycosylases are able to remove damaged bases from antiparallel G4 DNA formed in Na^+ solution (Zhou et al., 2013). Here, we studied the impact of hydantoin lesions on the thermostability of telomeric G4 DNA formed in K^+ solution as well as the removal of hydantoins from such structures. We have also investigated the removal of hydantoin lesions from quadruplexes formed by promoter sequences, using *VEGF* and *c-MYC* promoter sequences as models.

3.3. MATERIALS AND METHODS

Oligodeoxyribonucleotide synthesis and purification

Sequences of oligodeoxyribonucleotides (ODNs) used in this study are listed in Table 1. All 8-oxoG-containing ODNs were synthesized and deprotected by the DNA-peptide core facility at the University of Utah following the manufacturer's protocols (Glen Research, Sterling, Virginia). The crude samples were purified by semi-preparative ion exchange HPLC and desalted by dialysis against ddH_2O. The purified 8-oxoG ODNs were used in the synthesis of Sp and Gh. Synthesis of Gh and Sp was achieved by oxidation using K_2IrBr_6 in solution. Samples were purified by analytical ion-exchange HPLC and desalted by dialysis against ddH_2O. The detailed protocols for synthesis and purification of Gh and Sp can be found in Supplemental Information. Product purity was determined by analytical ion-exchange HPLC and product identity was determined by ESI-MS (Supplemental Figure 3-S1 and Figure 3-S2). Other ODNs used in this study, including the furan-containing ODNs, were purchased from Midland Certified Reagent Co. (Midland, TX), and were gel purified. All oligodeoxyribonucleotides were quantified by NanoDrop spectrophotometry using their extinction coefficients.

Circular Dichroism (CD) Analysis

G-quadruplex folding was characterized by CD spectroscopy using a JASCO spectrometer. First the G-quadruplex samples were annealed at a 10 μM concentration in 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7.4) and 100 mM KCl by heating the samples at 90 ^{o}C for 5 min followed by slowly cooling them

down to room temperature, after which they were placed at 4 ^{o}C for 48 hours prior to analysis. CD analysis of each sample was achieved on the annealed G-quadruplexes by scanning and averaging 10 scans of the CD spectrum. The data were background subtracted, normalized, and plotted as molar ellipticity vs. wavelength.

T_m Analysis

Thermal melting studies were conducted on the annealed G-quadruplex samples. The CD samples were diluted to a 3 μM G-quadruplex concentration using the buffer system described above. Next, the samples were placed in T_m cuvettes that were put into a UV-vis spectrometer (Shimadzu) equipped with a temperature control unit. The samples were thermally equilibrated at 20 ^{o}C for 5 min prior to commencing the heating cycle. The samples were heated at a rate of 0.5 ^{o}C/min and thermally equilibrated after each step for 1 min to achieve a final temperature of 100 ^{o}C that was then reversed back to room temperature via an identical decreasing temperature method. Absorbance readings at 260 and 295 nm were taken every 1 ^{o}C. The data were plotted as absorbance at 295 nm vs. temperature and the T_m values were determined from the first derivative of the curve as analyzed by the instrument's software (Shimadzu). The average and standard deviation values of quadruplicate trials are reported.

Enzyme purification

Cloning, expression, and purification of the glycosylase domain of the human NEIL3 glycosylase were recently published (Liu et al., 2012). Unless otherwise specified, NEIL3 used in this study was the glycosylase domain (NEIL3-GD) of the enzyme. NEIL1 and

NTH1 glycosylases were purified as previously described (Odell et al., 2010). NEIL2, OGG1 and APE1 were from our laboratory stocks and were purified as previously described (Bandaru et al., 2006). Because the cation identity is critical for folding of the quadruplex DNA, enzymes prepared in Na^+-containing buffer were dialyzed against K^+-containing buffer when assaying K^+-coordinated quadruplexes. Protein concentrations were determined by the BCA Protein Assay Kit (Thermo Scientific Pierce). The percentage of active glycosylase was determined by the Schiff base assay (for NEIL1, NEIL2, NEIL3 and OGG1) (Bandaru et al., 2006) or by the molecular accessibility method (for NTH1) (Blaisdell and Wallace, 2007), and all protein concentrations reported in this study were corrected for the active enzyme percentage.

DNA substrate preparation

Lesion-containing ODNs were ^{32}P-labeled at the 5' end by T4 polynucleotide kinase (NEB). The labeled ODNs were ethanol precipitated as previous described (Robey-Bond et al., 2008). Substrates typically contained 1 part hot ODN and 9 parts cold ODN. All G4-forming ODNs were annealed in quadruplex folding buffer (Qu buffer), which contains 20 mM HEPES-KOH (pH 7.4), 100 mM KCl and 1 mM EDTA. HEPES-NaOH (pH 7.4) and 100 mM NaCl were used when making Na^+-coordinated quadruplex DNA. To fold quadruplex DNA, the ODN in Qu buffer was heated at 95 ^oC for 5 minutes and slowly cooled to room temperature. The mixture was then stored at 4 ^oC overnight before being used for assays.

Native gel electrophoresis

G4 DNA substrates were prepared as described above. Samples were loaded with 5% glycerol on to a 16% acrylamide (29:1) native gel with 0.5X Tris-Borate-EDTA (TBE) and 100 mM KCl. The native gel was run in 0.5X TBE plus 100 mM KCl at 3 volts/cm overnight in 4 ℃, and the gel was then dried and exposed to a phosphorimager screen for detection.

Glycosylase/lyase activity assays

Glycosylase assays were done in the quadruplex reaction buffer, which contains 20 mM HEPES-KOH pH 7.4, 100 mM KCl, 1 mM EDTA, 0.1 mg/ml BSA and 1 mM DTT. In the case of Na^+-coordinated quadruplexes, HEPES-NaOH and NaCl were used in the buffer. Substrate concentration was typically 10 nM unless otherwise specified. Enzymes and substrates were incubated at room temperature or 37 ^oC as indicated. To measure glycosylase plus lyase activities, reactions were quenched by adding an equal volume of FE buffer (96% formamide, 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol) directly. To measure only the glycosylase activity, reactions were terminated by adding NaOH to a final concentration of 0.33 N and heated at 95 ℃ for 4 minutes. An equal volume of FE buffer was added to the reactions before loading on to a 12% urea gel for separation. The gel was dried and exposed on a phosphorimager screen. Finally, bands from the screen were scanned by Molecular Imager PharosFX Plus (Bio-Rad Laboratories) and quantified by Quantity One software (Bio-Rad Laboratories).

Single turnover kinetics

For single-turnover kinetics analysis of NEIL3-GD, 10 nM of substrate was incubated with 100 nM of enzyme in a total volume of 150 µl. An aliquot of 10 µl reaction mixture was taken out and quenched with 5 µl of 1 N NaOH. The same procedure was then carried out to obtain the gel images. The intensity of the bands was quantified by Quantity One and analyzed by GraphPad Prism 6 software. The catalytic rate (k_{obs}) was determined by fitting the data to a one phase association model.

3.4. RESULTS

Guanine oxidation alters the folding of telomeric quadruplexes and reduces their thermostability in KCl solution

All telomeric DNA sequences were folded in the quadruplex folding buffer containing K^+, in which the ODN without a lesion folds into a hybrid quadruplex structure, as previously determined (Fleming et al., 2013). The CD spectrum of each lesion-containing telomeric DNA was recorded in order to reveal the structure under the assay conditions. First, telomeric G4 DNA with a guanine lesion in an exterior quartet (position 9 and 11) gave similar CD spectra. These folds have maxima at 295 and 245 nm, and a minimum around 265 nm (Figure 3-2 A and C), which are consistent with an antiparallel fold that adopts a basket-like topology (Li et al., 2002). In addition, the thermal melting (T_m) studies for the G4 DNAs with lesions at either exterior quartet gave significantly reduced T_m values (-17 ^oC) for 8-oxoG, Gh, and the two Sp diastereomers (Figure 3-2D). Based on the CD spectra, the lesions at positions 9 and 11 convert the fold from a hybrid to a basket under identical conditions with a significant reduction in

thermal stability. Furthermore, 8-oxoG, Gh, and the Sp diastereomers are not capable of Hoogsteen H-bonding in the guanine quartet (Figure 3-1C); therefore, we propose that the damage induces the basket topology with two quartets and the lesion residing in a poorly defined context (Figure 3-2 A and C).

In contrast, the ODN with a guanine lesions in the center quartet (position 10) showed a dramatic change in the CD spectra recorded compared to the native hybrid fold (Figure 3-2B). Placement of damage in the center quartet gave a maximum peak at 263 nm and a minimum at 239 nm. The T_m values for all lesions in the middle quartet were ~30 ^oC lower than the native hybrid fold (Figure 3-2D). First, these observations were anticipated because 8-oxoG, Gh, and the Sp diastereomers cannot Hoogsteen base pair, and Gh and Sp are not planar, and therefore, cannot π-stack in the middle of a G4 fold (Figure 3-1C). Based on these observations, we propose that the damage is everted and the remaining Gs fold to a triplex-like structure with limited stability (Figure 3-2B). Consistent with this hypothesis are the CD profiles for triplex structures that were further characterized by optical tweezers (Koirala et al., 2012) and nanopore measurements (An et al., 2013). Taken together, introduction of lesions of G oxidation causes the human telomere G4 to adopt significantly different topologies than the non-damage-containing sequence with significant reduction in their thermal stability. Analogous results have been reported for 8-oxoG in a similar sequence (Vorlickova et al., 2012).

Next, the compacted secondary structures of these guanine lesion-containing G4 DNAs were assayed on a native gel (Figure 3-3A). Telomeric DNA with a guanine lesion

in an exterior quartet (position 9 or position 11) migrated faster than the single-strand random sequence (R) control and formed uniform bands, which suggested that these sequences form compact DNA structures, presumably G-quadruplexes. Telomeric DNA with a Gh, (*S*)-Sp or (*R*)-Sp in a middle quartet (position 10) migrated a little more slowly than the quadruplexes, agreeing with the prediction of a triplex DNA structure for these sequences. Additionally, a minor band was observed that appears to be single-stranded DNA in (*S*)-Sp-10 and (*R*)-Sp-10; this observation is consistent with the greatly reduced stability of a quadruplex when a damaged base is introduced into the middle quartet (Virgilio et al., 2012).

Hydantoin guanine lesions but not 8-oxoguanine are readily removed by glycosylases NEIL1 and NEIL3 from telomeric quadruplex DNA and triplex DNA

Previously, we demonstrated that OGG1, NTHL1, NEIL1, NEIL2, and NEIL3 DNA glycosylases are incapable of removing 8-oxoguanine from an antiparallel basket G4 folded in NaCl solution (Zhou et al., 2013). In the current study, repair of 8-oxoguanine in human telomere G4 was studied in KCl solution, which is more similar to cellular conditions. Similar to the Na^+ study, none of the DNA glycosylases, including OGG1, initiated repair of 8-oxoG in any of the three positions studied (9, 10, and 11) (Figure 3-3B). Note that these reactions were carried out with 20-fold excess of enzyme relative to substrate.

On the other hand, Gh in the quadruplex/triplex structure can be rapidly removed by human DNA glycosylases NEIL1 and NEIL3 (Figure 3-3C). Similar results were

observed for (*S*)-Sp and (*R*)-Sp in the quadruplex/triplex structure (Supplemental Figure 3-S2). However, NEIL2 and NTH1 did not excise any of the hydantoin lesions from quadruplex or triplex DNA; this result is in contrast to the hydantoins being good substrates for NEIL2 in single-stranded DNA (Hailer et al., 2005) and moderate substrates for Nth in duplex DNA (Hazra et al., 2001).

We also tested the glycosylase plus lyase activities of the NEIL glycosylases. Telomeric quadruplex DNA with Gh was used as substrate, and the reactions were stopped by formamide/EDTA buffer to visualize the lyase activity after base removal. NEIL1 and NEIL3 were able to hydrolyze abasic sites, primarily at positions 10 and 11 (Supplemental Figure 3-S3). However, the lyase activity of NEIL3 was low including the control ssDNA substrate, which is consistent with observations that mouse (Liu et al., 2012) and human NEIL3 (Krokeide et al., 2013) are primarily monofunctional glycosylases. In addition, NEIL2 showed no glycosylase/lyase product as predicted, since NEIL2 does not have glycosylase activity on Gh in quadruplex DNA in the first place (Supplemental Figure 3-S3 B and Figure 3-3 C).

Lesions at the preferred oxidation site in telomeric quadruplex DNA are resistant to excision by NEIL1 and NEIL3

Our previous study on quadruplex oxidation revealed that hydantoins were the major oxidation products, and identified the 5’ guanine of a GGG stretch as the most reactive site toward one-electron oxidants in the G4 context (Fleming and Burrows, 2013). With this information, the glycosylase repair efficiency toward G lesions was measured for the

hydantoin lesions specifically synthesized at each site of the $G_9G_{10}G_{11}$ run. We first measured the glycosylase activities of NEIL1 and NEIl3 on these substrates under multiple turnover conditions (Figure 3-4). These results show that NEIL1 and NEIL3 preferentially remove hydantoins from the middle and 3'-sites of a GGG stretch (position 10 and position 11), while damages at the 5'-site of a GGG (position 9) were not efficiently removed (Figure 3-4 A-C). Specifically, when 10 nM hydantoin (Gh, (*S*)-Sp or (*R*)-Sp)-containing G4 DNA was incubated with 4 nM enzyme for 30 minutes, about 50% of the lesions located at the middle or 3' sites were removed, while only about 15% of the 5' lesions were removed. These observations also support the conclusion that it is the position of the hydantoin in the G4 DNA, and not its chemical architecture, that dominates the removal efficiency (Figure 3-4 A-C).

We have previously shown that the sequence surrounding the lesion may significantly affect the glycosylase activity (sequence context effect) (Zhou et al., 2013). To determine if the reason why lesions at position 9 were inefficiently removed by NEIL1 and NEIL3 was due to sequence context effect, double-stranded substrates with the same sequences were assayed under the same condition. Figure 3-4D shows that NEIL1 removes Gh, (*S*)-Sp and (*R*)-Sp at the three positions with the same or comparable efficiency. As expected, NEIL3 exhibits a lower efficiency than NEIL1 (Figure 3-4E) on duplex DNA; however, there was no observable sequence context effect on the ability of NEIL3 to remove the Sp diastereomers. Although NEIL3 had higher activity on dsGh11 than dsGh9, dsGh9 was a better substrate than dsGh10, which does not explain the inefficient lesion removal from position 9 in the quadruplex. Comparison of the G4 and duplex results suggests that the

differential activity of NEIL1 and NEIL3 at positions 9, 10, 11 in G4 is not a result of sequence context, but rather the difference in the structures of the lesion-bearing G4 DNAs.

In order to better compare the base removal efficiency, kinetic rates of NEIL3 were measured under the single turnover conditions (enzyme in a 10-fold excess) (Figure 3-5). As observed above, the 5' hydantoin lesions were removed from the quadruple/triplex at the slowest rate by NEIL3 (Figure 3-5 A and B). For all three hydantoins, the time course studies revealed that NEIL3 removes the middle and 3' hydantoins much more rapidly (Figure 3-5A for (*S*)-Sp and Figure 3-S4 for (*R*)-Sp and Gh). Not surprisingly, the kinetic rates (k_{obs}) again showed that NEIL3 preferred the middle and 3' hydantoins over those in the 5' position, with differences ranging from 2- to 7-fold (Figure 3-5B). We also measured k_{obs} for NEIL3 using the corresponding duplex DNA under the same conditions (Figure 3-5 C and D). There was no significant difference in the removal of the ds(*S*)-Sp and ds(*R*)-Sp by NEIL3 among three positions studied, and the differences among dsGh9, dsGh10, and dsGh11 does not explain the inefficiency of removal of Gh9 in the quadruplex DNA context (Figure 3-5 C and D). Therefore, we conclude that hydantoin lesions present in the major site for damaging guanine (5', position 9) are not efficiently removed by NEIL1 or NEIL3 glycosylases, and that the structural differences of G4 DNA, but not the sequence context effects or lesion identity of the hydantoin, play the dominant role in the efficiency of damage removal from the quadruplexes.

An extra telomeric 5’-TTAGGG-3’ repeat allows alternative folding of the quadruplex and facilitates removal of the resistant 5’ hydantoin by the NEIL glycosylases

The high reactivity of the 5’ G toward oxidation leading to hydantoin products that are not properly removed by the BER glycosylases created a paradox. Therefore, we looked at a more relevant telomere context by adding a fifth 5’-TTAGGG-3’ repeat to those previously studied. Next, synthesis of 5-repeat ODNs with an 8-oxoG or a Gh at the 5’-G of the first, third, or fifth 5’-TTAGGG-3’ repeat was undertaken. The CD spectra for 5-repeat G4s with an 8-oxoG or a Gh was in contrast to the disruptive effect exhibited by the guanine lesions in the four-repeat quadruplex DNA. The CD data suggest that all 5-repeat G4 DNAs fold into hybrid-like structures, with the damage-containing repeat extruded from the fold when oxidized (Figure 3-6 A-C). Furthermore, the T_m values for all 5-repeat G4 DNAs were similar, which support the idea that the damage is not impacting the global G4 structure (Figure 3-6D). Based on these results, we propose that damage (8-oxoG or Gh) in the 5’-repeat is part of a 5’-tail on a hybrid fold, while damage in the 3’-repeat is part of a 3’-tail on a hybrid fold (Figure 3-6 A and C). Lastly, when the damage was placed in the middle repeat, the lesion-containing repeat is part of an extended edgewise loop that maintains the hybrid topology (Figure 3-6B).

The activities of NEIL1, NEIL2, and NEIL3 on the lesion-containing 5-repeat quadruplex DNA were then determined. Both NEIL1 and NEIL3 removed Gh (at the 5’ most site of a GGG triad) from all three 5-repeat quadruplexes (white bars) much faster than observed for the four-repeat quadruplex DNA (black bar) (Figure 3-6 E and G). For

example, under our assay conditions, NEIL1 removed more than 50% of the Gh from the three 5-repeat quadruplexes compared to 12.5% from the four-repeat quadruplexes. Surprisingly, while it was shown earlier that NEIL2 was not able to remove any hydantoins from the four-repeat quadruplexes, NEIL2 did remove Gh from the 5-repeat quadruplexes (compare Figures 6F and 3C). Thus it appears that the resistant lesions in quadruplex DNA may be removed through an alternative folding mechanism, which makes the lesion more accessible. This may be also true in cells considering the extensive availability of extra 5'-TTAGGG-3' repeats in telomeres.

APE1 cleaves furan at selected positions in Na^+-coordinated quadruplex DNA but not in K^+-coordinated quadruplex DNA

Abasic sites are one of the most abundant DNA lesions in genomes and their repair is essential for cell survival (Guillet and Boiteux, 2003; Lindahl and Nyberg, 1972). This led us to study the activity of APE1 on abasic sites in quadruplex DNA. To measure the activity of APE1 on quadruplex DNA, ODNs with furan (a stable abasic site analog) were used. We first examined the structures of furan-containing telomeric sequences. Furan was introduced 5', middle and 3' of the $G_9G_{10}G_{11}$ stretch (F9, F10, and F11). We also looked at furan in a loop region (F13). In K^+-containing buffer, all the furan-containing telomeric sequences presented the 295 nm maximum, indicating antiparallel quadruplex structures. However, F10 appeared to be a hybrid form of quadruplex (Figure 3-7A). Interestingly, there was no difference among the T_m values of F9, F10, or F11, which all exhibited a 20 ^oC drop in T_m compared to the non-damage-containing hybrid

quadruplex DNA (Figure 3-7B). This reduction of thermostability agrees with a previous report on abasic site-containing quadruplexes in K^+ solution, except that a greater drop in T_m was observed when a center G was replaced with an abasic site (Virgilio et al., 2012).

We tested APE1 activity on these furan-containing quadruplexes formed in KCl buffer, and we found no cleavage of furan in any position. We tried enzyme concentrations up to ten fold excess to substrate and there was still little detectable product (Figure 3-7C and 7H for quantification). In contrast, APE1 activity was robust on the corresponding duplex controls in K^+ buffer, which went to completion under the same reaction conditions (Figure 3-7 G and H).

Since secondary structure is required for the activity of APE1 on so called 'single-stranded' DNA (Fan et al., 2006), we tested its activity on quadruplexes in Na^+ solution. CD experiments were first carried out. All quadruplexes containing furan presented a dominant 295 nm antiparallel maximum, and in the case of F9 and F11, the G4s adopted a basket antiparallel structure in NaCl buffer, with the 295 nm maximum and 265 minimum (Figure 3-7D). F10 also appeared to be a basket quadruplex in Na^+, but the minimum at 265 nm was not as prominent. F9, F10, and F11 all gave a T_m drop of ~15 ^oC in buffered NaCl solution, but there was also no difference in T_m among the three. In addition, the loop F13 had the same T_m as telomeric DNA without a lesion (Figure 3-7E).

We then tested the incision activity of APE1 on furan in Na^+-coordinated quadruplexes. APE1 cleaved furan at position 9 and 10 in Na^+-coordinated quadruplexes (Figure 3-7F and 7H for quantification), although the activity was much slower than the

optimal duplex DNA structure (Figure 3-7G and 7H for quantification). The quadruplex containing F10 was the best substrate of the four in Na^+ buffer. By comparing the CD spectra of Na^+- and K^+- coordinated quadruplexes of F10, APE1 appeared to prefer the basket form of antiparallel quadruplex. Our results here are another example of secondary structure-dependent activity of APE1. We thus conclude that APE1 cleaves furan in Na^+-coordinated telomeric basket quadruplex DNA but not in K^+-coordinated hybrid quadruplex DNA.

DNA glycosylases cannot remove 8-oxoG and Gh from K^+-coordinated promoter quadruplexes/triplexes

Given the potential importance of quadruplexes in regulating gene transcription, we asked if glycosylases could remove guanine damages from the quadruplex DNA that potentially forms at promoter regions of critical genes. We chose the purine-rich nuclease hypersensitive elements of *c-MYC* and *VEGF* promoters as model sequences due to their critical cellular functions. Moreover, structures of quadruplexes formed by these two model sequences have been solved by NMR in KCl (Agrawal et al., 2013; Phan et al., 2004).

We initiated our structural studies and enzymatic activity assays in a buffer containing KCl. The *VEGF* promoter sequence we used (VEGF-Pu22) forms a propeller-type parallel quadruplex structure in buffered K^+ solution (Agrawal et al., 2013). An 8-oxoG or a Gh was introduced at positions 12 and 14, which are located in a loop and in a G-quartet core, respectively, in the above-mentioned structure. When the loop G (G12)

was replaced by 8-oxoG or Gh in the *VEGF* promoter quadruplex, the *VEGF* sequences showed a positive 265 nm peak and a negative 240 nm peak (Figure 3-8A), suggesting an all parallel, propeller-type quadruplex DNA structure. This is supported by the fact that there was no change in the T_m (Figure 3-8C). However, when a guanine in G-quartet (G14) was replaced by 8-oxoG or Gh, the CD spectrum red-shifted and it had a nearly identical spectrum to the triplex DNA we previously reported (Figure 3-8B and (An et al., 2013)), suggesting formation of triplex DNA in the K^+ buffer. There was also a T_m drop of 20 ^oC when G14 was substituted by 8-oxoG14 or Gh14 in the *VEGF* quadruplex DNA (Figure 3-8C), which supports the triplex conformation of these two substrates.

In the case of the *c-MYC* promoter quadruplex, we used the Myc-2345 sequence, which contains the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} G-tracks of the five-track 27-nt sequence (Pu27) of the *c-MYC* promoter (Phan et al., 2004). Phan *et al.*, show that the Myc-2345 sequence also forms a propeller-type parallel G-quadruplex (Phan et al., 2004). Interestingly, replacing G with either 8-oxoG or Gh at either position 8 (in an exterior G-quartet) or position 11 (in a loop) did not cause any observable difference in the CD spectrum. All Myc-2345 sequences showed a positive 265 nm peak and a negative 240 nm peak (Figure 3-S6 A and B), suggesting an all parallel, propeller-type quadruplex DNA that is the same as the non-damage-containing sequence (Phan et al., 2004). The T_m studies of the *c-MYC* promoter quadruplexes agreed with their CD spectra. The T_m values of the lesion-containing Myc-2345 quadruplexes had the same values as the non-damage-containing quadruplexes (Figure 3-S6 C). These data suggest that G8 will be pushed into a loop

when damaged and G11 will be used to form a new quadruplex quartet, which is thermodynamically favored.

Glycosylase activity assays were carried out on these substrates. In K^+ buffer, 8-oxoG in the *VEGF* promoter quadruplex DNA could not be removed by any of the human glycosylases, including OGG1 (Figure 3-8D), similar to the results obtained with the telomeric quadruplexes. In addition, the Gh in the *VEGF* promoter quadruplex could not be removed by any glycosylase (Figure 3-8D). The positive controls showed that the enzymes we used were active (Figure 3-8D, right), and mass spectrometry data (Table S1) and cleavage of duplex ODNs experiments (see Figure 3-9D and below) confirmed that the ODNs we used here contained the correct damages at the correct positions. In duplex DNA with the same sequences, 8-oxoG and Gh could be appropriately removed by OGG1 and NEIL1, respectively (Figure 3-9D, right). Similar results were observed for the *c-MYC* promoter quadruplex (Figure 3-S8). No glycosylase was able to remove 8-oxoG or Gh from the *c-MYC* promoter quadruplex DNA in K^+ solution (Figure 3-S8 A). We thus conclude that glycosylases cannot remove guanine oxidation damages from K^+-coordinated, parallel quadruplex/triplex DNA containing the *c-MYC* or the *VEGF* promoter sequences.

The NEIL glycosylases do have glycosylase and lyase activities on Gh in Na^+-coordinated promoter quadruplexes

Because the promoter sequences can adopt different quadruplex structures in K^+ and Na^+ buffers (Risitano and Fox, 2005), we extended our promoter quadruplex study to

include buffered Na^+ solutions. CD spectra and T_m studies of the *VEGF* promoter sequences were carried out. First, the T_m of Na^+ quadruplexes were lower than the K^+ quadruplexes of the same sequence (Figure 3-9C), indicating Na^+ quadruplexes were less stable. Second, the CD spectra were also very different between the Na^+ and K^+ quadruplexes (Figure 3-9 A and B). The Na^+ quadruplexes displayed an extra positive peak at 295 nm that was absent from the K^+ quadruplexes. The combination of 295 positive, 265 positive and 240 negative peaks suggested that there was a mixture of parallel and antiparallel strand orientations in the Na^+ buffer. A similar result was observed when a guanine was replaced by inosine in a telomeric quadruplex (Risitano and Fox, 2005).

To our surprise, we observed very different results when glycosylase activity assays were done in Na^+-coordinated quadruplex DNA. All three NEIL glycosylases were able to remove Gh from *VEGF* promoter quadruplex DNA efficiently at both positions (Figure 3-9 D and E, Gh12 and Gh14). NTH1 had only marginal activity. We also found that OGG1 had slow activity on 8-oxoG in the *VEGF* promoter quadruplex at position 12 (Figure 3-9D).

In order to answer the question as to whether the different activities of glycosylases on K^+- and Na^+- coordinated quadruplexes was an effect of cations, glycosylase activities of the five enzymes on *VEGF* duplex DNA (containing 8-oxoG12 or Gh12) were compared in buffered K^+ or Na^+ solutions (Figure 3-9G). NEIL1, NEIL2 and NTH1 showed no significant difference in activity on duplex-Gh12 in K^+ or Na^+ solutions, and

the cation used did not affect the activity of OGG1 on the duplex-8-oxoG12 substrate. NEIL3 showed very little activity on duplex DNA in both K^+ and Na^+ solutions as expected. These observations suggest that the *VEGF* promoter sequence adopts different quadruplex structures in K^+ and Na^+ that are recognized differently by the glycosylases. As shown above, the *VEGF* promoter sequence in Na^+ showed a mixture of antiparallel and parallel strand orientations, while in K^+ it presented an all-parallel quadruplex structure (8-oxoG12 and Gh12) or a triplex structure (8-oxoG14 and Gh14). We reason that NEIL glycosylases primarily recognize damages in a *VEGF* promoter quadruplex that has an antiparallel strand arrangement.

We also asked if the NEIL glycosylases have lyase activity on quadruplex DNA after base removal. The reactions were started with Gh-containing quadruplexes as substrates and were stopped by formamide/EDTA stoppage buffer without NaOH. NEIL1 and NEIL2 had efficient lyase activity on quadruplex DNA producing strand breaks after base removal (Figure 3-9F and Figure 3-S5 A). On the other hand, there was very little strand break product (13%) when NEIL3 was incubated with the Gh-containing *VEGF* promoter quadruplex (Figure 3-9F), where 52% of Gh had been removed generating AP sites (Figure 3-9E), indicating that NEIL3 has slow lyase activity on AP sites in the *VEGF* promoter quadruplex. When we added APE1 to the NEIL3 reaction, we did not see a significant increase in the strand incision product, indicating that APE1 also does not cleave AP sites in *VEGF* promoter quadruplexes (Figure 3-S5 A).

We have done the same sets of experiments using the *c-MYC* promoter quadruplex in Na^+ buffer and found that the CD spectra of the *c-MYC* promoter sequence were very different from *VEGF* promoter sequence. All *c-MYC* sequences with or without a lesion gave a 265 positive peak and a 240 negative peak, suggesting an all-parallel, propeller-type of quadruplex (Figure 3-S7 A and B). In addition, there was also no significant difference in melting temperatures among these quadruplexes (Figure 3-S7 C). Moreover, the activity assays showed that as with the telomeric and *VEGF* quadruplex structures, the glycosylases were unable to remove 8-oxoG from Na^+-coordinated *c-MYC* promoter quadruplex (Figure 3-S7 D). Although we did observe some activity of the NEIL glycosylases on Gh-containing *c-MYC* quadruplex substrates, the rate was much slower than on those formed by *VEGF* sequences (both glycosylase activity alone (Figure 3-S7 E) and glycosylase plus lyase activity (Figure 3-S7 F)). These data support the conclusion that the NEIL glycosylases primarily remove hydantoin lesions from promoter quadruplex DNA having an antiparallel strand arrangement.

3.5. DISCUSSION

We have studied the structures and thermostability of telomeric quadruplexes containing guanine oxidative damages (8-oxoG, (*S*)-Sp, (*R*)-Sp and Gh) at multiple positions. The guanine lesions reduce the thermostability of telomeric quadruplex DNA and alter its folding in a lesion position-dependent manner, while the lesion type does not play a significant role. The general rule is that oxidation in the middle quartet results in the most disruptive effect to the quadruplex structure, which is consistent with several

other studies when guanine was substituted with an abasic site or 8-oxoguanie (Szalai et al., 2002; Virgilio et al., 2012). Here we propose the most reasonable structure for each lesion-containing substrate, based on their CD spectra and supported by T_m studies. The CD spectra of lesion-containing telomeric quadruplexes were not all perfectly characteristic of a particular quadruplex structure due to their dynamic nature in solution. Moreover, the fact that 8-oxoG placed in an exterior quartet of the human telomere sequence led to a structure that was so poorly folded that suitable NMR signals could not be obtained (Lech et al., 2011) is consistent with our results.

Because guanine has the lowest redox potential of the native bases, 8-oxoG is one of the most prevalent oxidative lesions in cells (van Loon et al., 2010). 8-oxoG can alter telomere sequences by mispairing with A (Shibutani et al., 1991) and it also inhibits shelterin proteins from binding to telomere DNA (Opresko et al., 2005). Further oxidation of 8-oxoG, results in the formation of hydantoins that may block replication of telomere DNA (Aller et al., 2010). We show here and previously (Zhou et al., 2013) that 8-oxoG cannot be removed from telomeric quadruplex DNA by human DNA glycosylases, including OGG1, which provides one explanation of why telomeres contain more 8-oxoG lesions than the rest of the chromosome (O'Callaghan et al., 2011). Upon further oxidation 8-oxoG to hydantoins, oxidized guanines can then be removed by the NEIL1 and NEIL3 glycosylases, as shown in this study.

We also show that abasic sites in quadruplex DNA can be hydrolyzed by the lyase activity of NEIL1 and NEIL3, although the rate of NEIL3 is slow (Figure 3-S3). NEIL1

and NEIL3 exhibited most of their activities at position 10 and position 11, which mirrored their glycosylase activity that was presumably rate-limiting; therefore, one could not make direct comparison of the lyase activities among positions or between enzymes. We could not determine if NEIL2 has lyase activity on abasic sites in G4 DNA, since we started with Gh-containing quadruplex DNA, which is not a substrate for NEIL2 (Figure 3-3C).

We previously showed that the NEIL3 and NEIL1 glycosylases were the principal enzymes that removed hydantoin lesions from one position in telomeric quadruplex DNA (Zhou et al., 2013). We also showed (Fleming and Burrows, 2013) that oxidation of quadruplex DNA results in hydantoins as major products at the outer quartets (5' followed by 3' of GGG); however, our current study shows that the 5' hydantoins are removed much less efficiently by the NEIL glycosylases. The alternative folding model of the 5-repeat quadruplex provides a solution to this dilemma. When extra 5'-TTAGGG-3' repeats are available, which is always the case considering the long human telomere repeats, damage-containing 5'-TTAGGG-3' stretches may be looped out from the quadruplex core and processed by the NEIL glycosylases. Repair of 8-oxoG in quadruplexes may also utilize this alternative folding mechanism, when the looped out region is annealed to a complementary strand.

A number of recent studies have shown that BER enzymes are required for telomere homeostasis. Mouse embryonic fibroblast (MEF) cells that lack either NTH1 or OGG1 glycosylase show telomere defects including telomere shortening and loss, concurrent

with increased DNA base damages in telomere DNA (Lu and Liu, 2010; Vallabhaneni et al., 2013). We did not find significant activity of NTH1 or OGG1 on telomeric quadruplex DNA in the current study or in our previous study (Zhou et al., 2013), suggesting these enzymes function primarily on telomere duplex DNA. The same interpretation applies to APE1, except that we showed APE1 could process furan at selected positions in a Na^+-coordinated quadruplex DNA albeit at a slow rate. Madlener *et al.* have recently shown that APE1 is required for telomere maintenance (Madlener et al., 2013). Taken together, our data suggest that NEIL1 and NEIL3 are the glycosylases that remove damages from telomeric quadruplex DNA, leading us to speculate on their function in telomeres. We have also shown that knock down of NEIL3 in human cells results in telomere defects (Zhou, unpublished data), suggesting that repair of quadruplex damage may be required for telomere maintenance, given the fact that the activity of NEIL3 on duplex telomeric DNA is slow. It will be interesting to see if cells where NEIL1 is knocked down also develop telomere defects. Nevertheless, it is clear that the BER glycosylases are involved in the repair of telomere DNA (both duplex and quadruplex) to ensure their proper function.

Several bioinformatics studies reported the enrichment of potential quadruplex-forming sequences at promoters of genes (Huppert and Balasubramanian, 2005; Todd et al., 2005), which led to a model of transcriptional control via assembly and disassembly of quadruplex DNA (Lipps and Rhodes, 2009). This model is supported by transcription studies of a number of biologically important promoters, including *c-MYC* and *VEGF* (Balasubramanian et al., 2011; Siddiqui-Jain et al., 2002). BER enzymes, OGG1

glycosylase in particular, have been implicated in gene transcription regulation by altering local DNA secondary structure and chromosomal arrangement (Perillo et al., 2008). Gillespie's group showed that G4-forming sequences are targets of oxidation in hypoxia-induced signaling, after which the BER enzymes, OGG1 and APE1, are recruited to these damaged G4 sequences (Sedoris et al., 2012). These same researchers also found that oxidative modifications of the hypoxia response elements (HREs) coincided with the onset of mRNA accumulation of hypoxia-inducible genes, including the *VEGF* gene (Gillespie et al., 2010). Based on these observations, they hypothesized a model of controlled DNA damage and repair to regulate gene expression, by altering transcription factor binding and increasing DNA/chromatin flexibility (Gillespie et al., 2010; Ziel et al., 2005). However, no direct link has been made between damage repair and secondary structure changes.

Here, we provide biochemical evidence that links damage base removal followed by strand breakage to potential gene regulation. The folding of promoter quadruplex DNA is dependent on coordinating ions, which leads to differential accessibility of glycosylases to the lesions. The NEIL glycosylases were able to remove hydantoin lesions from Na^+-coordinated (antiparallel), but not from K^+-coordinated (parallel) quadruplex DNA, which leads us to hypothesize a switch model of transcription via the differential actions of glycosylases on lesions in quadruplex DNA structures (Figure 3-S8).

NEIL1, NEIL2 and NEIL3 remove damaged bases from Na^+-coordinated antiparallel quadruplex DNA, and NEIL1 and NEIL2 are able to cleave the abasic sites produced.

The strand break created by NEIL2 leads to the collapse of quadruplex DNA (Figure 3-S5B). Interestingly, NEIL2 is linked to transcription-coupled repair (Banerjee et al., 2011). Our data suggest that NEIL2 may also function as a transcription initiator for certain genes by removing quadruplex DNA structures at their promoters. We could not determine if the quadruplex structure collapses after NEIL1 lyase activity, because the substrate remained bound to the enzyme (Figure 3-S5B).

Considering that K^+ is the major cation in cells, the parallel quadruplex structure is presumably the dominant structure formed *in vivo*, which sets the promoter to an 'OFF' position. Conversely, using structure-specific antibodies, Schaffitzel *et al*. found that the antiparallel quadruplex was the dominant form found in *Stylonychia lemnae* macronuclei (Schaffitzel et al., 2001). In addition, quadruplex folding proteins may be involved in the parallel/antiparallel quadruplex switch model. For example, the telomere end-binding proteins (TEBPs) promote formation of antiparallel quadruplex DNA in *stylonychia* (Paeschke et al., 2005). On the other hand, the C-terminus of nucleolin promotes the formation of a parallel G-quadruplex at the *c-MYC* promoter and inhibits its promoter activity (Gonzalez and Hurley, 2010b). We argue that the local concentration of K^+ and Na^+ may vary around G4 sequences of interest, which may also dictate which quadruplex structure is formed, the accessibility of the lesion in the quadruplex to glycosylases, and eventually the status of the promoter.

In conclusion, we show here that the NEIL glycosylases remove oxidized guanine lesions from quadruplex DNA structures formed by telomere sequences as well as *VEGF*

and *c-MYC* promoter sequences. These data suggest that the NEIL glycosylases may function in telomere maintenance by initiating base excision repair in quadruplex DNA. Our data also provide a biochemical explanation as to how DNA glycosylases may function in gene regulation by acting on quadruplex DNA formed at promoter regions, although *in vivo* experimental data are necessary to support this model.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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3.7. TABLE AND FIGURE LEGENDS

Table 3-1. Oligodeoxyribonucleotide (ODN) sequences used in this study. Tel, telomere DNA sequence with four repeats; 5-repeat, telomere DNA sequence with five repeats.

Figure 3-1. Folding, CD spectra and guanine oxidation of quadruplex DNA. (A) Folding of parallel propeller, antiparallel basket and hybrid (type 2) quadruplex DNA. (B) Representative CD spectrum of each quadruplex DNA. In a parallel quadruplex, all four strands point in one direction and the neighboring strands are connected with double reversal loops. The CD spectrum of a parallel quadruplex features a 265 nm maximum and a 240 nm minimum. The basket antiparallel quadruplex DNA has neighboring strands running in opposite directions and connected with two lateral loops and a diagonal loop. This structure features a 295 nm maximum and a 265 nm minimum. A hybrid (type 2) quadruplex has mixed strand directionalities and presents a 295 nm maximum, a 270 nm shoulder and a 235 nm minimum (see ref. (Karsisiotis et al., 2011)). (C) Chemical structures of guanine (G), 8-oxoguanine (8-oxoG) and guanidinohydantoin (Gh) in the context of a G quartet.

Figure 3-2. Guanine oxidation alters quadruplex folding and reduces the thermostability of quadruplexes. All samples were annealed in quadruplex folding buffer containing K^+. CD spectra were recorded at 20 ^oC. (A) The CD spectra of telomeric DNA with an 8-oxoG, Gh or Sp lesion at the 5' position of a GGG triad (Position 9) (B) The CD spectra of telomeric DNA with a guanine lesion in the middle of a GGG triad

(Position 10). (C) The CD spectra of telomeric DNA with a guanine lesion 3' of a GGG triad (Position 11). CD spectra for the Sp-containing quadruplexes were obtained on a mixture of diastereomers. (D) Guanine lesions reduce the thermostability of telomeric quadruplex DNA. Mean and standard deviation of the T_m values calculated from four experiments are shown.

Figure 3-2. Hydantoin lesions but not 8-oxoguanine are readily removed by glycosylases from quadruplex/triplex DNA. (A) Native gel electrophoresis shows that guanine lesion-containing telomere ODNs (Tel sequences) form compacted secondary structures and migrate faster than single-stranded DNA. Qu, quadruplex structure formed by Tel sequence without a lesion. R25, a single-stranded DNA control with random sequence. (B) No glycosylase shows activity on 8-oxoG-containing quadruple/triplex DNA. In the positive control, duplex R-8-oxoG was used for OGG1, duplex R-Tg was used for NTH1 and single-stranded R-Gh was used for NEIL1, 2 and 3. (C) NEIL1 and NEIL3 but not NEIL2 remove Gh from quadruplex DNA. 10 nM of each substrate was incubated with 200 nM of each glycosylase at room temperature for 30 min, and the reaction was stopped by adding NaOH and heating.

Figure 3-4. Lesions at the preferred oxidation site in quadruplex DNA are resistant to excision by glycosylases. (A) A representative gel image of the glycosylase assays. (B, C) Quantification of the glycosylase activities of NEIL1 (B) and NEIL3 (C) on quadruplex/triplex DNA containing Gh, (*S*)-Sp and (*R*)-Sp at three positions. (D, E) Quantification of the glycosylase activities of NEIL1 (D) and NEIL3 (E) on the

corresponding duplex substrates. Average percentage of base removal and the standard deviation from three replicates are shown. All reactions contained 10 nM substrate and 4 nM active enzyme. All reactions were stopped by adding NaOH and heating after incubation at room temperature for 30 min.

Figure 3-5. Catalytic rates (k_{obs}) of NEIL3 glycosylase on quadruplex DNA confirms poor cleavage of the 5' lesion. (A) Representative time courses of NEIL3 on (*S*)-Sp-containing quadruplex/triplex DNA used for calculating k_{obs}. (B) Catalytic rates of NEIL3 on three hydantoin lesions at three different positions in quadruplex/triplex DNA. (C) Representative time courses of NEIL3 on (*S*)-Sp-containing duplex DNA used for calculating k_{obs}. (D) Catalytic rates of NEIL3 on three hydantoin lesions at three different positions in duplex DNA. All reactions contained 10 nM substrate and 100 nM active NEIL3 enzyme. Reactions were stopped by mixing with NaOH and heating after incubating at room temperature at the indicated time points.

Figure 3.6. Alternative folding of the quadruplex facilitates removal of the resistant 5' hydantoin. (A-C) CD spectra of folded 5-repeat telomeric sequences with 8-oxoG or Gh at the 5', middle or 3' TTAGGG repeat. (D) Melting temperature (T_m) of 5-repeat quadruplexes with 8-oxoG and Gh at the 5', middle or 3' TTAGGG repeat. (E-G) Quantification of Gh removal in 5-repeat quadruplexes by NEIL1 (E), NEIL2 (F), and NEIL3 (G). The 5-repeat quantification results were compared to the values obtained with 4-repeat quadruplexes with Gh at position 9 (5' of GGG triad). 10 nM of substrate and 4 nM of active enzyme were incubated at room temperature for 30 min.

Figure 3-7. APE1 cleaves furan at selected positions in Na^+-coordinated, but not K^+-coordinated, telomeric quadruplex DNA. (A-B) CD spectra and T_m of furan-containing quadruplex DNA in KCl solution. (C) A representative gel shows that APE1 does not cleave furan from K^+-coordinated quadruplex DNA. (D-E) CD spectra and T_m of furan-containing quadruplex DNA in NaCl solution. (F) A representative gel shows that APE1 cleaves furan from position 9 and 10 in Na^+-coordinated quadruplex DNA. (G) APE1 activity on furan-containing control duplex substrates. 10 nM of furan substrate and 1, 10, or 100 nM of APE1 were incubated at 37 ^oC for 30 min. (H) Quantification of strand incision activity of APE1 on furan in K^+-coordinated (black bars) and Na^+-coordinated (grey bars) quadruplex DNA or control duplex DNA.

Figure 3-8. DNA glycosylases cannot remove 8-oxoG or Gh from K^+-coordinated *VEGF* promoter quadruplexes/triplexes. (A,B) CD spectra of the *VEGF* promoter quadruplex in K^+ solution with and without 8-oxoG or Gh lesions at position 12 (A) or 14 (B). (C) The T_m of *VEGF* promoter quadruplex in K^+ solution with and without 8-oxoG or Gh lesions at position 12 or 14. (D) Glycosylases show no activity on K^+-quadruplexes with the *VEGF* promoter sequence. 10 nM substrate and 200 nM of enzyme were incubated at 37 ^oC for 30 min.

Figure 3-9. The NEIL glycosylases remove Gh from Na^+-coordinated promoter quadruplexes. (A, B) CD spectra of the *VEGF* promoter quadruplex in Na^+ solution with and without a lesion at position 12 (A) or 14 (B). (C) T_m of the *VEGF* promoter quadruplex in Na^+ solution with and without 8-oxoG or Gh lesions at position 12 or 14.

(D) A representative gel of a glycosylase assay on Gh in a Na^+-coordinated *VEGF* promoter quadruplex showing that the NEIL glycosylases remove Gh from quadruplex structures. (E) Quantification of glycosylase activity on Gh in the *VEGF* promoter quadruplex. (F) Quantification of glycosylase plus lyase activity on Gh-containing *VEGF* promoter quadruplexes. (G). Comparison of the glycosylase activity of each glycosylase on duplex DNA in K^+ and Na^+ reaction buffers where no significant difference was found. For reaction with quadruplex DNA, 10 nM substrate and 200 nM of enzyme were used. For duplex DNA controls, 10 nM substrate and 10 nM enzyme were used. Reactions were incubated at 37 ^oC for 30 min and quenched with NaOH and heating to measure glycosylase activity, or quenched with formamide/EDTA to measure glycosylase plus lyase activity.

3.8. TABLES AND FIGURES

Table 3-1. Oligodeoxyribonucleotide (ODN) sequences used in this study.

Name	Sequence	Description
Tel-9	5'-TAGGGTTAXGGTTAGGGTTAGGGTT-3'	X = OG, Gh, (*S*)-Sp or (*R*)-Sp at 5'
Tel-10	5'-TAGGGTTAGXGTTAGGGTTAGGGTT-3'	X = OG, Gh, (*S*)-Sp or (*R*)-Sp in the middle
Tel-11	5'-TAGGGTTAGGXTTAGGGTTAGGGTT-3'	X = OG, Gh, (*S*)-Sp or (*R*)-Sp at 3'
5-repeat-5'	5'-TAXGGTTAGGGTTAGGG-TTAGGGTTAGGGTT-3'	X = OG or Gh at the 5' repeat of the 5-repeat sequence
5-repeat-mid	5'-TAGGGTTAGGGTTAXGG-TTAGGGTTAGGGTT-3'	X = OG or Gh at middle repeat of the 5-repeat sequence
5-repeat-3'	5'-TAGGGTTAGGGTTAGGG-TTAGGGTTAXGGTT-3'	X = OG or Gh at the 3’ repeat of the 5-repeat sequence
F9	5'-TAGGGTTAFGGTTAGGGTTAGGGTT-3'	Furan at 5' of GGG
F10	5'-TAGGGTTAGFGTTAGGGTTAGGGTT-3'	Furan at the middle of GGG
F11	5'-TAGGGTTAGGFTTAGGGTTAGGGTT-3'	Furan at 3' of GGG
F13	5'-TAGGGTTAGGGTFAGGGTTAGGGTT-3'	Furan in a loop region
VEGF-12	5'-CGGGGCGGGCCXGGGGCGGGGT-3'	X = OG or Gh
VEGF-14	5'-CGGGGCGGGCCGGXGGCGGGGT-3'	X = OG or Gh
c-MYC-8	5'-TGAGGGTXGGGAGGGTGGGGAA-3'	X = OG or Gh
c-MYC-11	5'-TGAGGGTGGGXAGGGTGGGGAA-3'	X = OG or Gh
R25	5'-ATTGACTTCTCCACTTGCTATTGAC-3'	A random 25-mer used as a ssDNA control
R-Gh	5’TGTTCATCATGCGTC[Gh]TCGG-TATATCCCAT-3′	Control sequence with Gh
R-8-oxoG	5’-TGTCAATAGCAAG[8-oxoG]GGAGAA-GTCAATCGTGAGTCT-3’	Control sequence with 8-oxoG
R-Tg	5’-TGTCAATAGCAAG[Tg]GGAGAA-GTCAATCGTGAGTCT-3’	Control sequence with Tg

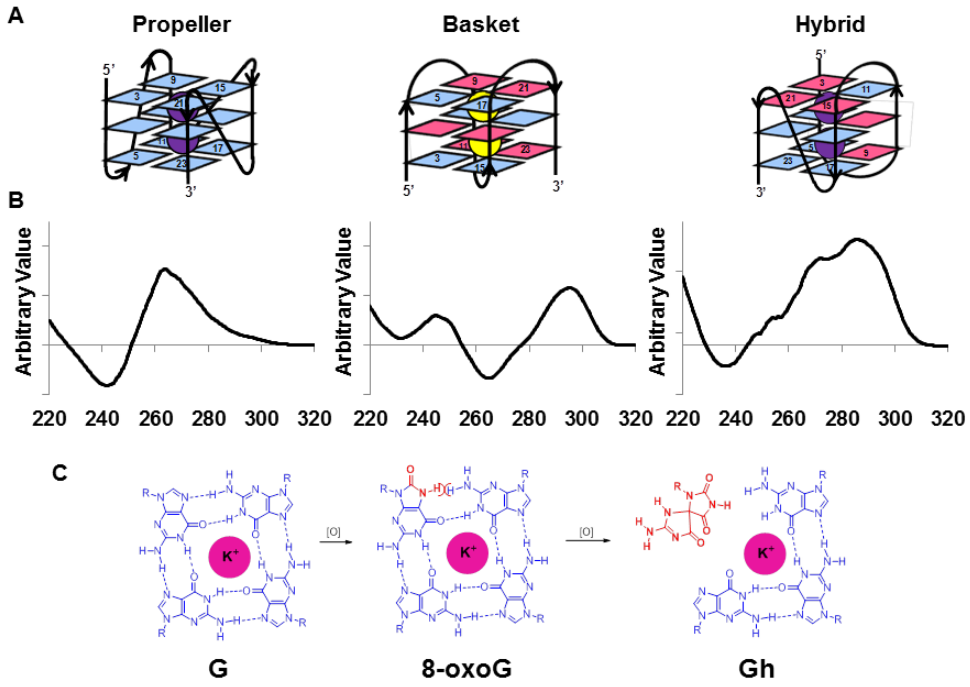


Figure 3-1. Folding, CD spectra and guanine oxidation of quadruplex DNA

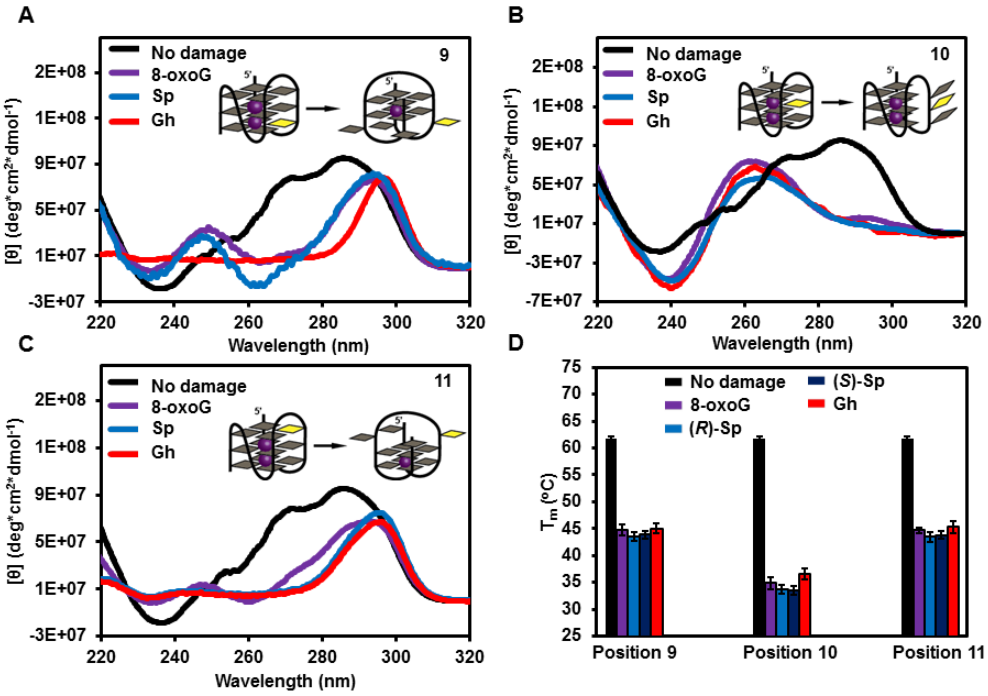


Figure 3-2. Guanine oxidation alters quadruplex folding and reduces the thermostability of quadruplexes.

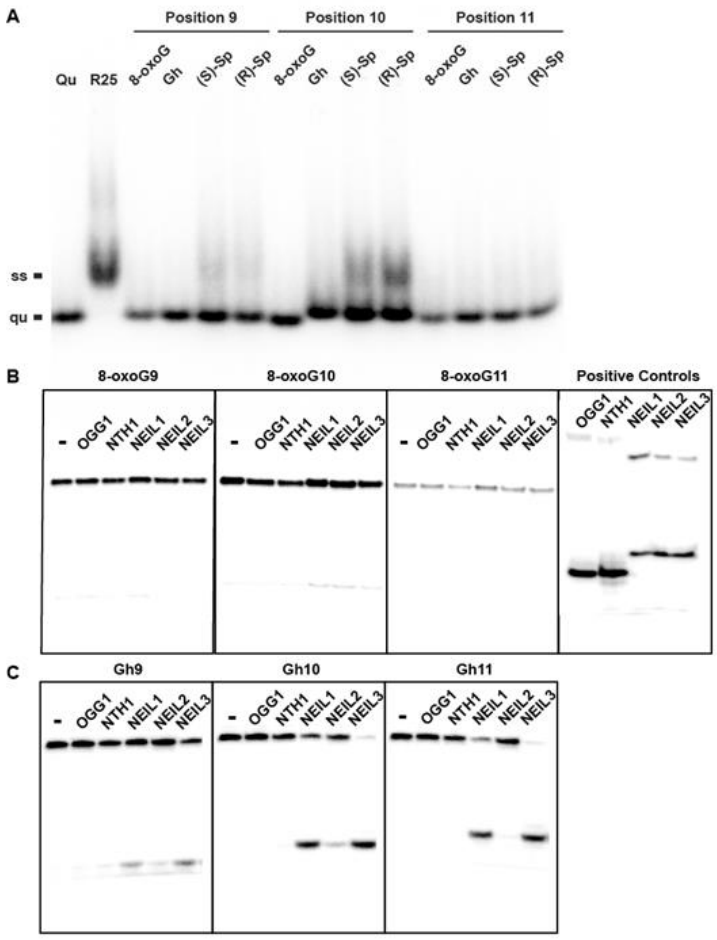


Figure 3-3. Hydantoin lesions but not 8-oxoguanine are readily removed by glycosylases from quadruplex/triplex DNA.

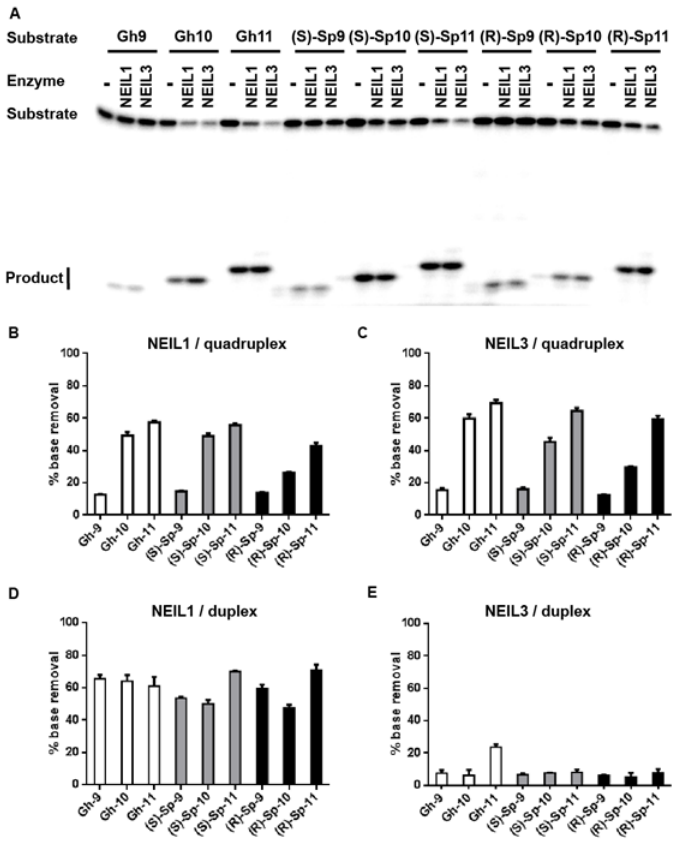
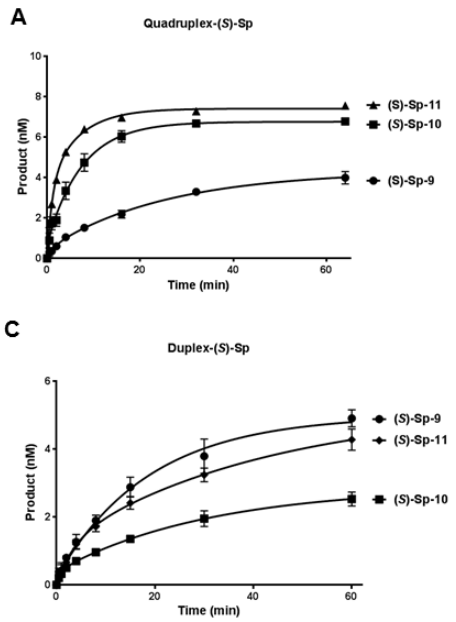


Figure 3-4. Lesions at the preferred oxidation site in quadruplex DNA are resistant to excision by glycosylases.



B Quadruplex, k_{obs} (min^{-1})

	9 (5')	10 (Middle)	11 (3')
Gh	0.05 ± 0.007	0.12 ± 0.004	0.29 ± 0.01
(*S*)-Sp	0.05 ± 0.004	0.15 ± 0.012	0.35 ± 0.02
(*R*)-Sp	0.07 ± 0.01	0.12 ± 0.004	0.14 ± 0.01

D Duplex, k_{obs} (min^{-1})

	9 (5')	10 (Middle)	11 (3')
Gh	0.089 ± 0.009	0.027 ± 0.005	0.166 ± 0.007
(*S*)-Sp	0.053 ± 0.004	0.045 ± 0.006	0.052 ± 0.004
(*R*)-Sp	0.060 ± 0.007	0.076 ± 0.005	0.074 ± 0.010

Figure 3-5. Catalytic rates (k_{obs}) of NEIL3 glycosylase on quadruplex DNA confirms poor cleavage of the 5' lesion.

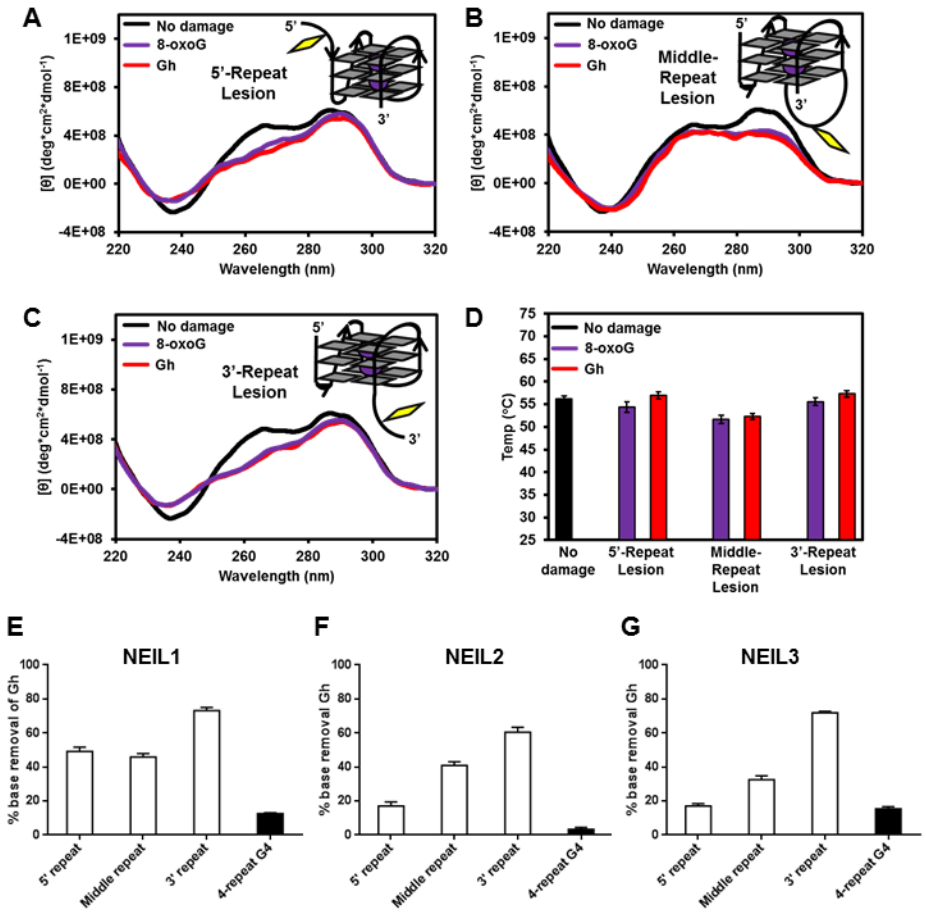


Figure 3-6. Alternative folding of the quadruplex facilitates removal of the resistant 5' hydantoin.

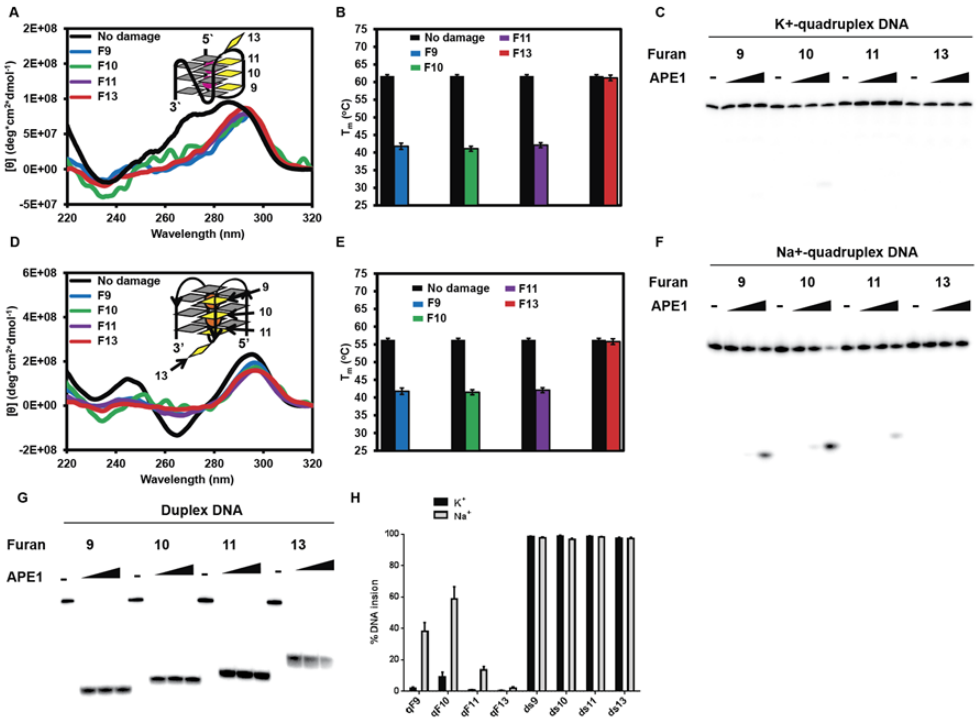


Figure 3-7. APE1 cleaves furan at selected positions in Na^+-coordinated, but not K^+-coordinated, telomeric quadruplex DNA.

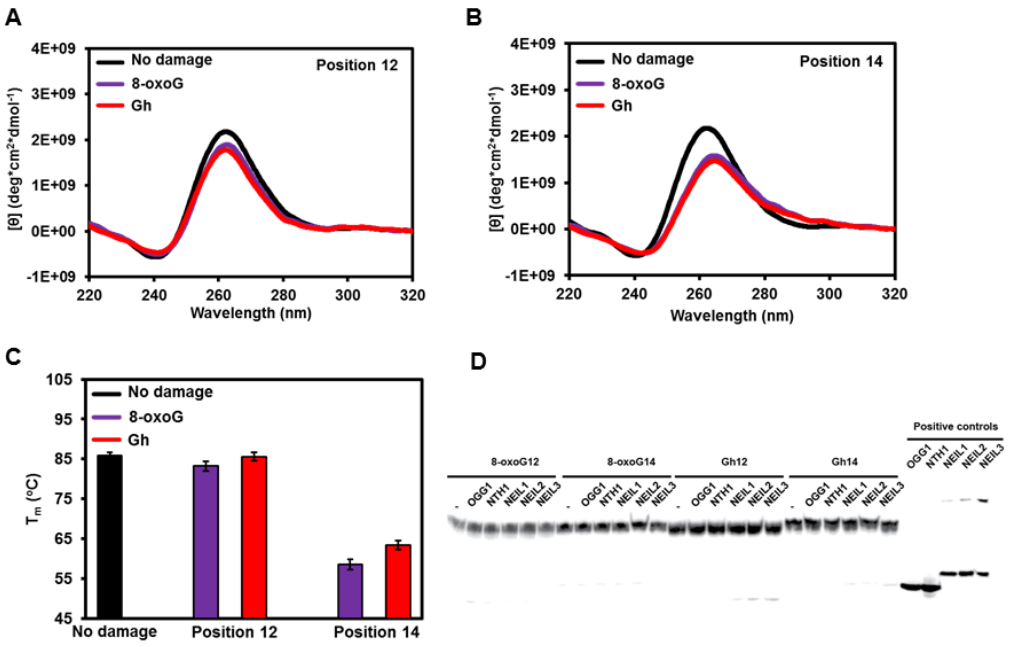


Figure 3-8. DNA glycosylases cannot remove 8-oxoG or Gh from K^+-coordinated *VEGF* promoter quadruplexes/triplexes.

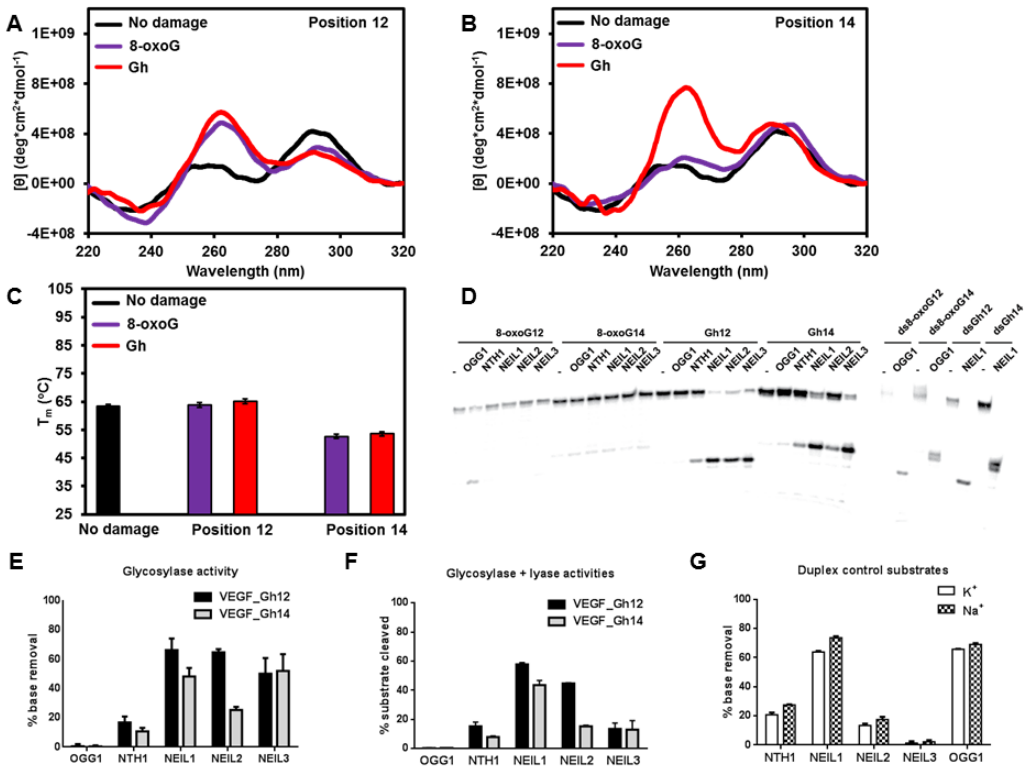


Figure 3-9. The NEIL glycosylases remove Gh from Na^+-coordinated promoter quadruplexes.

3.9. SUPPLEMENTAL INFORMATION

Supplemental Methods

Supplemental Table and Figure Legends

Supplemental References

Supplemental Table S1

Supplemental Figures S1-S8

Synthesis and Purification of Oligodeoxyribonucleotides (ODN)

All 8-oxoG-containing ODNs were synthesized and deprotected by the DNA-peptide core facility at the University of Utah following the manufacturer's protocols (Glen Research, Sterling, Virginia). The crude samples were purified by semi-preparative ion exchange HPLC running the following mobile phases: A = 1.5 M LiOAc (pH 7.0) in 10% MeCN and 90% ddH_2O, and B = 10% MeCN and 90% ddH_2O while running a flow rate of 3 mL/min and monitoring the absorbance at 260 nm. Purification salt was removed by dialysis against ddH_2O for 36 hr using a 3500 molecular weight cutoff (MWCO) membrane cassette. The purified samples were used in the synthesis of Sp and Gh via the following methods. Synthesis of Gh was achieved by mixing a 20 μM solution of 8-oxoG-containing ODN in ddH_2O at 20^oC with 120 μM K_2IrBr_6, while letting the reaction sit for 30 min. Synthesis of Sp was achieved by mixing a 20 μM solution of 8-oxoG-containing ODN in 10 mM NaP_i (pH 8.0) buffer preincubated at 45^oC, after which 120 μM K_2IrBr_6 was added, while letting the reaction sit for 30 min. The samples were purified using an analytical ion-exchange HPLC setup running the following mobile phases: A = 1 M LiCl, 25 mM Tris (pH 8.0) in 10% MeCN and 90% ddH_2O, and B = 10% MeCN and 90% ddH_2O while running a flow rate of 1 mL/min and monitoring the absorbance at 260 nm. Purification salts were removed by dialysis against ddH_2O for 36 hr using 3500 MWCO membrane cassettes. Product purity was determined by analytical ion-exchange HPLC and product identity was determined by ESI-MS (Figure S1 and Table S1). Oligodeoxyribonucleotides were quantified by NanoDrop spectrophotometry using their extinction coefficients.

Supplemental Table and Figure Legends

Table 3-S1. HPLC and ESI-MS analysis of the synthesized oligodeoxyribonucleotides. The (*) indicates that this value was determined on a mixture of the Sp diastereomers.

Figure 3-S1. HPLC analysis of the lesion-containing telomeric G4 and promoter G4 sequences. (A) HPLC traces of hydantoin-containing 4-repeat telomere sequences. The (*) indicates that the two peaks observed for Gh represent the interchangeable R and S diastereomers of Gh (Ye et al., 2003). (B) HPLC traces of 5-repeat telomere sequences with an 8-oxoG or a Gh. (C) HPLC traces of the *VEGF* promoter sequence with an 8-oxoG or a Gh at position 12 or position 14. (D) HPLC traces of the c-*MYC* promoter sequence with an 8-oxoG or a Gh at position 8 or position 11.

Figure 3-S2. NEIL1 and NEIL3 remove *(S)*-Sp and *(R)*-Sp from quadruplex/triplex DNA. NEIL1, human NEIL3 and mouse NEIL3, but not OGG1, NEIL2 or NTH1, remove (*S*)-Sp (A) and (*R*)-Sp (B) from quadruplex/triplex DNA. 10 nM of each substrate was incubated with 200 nM of each glycosylase at room temperature for 30 min, and the reaction was stopped by adding NaOH and heating.

Figure 3-S3. NEIL1 and NEIL3 exhibit lyase activity on an AP site in telomeric quadruplex DNA. 10 nM Gh-containing quadruplex DNA was incubated with 10 nM of enzyme at room temperature for 30 min. The reactions were stopped by formamide/EDTA buffer to visualize glycosylase plus lyase activities. Lyase activity of NEIL1 (A), NEIL2 (B), and NEIL3 (C) on abasic sites in telomeric quadruplex DNA after removal of Gh is shown (mean and standard deviation from three experiments).

Figure 3-S4. Reaction time courses for NEIL3 k_{obs} measurements. NEIL3 shows poor cleavage of the 5' lesions (Gh and (*R*)-Sp) in quadruplex DNA (A and B), which is not the case for duplex

DNA (C and D). All reactions contain 10 nM substrate and 100 nM active NEIL3 enzyme. Reactions were stopped by mixing with NaOH and heating after incubating at room temperature at the indicated time points.

Figure 3-S5. NEIL1 and NEIL2 show glycosylase plus lyase activities on promoter quadruplex DNA and the lyase activity disrupts quadruplex folding. 10 nM Gh-containing promoter quadruplex DNA was incubated with 100 nM of enzyme at 37^{o}C for 30 min. (A) The reactions were stopped by formamide/EDTA buffer to visualize glycosylase plus lyase activities on a urea denaturing gel. Quantifications of these images were shown in Figure 9F and Figure S7F. (B) The same reactions were mixed with glycerol (5% final) and load to a native gel to visualize quadruplex DNA disruption.

Figure 3-S6. Glycosylases cannot remove 8-oxoG or Gh from K^+-coordinated *c-MYC* promoter quadruplexes. The CD spectra of *c-MYC* promoter quadruplexes in K^+ solution with and without a lesion at position 8 (A) and position 11 (B). (C) T_m values of the *c-MYC* promoter quadruplexes in K^+ solution. (D) Glycosylase assays with K^+-quadruplexes with the *c-MYC* promoter sequence. 10 nM of substrate and 200 nM of enzyme were incubated at 37^{o}C for 30 min.

Figure 3-S7. The NEIL glycosylases remove Gh from the Na^+-coordinated *c-MYC* promoter quadruplexes. The CD spectra of *c-MYC* promoter quadruplexes in Na^+ solution with and without a lesion at position 8 (A) and position 11 (B). (C) T_m values of the *c-MYC* promoter quadruplexes in Na^+ solution. (D) Glycosylase activity on Na^+-quadruplexes with the *c-MYC* promoter sequence. (E) Quantification of glycosylase activity on Gh-containing quadruplexes with the *c-MYC* promoter sequence. (F) Quantification of glycosylase plus lyase activity on Gh-containing quadruplexes with the *c-MYC* promoter sequence. 10 nM of substrate and 200 nM of enzyme were incubated at 37^{o}C for 30 min.

Figure 3-S8. A model for promoter activation by the glycosylase action on promoter quadruplex DNA. When a quadruplex is present at a promoter (i.e., *VEGF* promoter), transcription of the downstream gene is off. Oxidative stress induces oxidative damages in the susceptible G-rich quadruplex DNA structure. The oxidation of guanine, Na^+/K^+ exchange, or quadruplex folding proteins may induce the quadruplex to a topology that is recognizable by glycosylases. Glycosylases remove the damaged base from the quadruplex DNA and break the DNA backbone, which causes collapse of the quadruplex DNA structure and allows activation of the promoter.

Supplemental References

1. Ye, Y., Muller, J. G., Luo, W., Mayne, C. L., Shallop, A. J., Jones, R. A., and Burrows, C. J. (2003) Formation of 13C-, 15N-, and 18O-labeled guanidinohydantoin from guanosine oxidation with singlet oxygen. Implications for structure and mechanism. *J Am Chem Soc* 125, 13926-13927

Table 3-S1. HPLC and ESI-MS analysis of the synthesized oligodeoxyribonucleotides.

Sample	Sequence	Calculated Mass, Da	Experimental Mass, Da
Tel	5'-TA GGG TTA GGG TTA GGG TTA GGG TT	7878.2	7878.4
Tel-9	5'-TA GGG TTA XGG TTA GGG TTA GGG TT		
X =			
Gh		7884.2	7884.6
(*S*)-Sp		7910.2*	7909.6
(*R*)-Sp			
8-oxoG		7894.2	7894.4
Tel-10	5'-TA GGG TTA GXG TTA GGG TTA GGG TT		
X =			
Gh		N.D.	N.D.
(*S*)-Sp		N.D.	N.D.
(*R*)-Sp		N.D.	N.D.
8-oxoG		N.D.	N.D.
Tel-11	5'-TA GGG TTA GGX TTA GGG TTA GGG TT		
X =			
Gh		N.D.	N.D.
(*S*)-Sp		N.D.	N.D.
(*R*)-Sp		N.D.	N.D.
8-oxoG		N.D.	N.D.
5-repeat	5'-TA GGG TTA GGG TTA GGG TTA GGG TTA GGG TT	9787.4	9787.2
5-repeat with lesions at 5' repeat			
X=	5'-TA XGG TTA GGG TTA GGG TTA GGG TTA GGG TT		
8-oxoG		9803.4	9803.2
Gh		9793.4	9793.6
5-repeat with lesions at middle repeat			
X=	5'-TA GGG TTA GGG TTA XGG TTA GGG TTA GGG TT		
8-oxoG		N.D.	N.D.
Gh		9793.4	9793.7
5-repeat with lesions at 3' repeat			
X=	5'-TA GGG TTA GGG TTA GGG TTA GGG TTA XGG TT		
8-oxoG		N.D.	N.D.
Gh		9793.4	9793.6
VEGF (Pu22)	5'-CG GGG C GGG CC GGGGG C GGG GT	6945.5	6945.8
Position 12			
X=	5'-CG GGG C GGG CC XGGGG C GGG GT		
8-oxoG		6970.5	6971.4
Gh		6960.5	6960
Position 14			
X=	5'-CG GGG C GGG CC GGXGG C GGG GT		
8-oxoG		6970.5	6971.2

Gh		6960.5	6960.8
c-MYC (Myc2345)	5'-TGA GGG TGGGG A GGG T GGGG AA	7040.6	7040.8
Position 8			
X=	5'-TGA GGG TXGGG A GGG T GGGG AA		
8-oxoG		7056.6	7056.8
Gh		7046.6	7047.1
Position 11			
X=	5'-TGA GGG TGGGX A GGG T GGGG AA		
8-oxoG		7056.6	7056.8
Gh		7046.6	7047.2

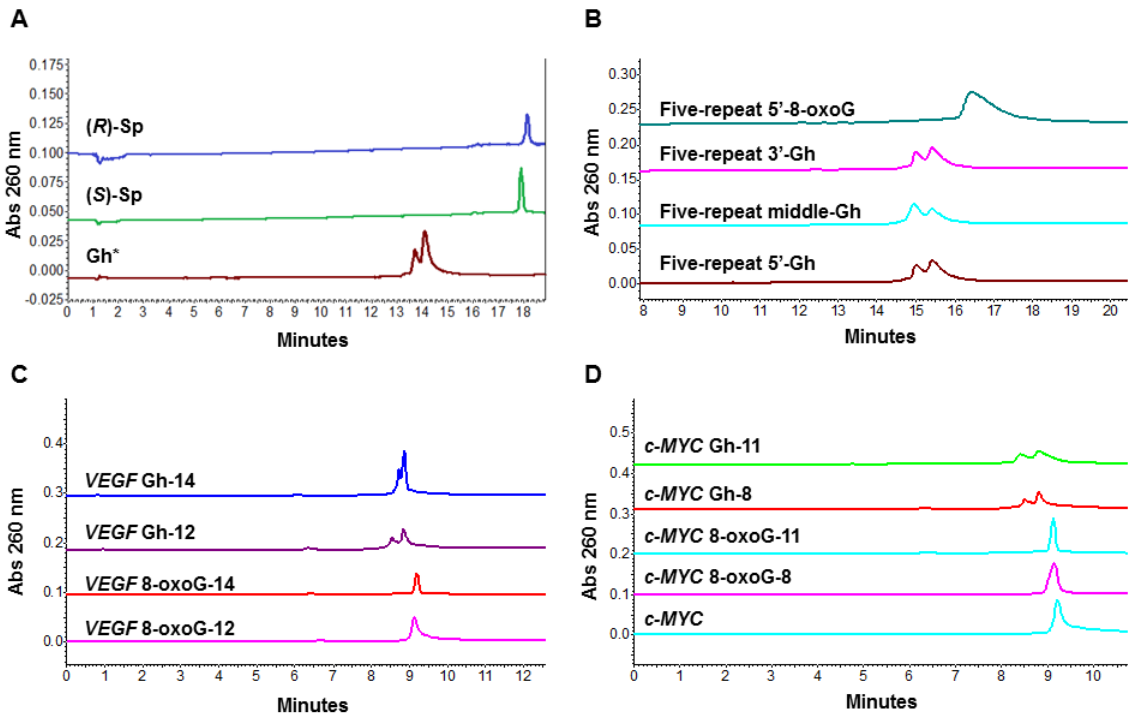


Figure 3-S1. HPLC analysis of the lesion-containing telomeric G4 and promoter G4 sequences.

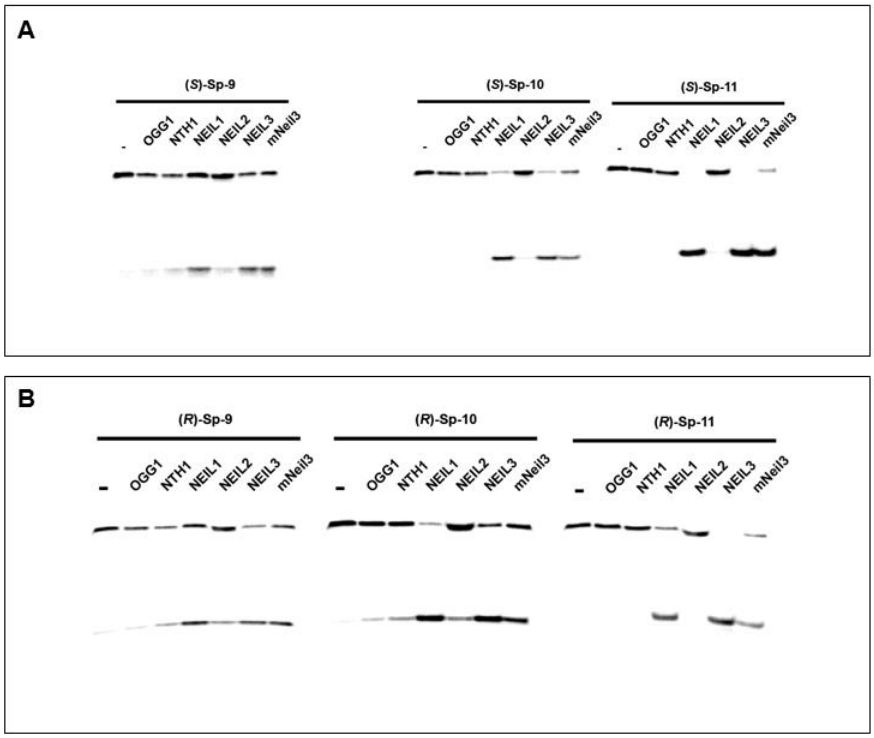


Figure 3-S2. NEIL1 and NEIL3 remove (S)-Sp and (R)-Sp from quadruplex/triplex DNA.

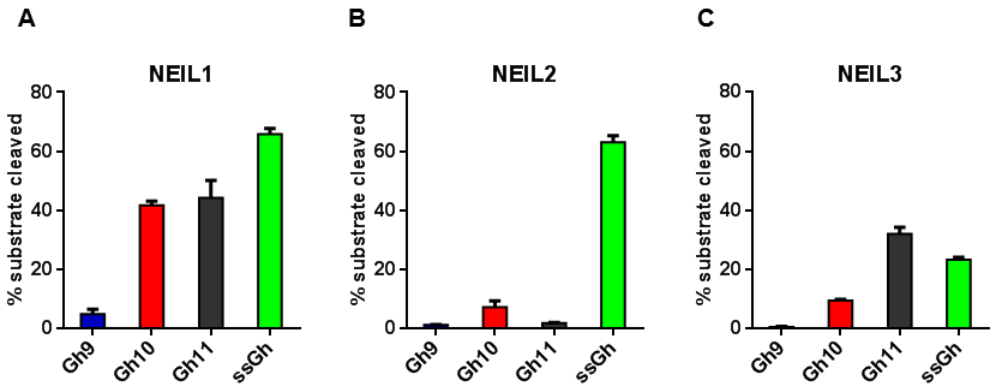


Figure 3-S3. NEIL1 and NEIL3 exhibit lyase activity on an AP site in telomeric quadruplex DNA.

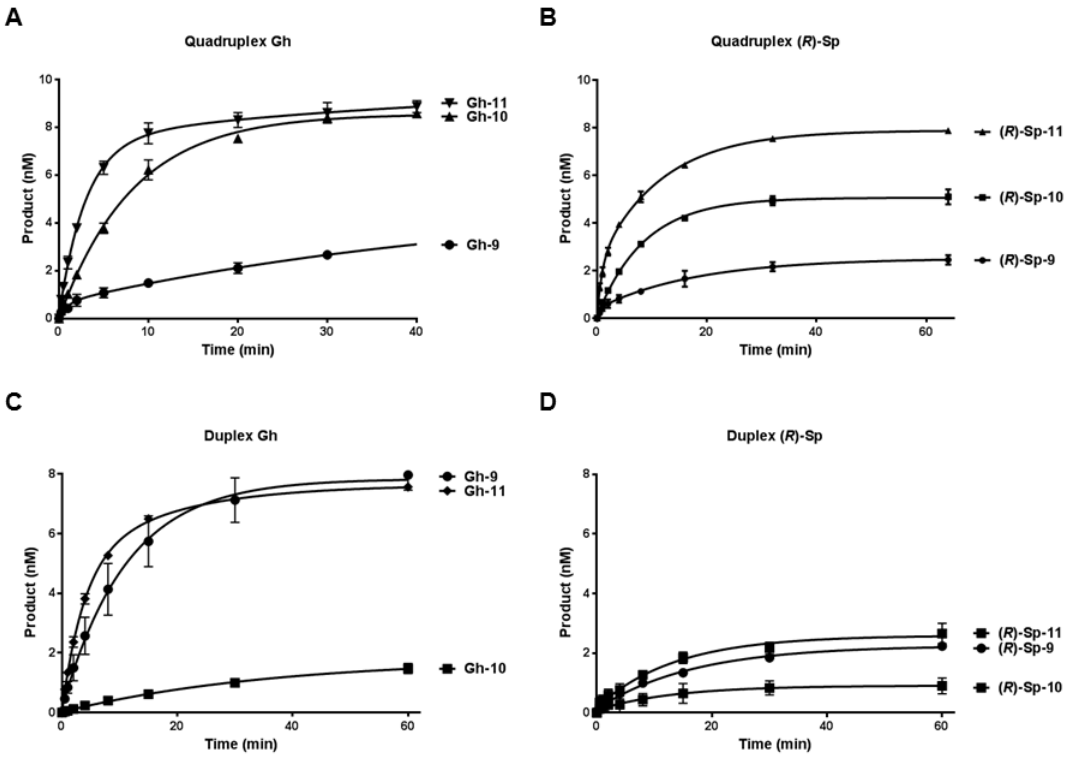


Figure 3-S4. Reaction time courses for NEIL3 kobs measurements.

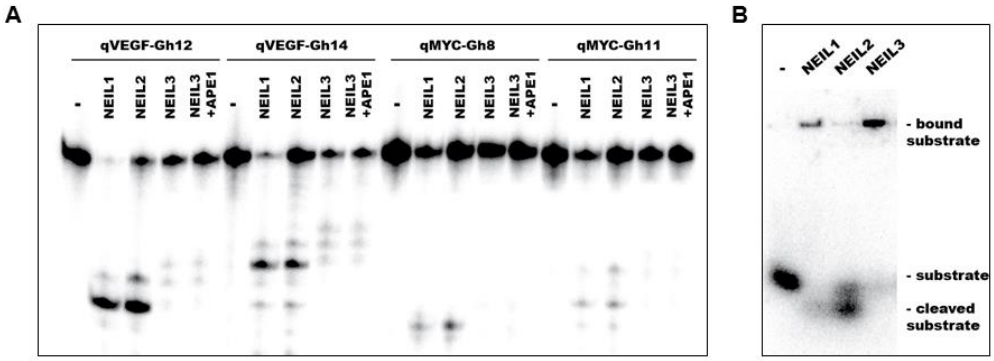


Figure 3-S5. NEIL1 and NEIL2 show glycosylase plus lyase activities on promoter quadruplex DNA and the lyase activity disrupts quadruplex folding.

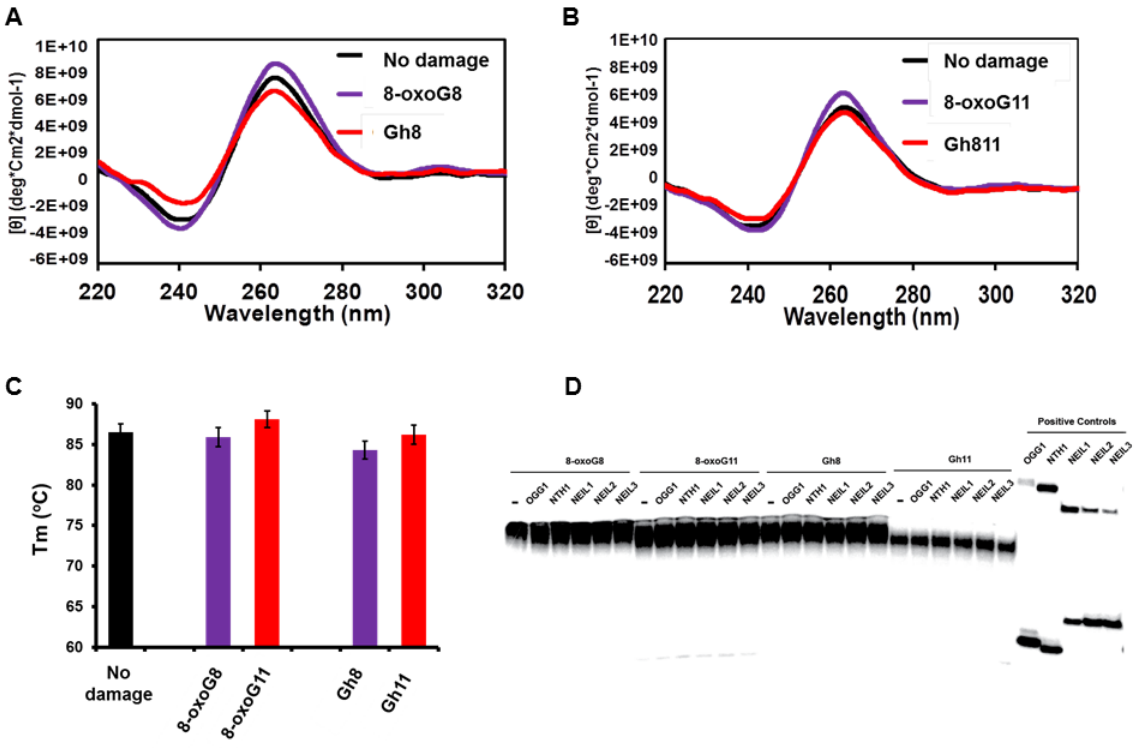


Figure 3-S6. Glycosylases cannot remove 8-oxoG or Gh from K^+-coordinated c-MYC promoter quadruplexes.

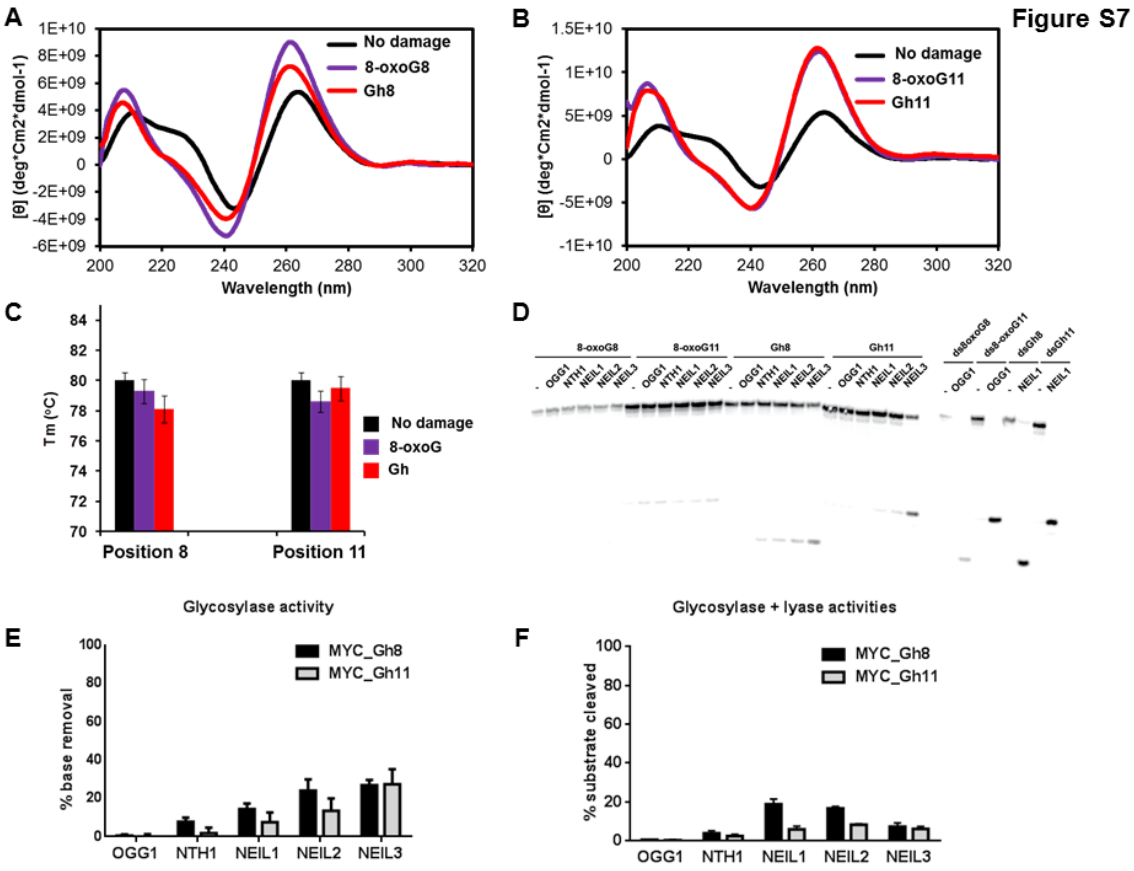


Figure 3-S7. The NEIL glycosylases remove Gh from the Na^+-coordinated c-MYC promoter quadruplexes.

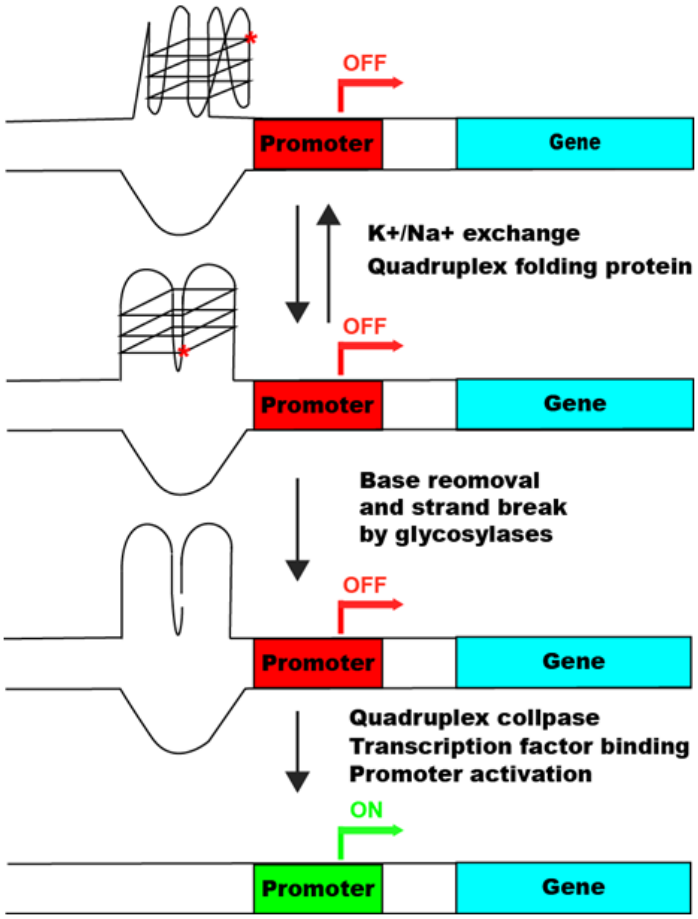


Figure 3-S8. A model for promoter activation by the glycosylase action on promoter quadruplex DNA.

CHAPTER IV: CONCLUSIONS, SIGNIFICANCE AND FUTURE DIRECTIONS

4.1. CONCLUSIONS

In this dissertation, G-quadruplex DNA structures that contain damaged DNA bases were studied as well as the removal of damages from these structures by DNA glycosylases. Tg- and 8-oxoG-containing antiparallel quadruplex DNA formed by telomere sequences in Na^+ solution were first examined. Interestingly, no glycosylase is able to remove 8-oxoG from quadruplex DNA and mouse Neil3 is the only DNA glycosylase that can remove Tg from a loop of antiparallel quadruplex DNA. However, NEIL1 and mNeil3, but not NEIL2, are able to excise Sp and Gh from telomeric quadruplex DNA in Na^+ solution. Also, mNeil3 shows a marked preference for Tg in the telomere sequence context.

Telomeric quadruplex DNA formed in K^+ solution was also examined and we found that hydantoin lesions reduce quadruplex stability and alter their folding. Similar to the results with Na^+-coordinated quadruplex DNA, 8-oxoG was not removed by any of the glycosylase but its oxidation products Gh and Sp were efficiently removed by NEIL1 and NEIL3. Another interesting observation was that hydantoin lesions at the most oxidation-prone position were not efficiently removed by either NEIL1 or NEIL3, but adding an extra TTAGGG repeat solved the dilemma by extruding the damage-containing repeat to a loop. In the presence of the fifth repeat, the lesion-containing repeat is extruded to a large loop that is more accessible to the NEIL glycosylases, including NEIL2.

Quadruplex DNA formed by promoter sequences was also studied. Promoter sequences of the *c-MYC* and *VEGF* genes were used as models because of their biological importance. DNA glycosylases were not able to remove Gh from K^+-coordinated promoter quadruplexes. However, the NEIL glycosylases are able to remove Gh from Na^+-coordinated promoter quadruplex DNA, especially those that adopt an antiparallel strand orientation. A model of quadruplex-dependent

4.2. SIGNIFICANCE

While base removal by DNA glycosylases has been well studied in double-stranded and single-stranded DNA, studies in this dissertation demonstrated, for the first time, that some DNA glycosylases can also remove damaged bases from quadruplex DNA structures. Since the G-quadruplex-forming sequences and the G-quadruplex DNA structures are highly susceptible to oxidation, repair of damages in quadruplex DNA structures is likely to be important for proper cell function. That NEIL glycosylases are able to remove damages from quadruplex DNA suggests that they may play a role in telomere maintenance. This may be particularly true for NEIL3, because this enzyme also prefers lesions in the telomere sequence context. DNA base damages are deleterious to telomeres and have to be removed to ensure the proper function of telomeres. An increasing number of studies have shown that DNA glycosylases and other BER enzymes play critical roles in telomere maintenance. The studies presented in this dissertation demonstrate that DNA glycosylases may be involved in telomere maintenance by removing base damages from quadruplex DNA.

Telomeres, by protecting the chromosome ends from fusion and DNA damage responses, plays critical role in genome stability. Research in maintenance of telomeres will help us understand the mechanisms of cancer and aging. I hope that results in this dissertation will inspire researchers to investigate the role of NEIL1 and NEIL3 DNA glycosylases in telomere maintenance and, ultimately, their function in cancer biology and aging prevention.

The NEIL glycosylases remove Gh and Sp from quadruplex DNA structures formed by promoter sequences, suggesting that they may function in the regulation of gene transcription. These studies extend our current understanding of DNA glycosylases as repair enzymes and show that DNA glycosylases may play other roles in cells besides their repair function. The observations presented in this dissertation provide the biochemical foundation for research on the biological functions of quadruplex DNA *in vivo*. We also proposed an oxidation-induced, quadruplex- and glycosylase-dependent mechanism for gene regulation. Since many oncogenes possess quadruplex-forming sequences at their promoters, understanding the role of these quadruplexes in gene transcription is important for cancer oncology.

4.3. FUTURE DIRECTIONS

Despite the interesting observations presented in this dissertation, more experiments need to be done to further support the proposed hypotheses. The results suggest that the NEIL glycosylases may play a role in telomere maintenance and regulation of gene transcription. Although the biochemical data support such a hypothesis, more studies have to be done before a solid conclusion can be drawn.

4.3.1. The Mechanism of the Marked Telomere Sequence Context Effect Exhibited by Neil3 Glycosylase

It will be interesting to understand the mechanism of the preference of Neil3 for Tg and 5-OHU in the telomere sequence context. The k_{obs} (Table 2-2) measured under single turnover conditions imply that a faster chemistry step is contributing to the preference for the telomere sequence. However, the question of whether binding of NEIL3 to different sequences plays a role was not addressed. Although the binding affinities of the catalytically inactive mNeil3 mutant (E3Q) to Tg-containing oligodeoxynucleotides (ODNs) with and without telomere sequence context were compared, the binding of mNeil3E3Q to all the substrates was so low that the binding parameters by EMSA (electrophoresis mobility shift assay) could not be determined. Nevertheless, visually there did not seem to be any difference in binding of mNeil3E3Q to ODNs with or without the telomere sequence context. A different approach could be used to obtain the parameters of enzyme/substrate binding. Michaelis–Menten enzyme kinetics would be a suitable approach. Catalytic constants (k_{cat}) at various substrate concentrations could be

measured and the Michaelis–Menten constant (K_m) determined. Comparison of the *K*m of ODNs with and without sequence context will answer the question as to whether binding contributes to the marked sequence context effect.

We argue that the reason why Neil3 preferentially removes Tg from the telomere sequence context resides in the different availability of the Tg in the telomere sequence compared to the random sequence. It has been known for quite some time that Tg is a helix-distorting lesion. In a crystal structure of a DNA polymerase with a Tg-containing DNA template, Aller *et al.* show that the methyl and hydroxyl groups present on the 5' position of thymine glycol severely altering the stacking of the 5' base and in the case of guanine, a hydrogen bond is formed with Tg (Aller et al., 2007). Our random sequence (5'G-Tg-G3') contains a G at 5' of Tg but the telomere sequence (5'T-Tg-G3') does not. Thus the Tg in the context of the "random sequence" would be less able to be extruded into the Neil3 substrate binding pocket than the one in telomere sequence context. It will be interesting to ask if the hydrogen bond that supports the extrahelical Tg contributes to the accessibility of the lesion. Substitution of the T at 5' (of Tg) with a G in the telomere sequence would be a perfect substrate to study. A crystal structure of Neil3 with a bound Tg-containing ODN would elucidate the structural basis for the sequence context effect. However, this may be difficult to achieve, since we have shown that the binding of Neil3 to Tg-containing substrates is poor.

Collectively, our data show that Neil3 is very sensitive to sequence context surrounding the lesion compared to the other four glycosylases. The sequence context

effect is well accepted but its mechanism is poorly understood. One of the hypotheses is that the local thermal stability governs the spontaneous opening of the duplex (DNA breathing) and thus the activity of Neil3, since Neil3 prefers single-stranded DNA. This could be further tested by measuring the rates of excision by Neil3 on substrates with a combination of sequences around the lesion. These experiments will address if DNA breathing contributes to the sequence context effect of Neil3 in duplex DNA and will also determine the effective range of the sequence context effect.

4.3.2. Removal of Damaged Bases from Quadruplex DNA Structures

We have studied base removal in G-quartets and in a loop region in Na^+ solution, but we have not investigated how DNA glycosylases access lesions in loop regions in K^+ solution. Since telomere sequences form antiparallel quadruplexes in Na^+ solution and form parallel or hybrid quadruplexes in K^+ solution, the conformation of the connecting loops are different in the two salts. Thymine damages (i.e. Tg) and adenine damages (i.e. 8-oxoA) in a loop region in a K^+-coordinated quadruplex would be suitable substrates to study this.

The human telomeres contain kilobases of TTAGGG repeats (McElligott and Wellinger, 1997). However, current research is mostly done with sequences containing four of these repeats. Therefore it is biologically meaningful to study quadruplexes formed by more than four repeats. Quadruplex folding by sequences longer than four repeats will be helpful for understanding the organization of secondary structures *in vivo.* In addition, we have done these experiments with damage-containing five repeat

sequences in K^+ solution, and similar experiments in Na^+ solution will provide the field with a more complete picture. Longer sequences such as an eight-repeat or more should be studied. Base damages can be introduced to these sequences to study their removal by DNA glycosylases.

Many promoter quadruplex-forming sequences harbor more than four G-runs that are capable of forming G-quartets (*i.e.* the *VEGF* and *c-MYC* promoter studied here). We have studied the truncated versions of the *VEGF* and *c-MYC* promoter sequences that only contain four G-runs. Studies with the wild type sequences will provide information such as which G-runs may be involved in the formation of G-quartets *in vivo*. Enzymatic studies with lesions in the quadruplex formed by the wild type sequences are critical, since the removal of damages in G-quartets and loop regions can be completely different. For example, if a lesion-containing G-run is extruded from the G-quartets, it will possess characteristics of single-strand DNA rather than a more compact quadruplex DNA structure and thus a more promising substrate for some glycosylases (i.e. NEIL2 and NEIL3).

4.3.3. Studies of Quadruplex DNA *in vivo*

Although accumulating evidence shows that quadruplex DNA structures exist *in vivo*, more efforts are needed to close the debate. Currently, the best evidence has been obtained using quadruplex-specific antibodies in fixed cells. Antibodies that are specific for different topologies of quadruplex DNA are urgently needed. In addition,

fluorophore-conjugated compounds that specifically bind to quadruplex DNA will also be invaluable; especially those that could be used in live cell imaging.

However, much can be accomplished with quadruplex-specific antibodies and compounds. Biffi *et al.* demonstrated that quadruplex DNA exists in human cells and that quadruplex DNA is present in telomeres (Biffi et al., 2013). Given the implication that quadruplexes are involved in telomerase regulation, it will be interested to study the dynamics of quadruplex DNA in telomeres during cell cycle and also during the administration of quadruplex-stabilizing reagents. These experiments will provide a solid link between the inhibition of telomerase activity by quadruplex DNA and the anticancer properties of the quadruplex-stabilizing drugs.

In addition, direct monitoring of the quadruplex DNA structures at promoter regions can be extremely helpful for understanding the role of such structures in gene regulation. This can be done by simultaneously monitoring quadruplex structures at promoter regions and the transcription of the downstream gene. With appropriate antibodies or compounds, such an approach can be extended to study the function of quadruplex DNA during DNA replication.

4.3.4. Studies of Hydantoin Lesions *in vivo*

We show in Chapters II and III that 8-oxoG in quadruplex DNA is not efficiently removed by any DNA glycosylase, including OGG1. Our data suggest that 8-oxoG may be further oxidized to Gh and Sp before being removed by NEIL1, NEIL3, and potentially NEIL2. However, the existence of Gh and Sp in cells is still under

investigation and unclear. Antibodies that are specific for the hydantoins with a high affinity will open a new chapter in DNA damage and repair.

With these antibodies, many biochemical and cell-based assays can be done to address critical questions including whether hydantoin lesions are present in cells. Although the formation of hydantoin lesions is likely to occur *in vivo* due to the low redox potential of 8-oxoG, there is no solid evidence that these lesions exist *in vivo.* Secondly, antibodies specific for hydantoins would allow researchers to examine how the levels of hydantoin lesions change with oxidative stress. In addition, it will be particularly interesting to study if the hydantoin levels in different regions of the genome vary. One may speculate that G-rich regions such as the telomeres will have higher levels of hydantoin lesions than other regions, similar to results obtained with 8-oxoG (O'Callaghan et al., 2011; Vallabhaneni et al., 2013; von Zglinicki, 2002; Wang et al., 2010). Lastly, since our data show that NEIL3 removes hydantoin lesions from quadruplex DNA much faster than from duplex DNA, it will be interesting to measure the levels of hydantoin lesions in telomeres upon NEIL3 depletion.

4.3.5. Function of DNA Glycosylases in Telomere Maintenance

We have studied the base removal and abasic site cleavage steps of the base excision repair pathway and the results suggest that the NEIL glycosylases may play a role in repair of telomere base damages and thus telomere maintenance *in vivo*. We have studied telomere abnormalities in cells where NEIL3 is knocked down. Using fluorescence *in situ* hybridization (FISH), we have shown that the NEIL3 knockdown cells have an increased

frequency of telomere defects including telomere loss and fusion (unpublished data). Moreover, our data also show that NEIL3 interacts with the shelterin protein TRF1. It will be interesting to compare the activity of NEIL3 in the presence and absence of TRF1 and also the localization of TRF1 in the presence and absence of NEIL3. Because NEIL3 prefers Tg in both single-stranded and double-stranded telomere DNA, it will also be important to understand how POT1 and TRF2 affect the activity of NEIL3.

The experiments discussed above can be extended to NEIL2 and, in particular, to NEIL1, since we have shown that NEIL1 removes hydantoins from quadruplex DNA and prefers Gh in the telomere sequence context. Telomere morphology studies of the NEIL glycosylase-depleted cells using FISH will provide valuable information. Quantitative FISH (qFISH) can be applied to study the function of the NEIL glycosylases in telomere length maintenance. Multiple base excision repair enzymes have been documented to be involved in telomere maintenance, including OGG1, NTH1, UDG, APE1 and Polβ ((Lu and Liu, 2010; Vallabhaneni et al., 2013; Vallabhaneni et al., 2015; Wang et al., 2010) and see Chapter I) and experiments proposed above will answer the question as to whether the NEIL glycosylases also function in telomere maintenance.

4.3.6. Function of DNA Glycosylase in Gene Regulation

In Chapter III, we proposed a mechanism of how DNA glycosylases can act on damage-containing quadruplex DNA at telomeres and regulate gene transcription (Figure 3-S8). This is a speculation based on our biochemical data that demands direct evidence in cells. It is critical to demonstrate that the NEIL glycosylases are able to switch on

transcription of a damage-containing, quadruplex-forming promoter. To test the hypothesis, the first step is to design a plasmid that contains a promoter that is able to form quadruplex DNA. The *VEGF* promoter and the *c-MYC* promoter are suitable for this purpose. Next, a short piece of oligodeoxynucleotide that contains a base damage (i.e. Gh) can be introduced into the plasmid to replace the original sequence using molecular cloning techniques. Such a plasmid can then be used in an *in vitro* transcription assay and the amount of transcribed mRNA in the presence and absence of NEIL glycosylases can be determined.

A reporter gene such as the luciferase gene inserted after the promoter will allow us to measure transcriptional activity in cells. A plasmid that expresses a NEIL glycosylase or a control plasmid can be co-transfected into cells. If the model we proposed in Figure 3-S8 is true, we should see increased transcriptional activity when a NEIL glycosylase is present. A reporter construct without damage in its promoter and a construct that contains a damage but is not able to form quadruplex DNA will be good controls.

The role of the NEIL glycosylases in gene regulation can be studied with more advanced gene expression profiling technologies such as RNA-seq. The expression profile of cells where a NEIL glycosylase is knocked down (or overexpressed) can be mapped to the profile of genes with quadruplex-forming sequences. The overlap of two profiles will elucidate the function of the NEIL glycosylases in transcriptional gene regulation.

4.4. CLOSING REMARKS

The results in this dissertation show, the first time, that DNA glycosylases can remove base damages in G-quadruplex DNA structures formed by both telomere sequences and promoter sequences, suggesting that glycosylases may play a role in telomere maintenance and gene regulation. Although increasing studies show that quadruplex DNA structures do exist in cells, such existence is still under debate. More studies using quadruplex-specific antibodies and other compounds are required to fully address this issue. Hydantoin lesions fall under the same scenario as quadruplex DNA structures, and their existence and biological function in cells also need further investigation.

It will be interesting to further study the mechanism underlying some of the observations reported in this dissertation, including the marked preference of NEIL3 for the telomere sequence. Additional work also needs to be done regarding how DNA glycosylases remove damages from the quadruplex DNA structures especially how DNA glycosylases and AP endonucleases function in quadruplex DNA formed by promoter sequences that have more than four G-runs.

Lastly, the function of the NEIL glycosylases in telomere maintenance and gene regulation needs to be tested *in vivo*. Monitoring telomere defects in cells where a NEIL glycosylase is knocked down will provide invaluable information. In addition, the role of NEIL glycosylases in gene regulation can be tested biochemically with a designed plasmid or in cells using a reporter system. Also, RNA-seq combined with bioinformatics

analysis of the quadruplex-forming sequences at promoters will define the role of the NEIL glycosylases in gene regulation at a genome-wide level.

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