Modulation of Base Excision Repair by Nucleosomes

Ian Odell

University of Vermont

Follow this and additional works at: https://scholarworks.uvm.edu/graddis

Recommended Citation
Odell, Ian, "Modulation of Base Excision Repair by Nucleosomes" (2010). Graduate College Dissertations and Theses. 170.
https://scholarworks.uvm.edu/graddis/170
MODULATION OF BASE EXCISION REPAIR BY NUCLEOSOMES

A Dissertation Presented by

Ian Odell

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Microbiology and Molecular Genetics

January, 2011
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Microbiology and Molecular Genetics.

Dissertation Examination Committee:

David S. Pederson, PhD
Susan S. Wallace, PhD
Sylvie Doulié, PhD
Scott Morical, PhD
Stephen Everse, PhD
Domenico Grasso, PhD

Advisor
Chairperson
Dean, Graduate College

Date: October 20, 2010
ABSTRACT

DNA in eukaryotes is packaged into nucleosomes, which present steric impediments to many of the factors and enzymes that act on DNA, including DNA repair enzymes. Within the nucleosome, DNA remains vulnerable to oxidative damage that can result from normal cellular metabolism, ionizing radiation, and various chemical agents. Oxidatively damaged DNA is repaired in a stepwise fashion via the base excision repair (BER) pathway. Other DNA repair pathways, including Nucleotide Excision Repair (NER), Mismatch Repair (MMR), Homologous Recombination (HR), and Non-homologous End-Joining (NHEJ) are all thought to require nucleosome remodeling or disruption. In contrast, it was reported that the first step of BER does not require or induce nucleosome disruption. For example, the human DNA glycosylase hNTH1 (human Endonuclease III) was discovered to excise thymine glycol lesions from nucleosomes without nucleosome disruption, and could excise optimally oriented lesions with an efficiency approaching that seen for naked DNA (Prasad, Wallace, and Pederson 2007). To determine if the properties of hNTH1 are shared by other human DNA glycosylases, we compared hNTH1 with NEIL1, a human DNA glycoylase that also excises thymine glycol from DNA, with respect to their activities on nucleosome substrates. We found that the cellular concentrations and apparent $k_{cat}/K_M$ ratios for hNTH1 and NEIL1 are similar. However, NEIL1 and hNTH1 differ in that NEIL1 binds undamaged DNA far more avidly than hNTH1. After adjustment for non-specific DNA binding, hNTH1 and NEIL1 proved to have similar intrinsic activities towards nucleosome substrates. We next wanted to examine the effects of nucleosomes on enzymes that catalyze the remaining steps in BER. We therefore assembled the entire four-step BER reaction with model, lesion-containing nucleosomes. The rates of substrate processing during the first three steps in BER, catalyzed by a DNA glycosylase, AP endonuclease, and DNA Polymerase β (Pol β), varied with the helical orientation of the substrate relative to the underlying histone octamer. In contrast, the rate of action by DNA Ligase III-α (in association with XRCC1) was independent of lesion orientation. These results are consistent with structural studies of BER enzymes and the previously proposed DNA unwrapping model for how BER enzymes gain access to lesions in nucleosomes (Prasad, Wallace, and Pederson 2007). During these investigations, we also discovered a synergistic interaction between Pol β and Ligase III-α complexed with XRCC1 that enhances the repair of lesions in nucleosomes. Together, our results support the hypothesis that DNA glycosylases have evolved to function in specific cellular environments (e.g. NEIL1 may function exclusively during DNA replication), but also possess DNA binding motifs and mechanisms of substrate recognition that impart a similar intrinsic activity on nucleosomes. In addition to hNTH1 and NEIL1, we have discovered that lesion orientation is also an important factor to the activities of APE and Pol β and that the complete BER reaction can occur without requiring or inducing nucleosome disruption. Finally, protein-protein interactions between XRCC1 and Pol β may be important for the efficient in vivo repair of lesions in nucleosomes.
CITATIONS

Material from this dissertation has been published in the following form:

Odell I.D., Newick K, Heintz N.H., Wallace S.S., Pederson D.S. (2010). Non-specific DNA binding interferes with the efficient excision of oxidative lesions from chromatin by the human DNA glycosylase, NEIL1. DNA Repair, 9,134-143.
ACKNOWLEDGEMENTS

I am lucky to have been surrounded by many supportive faculty and colleagues during my PhD. I started in Susan Wallace’s lab, where Scott Kathe trained me during the summer of 2007. After a year in the Wallace lab, David Pederson kindly accepted me into his lab, where Amalthiya Prasad and Joy-El Barbour taught me how to assemble nucleosomes. I am grateful to David for his mentorship and support; he personifies someone whose door is always open. I am also thankful to David for teaching me how to communicate like a scientist, in both my presentation and writing skills. I would like to thank everyone mentioned above, the MD/PhD program for their continual support, as well as my thesis committee, David Pederson, Susan Wallace, Sylvie Doublié, Scott Morrical, and Stephen Everse, for their guidance. Finally, I am also greatly thankful to my future wife, Anahí Fernández Cuppari (soon to be Anahí Odell) for her patience, love and understanding.
TABLE OF CONTENTS

CITATIONS .......................................................................................................................... ii

ACKNOWLEDGEMENTS ................................................................................................. iii

LIST OF TABLES ............................................................................................................... viii

LIST OF FIGURES ............................................................................................................. ix

CHAPTER 1: INTRODUCTION ......................................................................................... 1

1. Chromatin Structure and Dynamics........................................................................... 1

  1.1. Nucleosome Structure ......................................................................................... 1

  1.2. Histone Post-Translational Modifications ......................................................... 3

  1.3. Histone Variants ................................................................................................ 5

  1.4. ATP-dependent Nucleosome Remodeling ....................................................... 6

  1.5. Spontaneous Accessibility of Nucleosomal DNA .......................................... 8

2. DNA Repair in Chromatin ......................................................................................... 9

  2.1. DNA Repair Pathways ......................................................................................... 9

  2.2. Double Strand Break Repair: HR and NHEJ ................................................. 10

  2.3. NER ................................................................................................................... 10

  2.4. MMR .................................................................................................................. 12

3. Base Excision Repair ............................................................................................... 13

  3.1. Initiation of BER by DNA Glycosylases ......................................................... 14

  3.2. The Role of APE in BER ................................................................................. 19

  3.3. The Role of DNA Polymerase β in BER ....................................................... 22

  3.4. The Role of DNA Ligase III and XRCC1 in BER .......................................... 25

  3.5. Long-patch Base Excision Repair ................................................................. 28

4. Base Excision Repair in Nucleosomes .................................................................... 29

  4.1. Oxidative DNA Damage in the Nucleus ....................................................... 29
4.2. BER on Nucleosomes in vitro .................................................................30

5. Figure Legends .........................................................................................37

CHAPTER 2: NON-SPECIFIC DNA BINDING INTERFERES WITH THE EFFICIENT
EXCISION OF OXIDATIVE LESIONS FROM CHROMATIN BY THE HUMAN DNA
GLYCOSYLASE, NEIL1. ..................................................................................45

1. Introduction ..............................................................................................48

2. Experimental Procedures ..........................................................................51

2.1 In vivo concentrations of hNTH1 and NEIL1 ...........................................51

2.2 DNA and nucleosome substrates ............................................................51

2.3 Expression and purification of NEIL1 and hNTH1 .................................52

2.4 Enzyme assays .......................................................................................54

2.5 Determination of non-specific binding affinity .......................................55

3. Results .......................................................................................................57

3.1 Cellular abundance of NEIL1 and hNTH1 .............................................57

3.2 Impact of non-specific DNA binding on the capacity of hNTH1 and NEIL1
to excise oxidative lesions from naked DNA ............................................58

3.3 Comparison of hNTH1 and NEIL1 on nucleosome substrates ..............62

4. Discussion ................................................................................................65

5. References ................................................................................................68

6. Figure Legends .........................................................................................71

CHAPTER 3: SUBSTRATE HAND-OFF DURING BASE EXCISION REPAIR OF
OXIDATIVE LESIONS IN NUCLEOSOMES .................................................82

1. Introduction ..............................................................................................85

2. Experimental Procedures ..........................................................................88

2.1. Construction of DNA containing BER lesions and intermediates ..........88
APPENDIX F: PRELIMINARY STUDIES WITH HISTONE H3.3 CONTAINING NUCLEOSOMES. .................................................................................................................................156
LIST OF TABLES

Chapter 1-Table 1. Different Classes of Modifications Identified on Histones ............40
Chapter 1-Table 2. Functions of ATP-dependent Chromatin Remodeling Families ...40
Chapter 1-Table 3. Activity of BER enzymes on nucleosomal substrates ...............41
Chapter 2-Table 1. Kinetic constants for NEIL1 and hNTH1..................................75
Chapter 3-Table 1. List of DNA sequences used to generate BER substrates.........113
Chapter 3-Table 2. Enzyme concentrations and incubation times for repair of naked DNA, Tg-out, Tg-in, and Tg(601) nucleosomes. .............................................................113
# LIST OF FIGURES

**Chapter 1-Figure 1.** Canonical core histones and their variants ........................................ 42  
**Chapter 1-Figure 2.** Summary of equilibrium constants for site exposure ............................ 42  
**Chapter 1-Figure 3.** A model for the functions of chromatin remodeling complexes at DNA double-strand breaks in budding yeast ................................................................. 43  
**Chapter 1-Figure 4.** hNTH1 and NEIL1-initiated Base Excision Repair ............................... 44  
**Chapter 2-Scheme 1.** ......................................................................................................... 75  
**Chapter 2-Scheme 2.** ......................................................................................................... 75  
**Chapter 2-Figure 1.** In vivo concentrations of hNTH1 and NEIL1 ....................................... 76  
**Chapter 2-Figure 2.** Adjustment of hNTH1 and NEIL1 concentrations to compensate for differences in enzyme efficiency and non-specific DNA binding ........................................... 77  
**Chapter 2-Figure 3.** NEIL1 and hNTH1 exhibit similar intrinsic activities toward nucleosomes with ‘outward-facing’ Tg lesions, but the capacity of NEIL1 to excise lesions from sterically occluded sites in nucleosomes is limited by its high non-specific DNA binding ................................................................................................................... 78  
**Chapter 2-Figure 4.** Both hNTH1 and NEIL1 remove sterically accessible lesions from nucleosomes without requiring or inducing nucleosome disruption ........................................ 79  
**Chapter 2-Figure 5.** Both hNTH1 and NEIL1 remove sterically accessible lesions from nucleosomes without detectably altering nucleosome position ............................................ 80  
**Chapter 3-Figure 1.** Reconstitution of complete base excision repair reactions with model nucleosomes ....................................................................................................................................... 114  
**Chapter 3-Figure 2.** Influence of lesion orientation on the rate of each step of BER ........ 118  
**Chapter 3-Figure 3.** Characterization of abasic site repair on nucleosomes ....................... 120  
**Chapter 3-Figure 4.** Fate of lesion-containing nucleosomes during BER ............................. 122  
**Chapter 3-Figure 5.** Nucleosome binding by LigIIIα/XRCC1 enhances the rate of nucleotide extension by Pol β ...................................................................................................................... 125  
**Chapter 3-Supplementary Figure 1.** Increasing concentrations of LigIIIα/XRCC1 more efficiently ligate nick containing nucleosomes .................................................................................. 126  
**Appendix A-Figure 1.** Stimulation of NEIL1 by XRCC1 .................................................... 148  
**Appendix C-Figure 1.** Stimulation of hNTH1 Tg excision by chromatin competitor .......... 150  
**Appendix F-Figure 1.** H3.3 nucleosomes are stable for extended incubations in buffers containing 100 mM salt ......................................................................................................................... 159  
**Appendix F-Figure 2.** DNase I digestions of H3.3 containing nucleosomes ....................... 160  
**Appendix F-Figure 3.** Rate of Tg-46 excision by 100 nM hNTH1 with H3.1 and H3.3 containing nucleosomes ...................................................................................................................... 161
CHAPTER 1: INTRODUCTION

The packaging of genomic DNA in eukaryotes by histones and other functional proteins limits its accessibility to nuclear processes. As a result, the cell has evolved mechanisms that regulate DNA accessibility by modifying the packaging structure at various levels, which permits access to the salient information while keeping the rest archived away. Nevertheless, all DNA remains vulnerable to oxidative damage from cellular metabolism. To avoid the mutagenic and cytotoxic effects of oxidative DNA damage, cells evolved the Base Excision Repair (BER) pathway. The following introduction begins with an overview of factors that influence chromatin structure and current models for how DNA repair pathways access and repair DNA in chromatin, followed by a more detailed review of BER and studies of BER in chromatin.

1. Chromatin Structure and Dynamics.

1.1. Nucleosome Structure. The information in a human genome is encoded within ~2 m of DNA, which is compacted between 1,000 and 10,000-fold, depending on the phase of the cell cycle. This compaction is accomplished by an array of proteins that, together with the DNA, is called chromatin. The most abundant of the chromatin proteins are histones, whose general structure consists of a globular core containing the conserved histone fold domain (HFD) flanked by disordered N- and C-terminal tails (Figure 1). There are four canonical core histones, H2A, H2B, H3, and H4, which interact through their histone fold domains. Two copies each of H3 and H4 bind together to form an (H3-H4)_2 tetramer and H2A and H2B bind each other as a dimer. With the help of histone chaperones, two H2A-H2B dimers and one (H3-H4)_2 tetramer assemble together with DNA to form the fundamental unit of chromatin, the nucleosome. The globular domains of the (H3-H4)_2 tetramer and two H2A-H2B dimers make up the core of the nucleosome,
around which 147 bp of DNA is wrapped in 1.65 superhelical turns (Luger et al. 1997; Richmond and Davey 2003) (for reviews, see (Luger and Richmond 1998, 1998)). This structure contributes a 5-fold level of DNA compaction, but also sterically occludes it on the inner surface of the superhelix and constrains the overall DNA conformational flexibility. As a result, there are two conflicting, but mutually important functions of nucleosomes. First, they package and compact the DNA in an organized fashion as well as neutralize the negative charges in the phosphodiester backbone. Second, nucleosomes, histone chaperones, and chromatin remodeling agents must collaborate with one another so that the information contained within the DNA is made available at appropriate times in the cell cycle or during development.

Eukaryotes contain a diverse set of tools to regulate DNA accessibility. In addition to the four canonical histones, they contain histone variants (cf. Section 1.1.3.) that affect chromatin structure and stability when incorporated into nucleosomes. Histones are also subject to reversible post-translational modifications (cf. Section 1.1.2.), which can similarly affect chromatin structure, as well as function to recruit specific protein factors. Eukaryotes have the means to disassemble and/or move nucleosomes in order to expose regions of DNA to factors that would otherwise be inhibited by nucleosomes (cf. Section 1.1.4.); as well, the intrinsic dynamics of nucleosomes facilitates access of DNA binding proteins to occluded DNA (cf. Section 1.1.5.).

Pioneering studies of radiation-induced chromosomal translocations provided some of the earliest evidence of distinct DNA accessibility in different regions, such that epigenetic “on/off” transcriptional states depend on the position of a gene in either accessible (euchromatin) or inaccessible (heterochromatin) regions of DNA (Muller
However, a simple “on/off” relation between actively transcribed and silent genes does not encompass the complexity of chromatin structure. In one extreme, some transcriptionally inert chromosome regions remain condensed during interphase and are visible as Barr bodies, such as the inactive X chromosome in females (Brown 1966). The other extreme includes genes that are constitutively expressed, such as β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which exist in euchromatin and contain cis-regulatory elements within nucleosome-depleted promoters (for review, see (Cairns 2009)). In the middle of the two extremes lie inducible genes, such as the yeast GAL1-10 genes, which are rapidly and specifically activated in the presence of galactose, but are repressed in the presence of other carbon sources (Johnston 1987; Bash and Lohr 2001). They are located in euchromatin, but unlike constitutively active genes, they contain nucleosomes covering their transcription start site (TSS), the regions flanking the TSS, and most of the binding sites for transcriptional activators. Together, these examples demonstrate a hierarchy of gene accessibility and chromatin structure, but how do eukaryotes regulate all of it?

1.2. Histone Post-Translational Modifications. An important mechanism in the regulation of DNA accessibility involves the post-translational (PT) modification of histones (for reviews, see (Mersfelder and Parthun 2006; Kouzarides 2007)), in which chemical moieties such as acetate, phosphate, or ubiquitin are added to amino acid side chains on histones. Table 1 lists the eight types of histone modifications known to date, and it appears that all histone modifications can be reversed by enzymes that catalyze their removal. On a fundamental level, the modifications alter the character and

---

1 Carbamylation was once proposed to be a type of histone modification, but it has since been identified as an artifact resulting from degradation of urea in alkaline solution to iso-cyanate, which covalently modifies lysine residues (McCarthy et al. 2003; Shechter et al. 2007).
reactivity of the amino acid to which they are added and results in a number of effects. Many sites in the N-terminal tail domains of the core histones are subject to PT-modifications and primarily appear to alter the affinity of non-histone proteins that interact with chromatin, such as heterochromatin protein 1 (HP1) binding to methylated lysine 9 on histone 3 (H3K9Me) (Jacobs and Khorasanizadeh 2002). On the other hand, modifications in the histone globular domain cluster into three distinct classes: those located on the solute accessible face, those on the histone lateral surface, and those on the histone-histone interfaces. The most well characterized modification on the nucleosome face is methylation of H3K79, which is required for proper formation of silent chromatin, probably by preventing the binding of SIR proteins to regions of euchromatin (van Leeuwen and Gottschling 2002; Ng et al. 2003). On the lateral surface, H3K56 is positioned at the DNA entry-exit point on the nucleosome dyad, and its acetylation alters the rate of spontaneous nucleosome unwrapping (Buning and van Noort 2010). Finally H4K91 is located at the interface between the (H3-H4)_2 tetramer and the H2A-H2B dimer, and its acetylation has been proposed to weaken octamer stability by preventing salt bridge formation because the K91A mutation in H4 results in defects in chromatin assembly (Ye et al. 2005).

More generally, histone modifications can act as an epigenetic marking system to regulate specific functional outputs, as described in three predictions by the histone code hypothesis (Jenuwein and Allis 2001). First, modification of histone tails alters interaction affinities for chromatin-associated proteins (i.e. H3K9 methylation). The first examples of this to be discovered were protein domains in chromatin associatedbinding proteins that recognize specific histone modifications. The bromodomain specifically binds acetylated histones, the chromodomain and PhD finger motif bind methylated sites on histones, and
proteins that contain a 14-3-3 domain bind phosphorylated sites. Second, modifications on the same or different tails may be interdependent and thus generate a specific combination of modifications on the same nucleosome. A classic example of this is the methylation of H3K9, which inhibits the phosphorylation of H3S10, and vice versa. The phosphate group on H3S10 also promotes the acetylation of H3K9 and/or H3K14. Together, these modification states represent a nucleosome switch that can be targeted by a cellular signaling cascade, such as the MAP kinase cascade, and correlates with induction of target genes harboring the H3S10 associated modifications (Clayton et al. 2000). Third, distinct qualities of higher order chromatin structure, such as euchromatin and heterochromatin, depend on the local concentration and combination of differentially modified nucleosomes. An example of this is H3K16 acetylation, which negatively affects the formation of the 30 nm fiber (Shogren-Knaak et al. 2006). Despite rapid progress in the elucidation of this complex system of landmarks, the control of chromatin and gene activity through epigenetic marks is still an intense area of investigation.

1.3. Histone Variants. Another mechanism that alters the accessibility of DNA in the nucleosome is the replacement of canonical histones from the octamer core with histone variants. These are distinct non-allelic forms of major-type histones, whose expression patterns are not restricted to S phase and have been associated with specific nuclear processes (Figure 1). Nucleosome assembly, re-assembly during transcription, and disassembly or eviction is accomplished by histone chaperones (for review, see (Park and Luger 2008)). Histone chaperones can be grouped by their specificity for H2A-H2B dimers or H3-H4 dimers, but they vary widely in their specificity for canonical versus variant histone-containing dimers. For example, the histone chaperone Chz1 preferentially binds H2A.Z-H2B dimers over H2A-H2B, whereas the chaperone NAP1
not only binds canonical and variant-containing H2A-H2B dimers with high affinity, but also (H3-H4)$_2$ tetramers and histone H1 as well (McBryant et al. 2003; Luk et al. 2007). Incorporation of histone variants can result in structural changes in the nucleosome that alter its stability, as well as the formation of higher order structures. For example, H2A.Z stabilizes the nucleosome and higher order structures in vitro (Fan et al. 2002; Park et al. 2004; Hoch, Stratton, and Gloss 2007), whereas H2A.Bbd incorporation results in a less compact nucleosome with higher accessibility to transcription factor binding (Bao et al. 2004; Gautier et al. 2004). H2A.Bbd lacks the carboxy-terminal tail and signature acidic surface found in the canonical H2A and, as a result, inhibits the folding of 30 nm fiber-like structures. Interestingly, H3.3/H2A.Z double variant containing nucleosomes are unusually sensitive to salt dependent disruption (Jin and Felsenfeld 2007), in contrast to the stable H2A.Z single variant nucleosomes mentioned above. In addition to nucleosome structure, histone variants can alter nucleosome susceptibility to sliding or chromatin remodeling. For example, incorporation of MacroH2A interferes with nucleosome remodeling by SWI/SNF (Angelov et al. 2000). Finally, in some cases the differences in primary amino acid sequence provide altered sites for PT modifications. A notable example of this is for H2A.X, which is phosphorylated at double strand breaks, which recruits chromatin remodeling factors and DNA repair proteins (Rogakou et al. 1998; Morrison et al. 2004; Tsukuda et al. 2005).

1.4. ATP-dependent Nucleosome Remodeling. In addition to histone secondary modifications and the incorporation of histone variants, cells have nucleosome remodeling agents that alter nucleosome positioning and thereby alter the accessibility of DNA sequences. A process commonly referred to as “nucleosome sliding” changes nucleosome position such that a DNA segment that is inaccessible due to histone
contacts is moved to an accessible region of free linker DNA between nucleosomes. In addition to creating access to a DNA site, this process can alter the spacing between nucleosomes. Distinct from histone chaperones, there are at least eight families of ATP-dependent nucleosome remodeling complexes (Table 2) and they all adjust nucleosome positioning, albeit through different structural intermediates (for review, see (Fan, Narlikar, and Kingston 2004)). ATP-dependent remodeling complexes are large (greater than 1 MDa) and contain 11-15 subunits. The most prominent families are SWI/SNF and ISWI, named after yeast genetic studies for sucrose non-fermenters or defective in mating type switching and imitation switch, respectively. Most organisms have at least two versions of SWI/SNF complexes, each of which contain a central subunit that has both ATPase activity and remodeling activity as an isolated protein. Domain swapping experiments have shown that the ATPase domain in particular determines the nature of the remodeled product and the accessibility of sites in the DNA during the remodeling process (Fan, Narlikar, and Kingston 2004). It is not clear exactly what nucleosome structural perturbations occur during the remodeling process, but it is likely that perturbed nucleosomes are structurally different from the canonical nucleosome. This is evidenced by an increase in DNA accessibility to restriction enzymes during the remodeling process, which varies for different remodeling enzymes. For example, when incubated with mononucleosomes assembled with a 202 bp sequence of DNA, SNF2h, a central subunit to the human ISWI complex, increases the accessibility of sites near the edge of the nucleosome, whereas BRG1, which is a central subunit to the human SWI/SNF complex, increases the accessibility of DNA throughout the length of the DNA fragment (Fan, Narlikar, and Kingston 2004). These observations also imply that the nucleosomal DNA is perturbed by the remodeling complexes more so than the octamer.
In some cases, remodeling complexes appear to function together with histone chaperones. For example, RSC disassembles nucleosomes in the presence of the chaperone NAP1, but not ASF1 (Lorch, Maier-Davis, and Kornberg 2006). ATP-dependent nucleosome remodelers play a large role in both transcription and DNA repair. The observation that formation of the transcription pre-initiation complex and subsequent transcription are inhibited by nucleosomes (Knezetic and Luse 1986; Lorch, LaPointe, and Kornberg 1987; Workman and Roeder 1987)) highlights the importance of histone eviction and/or nucleosome remodeling.

1.5. Spontaneous Accessibility of Nucleosomal DNA. In addition to each of the tools the cell uses to regulate DNA accessibility within chromatin, nucleosomes themselves are dynamic entities (Thastrom et al. 1999). In one study by the Widom group, they titrated LexA protein with nucleosomes containing the LexA target site near the edge of the nucleosome and observed a corresponding concentration dependent increase in target DNA binding by LexA, as measured by a drop in FRET emission between a DNA bound Cy3 and histone bound Cy5 (Li and Widom 2004). Additionally, by incubating nucleosomes with difference restriction enzymes that have target sites located at different distances from the dyad axis, Widom’s group also noticed that accessibility to those target sites decreased exponentially with their proximity to the dyad (Figure 2) (Anderson and Widom 2000). Based on these results, Widom and colleagues proposed that DNA transiently unwraps off the surface of the histone octamer, allowing DNA binding proteins access to buried sites in the nucleosome. In later studies, they measured the frequency (~4/sec) and lifetime (~10-50 msec) of DNA dissociation off the histone surface (Li et al. 2005), and importantly, found evidence for spontaneous site exposure on folded chromatin fibers (Poirier et al. 2008). The model of “nucleosome
unwrapping” helps explain how chromatin remodeling factors can be recruited to specific nucleosomes. The histone code hypothesis postulates that PT modification of histone tails acts as a signal to recruit further nucleosome modifying proteins, but how do the histone modifiers know where to go? There is increasing evidence that DNA binding by site-specific DNA-binding proteins precedes nucleosome remodeling (Peterson and Logie 2000; Narlikar, Fan, and Kingston 2002). By accessing their own target sites during episodes of unwrapping, sequence specific DNA-binding proteins such as transcription factors could act as the initial signal to recruit the appropriate histone modifiers and nucleosome remodelers.

2. DNA Repair in Chromatin.

2.1. DNA Repair Pathways. In addition to transcription, there are many other nuclear processes that require access to specific DNA target sites, such as DNA replication, recombination, and repair. DNA is constantly damaged in many ways; it is cross-linked, broken, and oxidized. If not repaired accurately, DNA damage can lead to mutations and genomic instability, and overwhelming DNA damage can result in cell death, whereby the cell enters a state of irreversible growth arrest (replicative death), or triggers apoptosis. To combat these damages and their effects, cells have evolved several systems to detect DNA damage, signal its presence and mediate its repair. The pathways that mediate DNA repair include Homologous Recombination (HR), Non-Homologous End Joining (NHEJ), Nucleotide Excision Repair (NER), Mismatch Repair (MMR), and Base Excision Repair (BER). Each of these pathways employs specific mechanisms and/or factors that promote access to and repair of damaged DNA in chromatin.
2.2. Double Strand Break Repair: HR and NHEJ. The most serious kind of DNA damage is a double strand break (DSB), just one of which is lethal to a cell (Bonura and Smith 1975; Bonura, Smith, and Kaplan 1975). Depending on the phase of the cell cycle, DSBs are repaired by either HR or NHEJ (for reviews, see (Daley et al. 2005; San Filippo, Sung, and Klein 2008; Jackson and Bartek 2009)). Although NHEJ is more error-prone than HR, it operates in any phase of the cell cycle, whereas HR uses the sister-chromatid sequence as the template for repair and consequently only operates during S and G2 (Jackson and Bartek 2009). Initiation of each of these pathways involves a complex set of damage recognition and signaling events (Figure 3). Key events in both include the recognition of double strand breaks and phosphorylation of the histone variant H2A.X at serine 139 (γ-H2A.X). In NHEJ, the DSB is recognized by the Ku70-Ku80 heterodimer, whereas in HR, the DSB is recognized by the MRN complex (Mre11-Rad50-Nbs1) (Usui, Ogawa, and Petrini 2001; Bao and Shen 2007). MRN is required for DNA strand resection as well as recruitment of the INO80 ATP-dependent chromatin remodeling complex (Tsukuda et al. 2005), which induces nucleosome eviction near the site of the DSB. In both pathways, phosphorylation of H2A.X is mediated by ATM and ATR (Rogakou et al. 1998; Burma et al. 2001; Morrison et al. 2004; Tsukuda et al. 2005). γ-H2A.X spreads over megabase chromatin domains and is required for stable accumulation of repair proteins (Celeste et al. 2003). Together, these events recruit DSB repair machinery for stable HR or NHEJ and permit them to access and repair DSBs by temporarily disrupting the nucleosomes.

2.3. NER. As with DSB repair, NER also involves the recruitment of ATP-dependent chromatin remodeling factors to sites of DNA damage. NER is responsible for repair of damages induced by UV irradiation, of which the two major classes are cyclobutane...
pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PPs). 6-4PPs show a relatively random distribution around the nucleosome, but CPDs most commonly occur at sites where the phosphate backbone is farthest from the core histone surface (Gale and Smerdon 1990; Brown et al. 1993). However, no site within the nucleosome is completely resistant to UV-induced damage formation. The earliest evidence for ATP-dependent chromatin remodeling during NER comes from a 1978 study in which Smerdon and Lieberman UV-irradiated fibroblasts that had been pulse-labeled with $[^3]$H-dThd, and observed that initial repair synthesis occurred in micrococcal nuclease (MNase) sensitive regions of DNA (Smerdon and Lieberman 1978). Over time, MNase resistance returned and correlated with the appearance of nucleosomes, suggesting that the nucleosome architecture was altered during NER. More recently, Smerdon's laboratory co-purified the two subunits of the yeast NER damage-recognition heterodimer Rad4-Rad23 (human XPC-HR23B complex) in complex with V5-His$_6$-tagged Snf6 by cross-linking them with formaldehyde just after UV irradiation (Hall and Struhl 2002; Gong, Fahy, and Smerdon 2006). Additionally, they observed increased sensitivity of a yeast snf6 deletion mutant to UV-irradiation, suggesting that the SWI/SNF chromatin remodeling complex participates in altering nucleosome architecture during NER.

Nucleosomes in the yeast HML locus have been mapped to single nucleotide resolution and contain a single EcoRV site located within a positioned nucleosome (Weiss and Simpson 1998). To monitor for changes in nucleosome structure, Gong et al. UV-irradiated purified chromatin from wild type and snf6 yeast, then digested it with EcoRV (Gong, Fahy, and Smerdon 2006). After UV-irradiation, they observed ~10-fold increase in digestion by EcoRV of its HML target in chromatin purified from wild type yeast, but
not the snf6 mutant strain, indicating a SWI/SNF-dependent increase in DNA accessibility upon UV treatment. A follow-up study by Ray et al. confirmed the SWI/SNF-XPC interaction by coimmunoprecipitation with human SNF5 (hSNF5) (Ray et al. 2009). Ray et al. also observed that hSNF5 depletion by siRNA did not affect UV-induced XPC focus formation, suggesting that damage recognition precedes SWI/SNF recruitment. After damage recognition by XPC-HR23B, SWI/SNF appears to play a role in recruitment and activation of ATM and thereby promotes phosphorylation and activation of downstream NER repair machinery (Ray et al. 2009).

2.4. MMR. In contrast to DSB repair and NER, the mismatch recognition heterodimer made up of human MutS homologues 2 and 6 (hMSH2-hMSH6) directly disrupts nucleosomes in an ATP-dependent manner, seemingly in lieu of recruitment of ATP-dependent nucleosome remodeling agents (Javaid et al. 2009). Mismatched nucleotides result from polymerase incorporation errors, heteroallelic recombination and certain kinds of chemical and physical damage (Friedberg 2006). In eukaryotes, MutS homologues recognize a mismatch, followed by strand incision by a human Mut L homologue (MLH1) containing complex, and strand excision by human exonuclease 1 (EXO1) (Modrich 2006). In the study by Javaid et al, they reconstituted nucleosomes with a DNA sequence containing 147 bp of the Xenopus 5S rDNA nucleosome positioning sequence linked to a lacO sequence, G:T mismatch, and 3' biotin with octamers assembled from recombinant histones (Javaid et al. 2009). When they incubated these nucleosomes with the hMSH2-hMSH6 heterodimer, they observed ATP-dependent nucleosome disruption. The addition of ATP to hMSH2-hMSH6 reduces its affinity for the mismatch, thereby allowing it to dissociate and act as a sliding clamp (Selmane et al. 2003). Because disruption by hMSH2-hMSH6 was abrogated by the
addition of Lacl protein, which blocks the diffusion of the hMSH2-hMSH6 sliding clamp along the DNA into the nucleosome, it appears that sliding by hMSH2-hMSH6 into the nucleosome is responsible for nucleosome disruption (Mendillo, Mazur, and Kolodner 2005). It has been shown that *E. coli* MutS similarly binds to mismatches, followed by recruitment of MutL and formation of a mismatch ternary complex, which then may signal the subsequent repair steps (Selmane et al. 2003). By blocking the iterative cycles of hMSH2-hMSH6 loading and clamp formation, it would be interesting to test if the subsequent steps of MMR initiation alter the hMSH2-hMSH6 induced nucleosome disruption. It would also be interesting to know if nucleosome disruption is observed on a dinucleosome substrate, which would be a more natural model than the Biotin-Streptavidin 3’-block (Javaid et al. 2009). Further studies of MMR on chromatin will be interesting because it appears to be independent of the ATP-dependent remodelers that DSB and NER utilize to access their target sites in chromatin.


Oxidative DNA damage results from exposure of DNA to reactive oxygen species (ROS), which are the oxidant byproducts of aerobic cellular metabolism, as well as environmental agents such as γ-irradiation. The most reactive ROS is the hydroxyl radical, which reacts with DNA at various positions to yield apurinic/apyrimidinic (AP) sites, single strand breaks, and a plethora of oxidative base damages (Breen and Murphy 1995). Base Excision Repair enzymes recognize these damages, and replace them with the undamaged bases in an error-free fashion. The following sections will describe the steps of BER that culminate in the efficient repair of oxidative DNA damage,
the consequences of missing or mutated BER proteins, and the intracellular location and key structural features of BER components.

### 3.1. Initiation of BER by DNA Glycosylases

In its simplest form, BER consists of four enzymatic steps (for reviews see (Wallace et al. 2003; Wilson, Sofinowski, and McNeill 2003; Almeida and Sobol 2007; David, O'Shea, and Kundu 2007; Hegde, Hazra, and Mitra 2008)). The first step is the recognition and excision of a damaged base by either a mono- or bi-functional DNA glycosylase (Figure 4). Each DNA glycosylase excises a particular set of biological substrates from DNA. For example, the uracil created by hydrolytic deamination of cytosine is removed by the monofunctional glycosylases UNG or SMUG1. Oxidized bases in particular are mostly excised by bifunctional DNA glycosylases, which first cleave the N-glycosyl bond, and then in a second enzymatic step, cleave the phosphodiester backbone. In general, the bifunctional DNA glycosylases can be grouped based on whether they recognize oxidized purines or oxidized pyrimidines (for review, see (Zharkov, Shoham, and Grollman 2003)). Of the oxidized purines, 7,8-dihydro-8-oxo-2'-deoxyguanine (8-oxoG) is the most common; it can base-pair with adenine in place of cytosine, thus generating G to T transversion mutations after replication (Kasai et al. 1984; Shibutani, Takeshita, and Grollman 1991). The bifunctional glycosylase human 8-oxoguanine DNA glycosylase (hOGG1) can efficiently excise 8-oxoG and related damages. Of the oxidized pyrimidines, the most common oxidation product of thymine, thymine glycol (Tg), is poorly mutagenic, probably because it strongly blocks elongation by both repair and replicative polymerases (Ide, Kow, and Wallace 1985; Clark and Beardsley 1986; Hayes and LeClerc 1986; McNulty et al. 1998). Members of two different families of DNA glycosylases, human endonuclease III (hNTH1) in the HhH-GPD superfamily and human endonuclease VIII-
like 1 (NEIL1) in the Fpg/Nei family, excise oxidized pyrimidines such as thymine glycol. Interestingly, further oxidation of 8-oxoG produces lesions that are an order of magnitude more mutagenic (Henderson et al. 2003), but these are excised by NEIL1, not hOGG1. Two final facets of DNA oxidation include the oxidation of purine nucleoside triphosphates, such as dGTP to 8-hydroxy-dGTP, which is rapidly degraded by human MutT homologue (hMTH1), and excision of misincorporated adenine opposite 8oxG, which is completed by human MutY homologue (MUTYH). In concert, DNA glycosylases recognize and excise oxidized bases on multiple fronts.

As summarized above, each DNA glycosylase excises a particular set of biological substrates. However, their substrate specificities also substantially overlap with each other. This is particularly evident in the lack of strong phenotypes observed in mice that have homozygous gene deletions for a single DNA glycosylase. For example, $Ogg1^{-/-}$ mice showed increased levels of 8-oxoG in their genomes, but did not develop malignancies or show marked pathological changes in their tissues (Klungland et al. 1999). Likewise, $Nth^{-/-}$ mice showed no detectable abnormality. $Neil1^{-/-}$ mice unexpectedly exhibited symptoms of metabolic syndrome, suggestive of defective DNA repair in mitochondria, but no other gross abnormalities (Takao, Kanno, Shiromoto, et al. 2002; Vartanian et al. 2006). The role of these glycosylases in preventing carcinogenesis became much clearer when they were knocked-out in tandem. $Myh^{-/-}Ogg1^{-/-}$ mice developed pulmonary and ovarian tumors and lymphomas, and $Nth^{-/-}Neil1^{-/-}$ mice developed pulmonary and hepatocellular carcinomas (Xie et al. 2004; Chan et al. 2009). Because codons 12 and 13 of the $K-ras$ oncogene are activating hot-spots for lung tumorigenesis (Bos 1989; Johnson et al. 2001), tumors in the double-knockout mice were analyzed for mutations there. Tumors in the $Myh^{-/-}Ogg1^{-/-}$ mice contained G
to T transversions, as expected from misinsertion of adenine opposite 8-oxoG. In contrast, Nth⁻/⁻ Neil1⁻/⁻ tumors contained G to A transitions, consistent with misinsertion of thymine opposite the deamination product of oxidized cytosine during replication (Kreutzer and Essigmann 1998). Together, the phenotypes of the knock-out mice highlight the overlapping substrate specificities of DNA glycosylases as well as their role in preventing carcinogenesis.

Because both nuclear and mitochondrial genomes must faithfully be repaired, proper localization of DNA glycosylases is important in avoiding the carcinogenesis described above. Mouse NEIL1 was observed in immunofluorescence studies to localize to the nucleus, while comparisons of mitochondrial extracts from the wild type and Neil1⁻/⁻ mice suggested that NEIL1 is present in mitochondria as well (Takao, Kanno, Kobayashi, et al. 2002; Hu et al. 2005). Accordingly, the Neil1⁻/⁻ mice that were vulnerable to development of metabolic syndrome exhibited increased mitochondrial DNA strand breaks, polymerase blocking lesions, and DNA deletions (Vartanian et al. 2006). Finally, a NEIL1 mutant found in a patient with gastric cancer had lost appropriate nuclear localization as a result of a splicing abnormality that deleted part of the nuclear localization signal (NLS), suggesting an important role for NEIL1 in the nucleus as well (Shinmura et al. 2004). In contrast, mouse and human NTH localize to different cellular compartments. Most mouse NTH is sorted to the mitochondria, but human NTH is exclusively sorted to the nucleus as a result of distinct N-terminal localization signals (Ikeda et al. 2002). Alternative splicing of hOGG1 mRNA results in protein products that share a common N-terminus, but possess unique C-termini (Nishioka et al. 1999), which determine whether they localize to the nucleus or mitochondria.
In general, all DNA glycosylases share the common features of binding the lesion-containing strand of DNA and extruding the damaged base from the duplex such that the base enters an extrahelical recognition pocket in the enzyme (for review, see Fromme, Banerjee, and Verdine 2004). Glycosylases in the Fpg/Nei and HhH-GPD superfamilies contain multiple domains with the active site located in an interdomain junction. In contrast, monofunctional glycosylases in the UDG and AAG superfamilies are compact, single-domain enzymes with relatively small DNA-interaction surfaces. Structural studies of DNA-bound hOGG1, and endonuclease VIII (Nei) and endonuclease III (Nth) (the prokaryotic homologues of NEIL1 and hNTH1) demonstrate that each of these glycosylases binds the minor groove of DNA and bends the DNA between 45° and 70° (Bruner, Norman, and Verdine 2000; Zharkov et al. 2002; Fromme and Verdine 2003). Glycosylase-induced DNA bending is thought to facilitate flipping of the damaged base from the duplex (Wallace et al. 2003). Despite having only 25% sequence identity with E. coli Nei, one can superimpose the structure of NEIL1 onto the DNA-bound structure of E. coli Nei, indicating high structural similarity (Doublie et al. 2004). In addition, both of these enzymes can be superimposed onto the unliganded T. thermophilus Fpg, suggesting that there is minimal movement between protein domains upon DNA binding (Doublie et al. 2004). The human forms of each bifunctional glycosylase contain disordered tails that serve specialized roles. The N-terminal tail of hNTH1 reduces its rate of product release, but also enables it to homodimerize at higher enzyme concentrations (Liu, Choudhury, and Roy 2003). hNTH1 dimerization increases the rate of product release by 11-fold, and by this means reverses the inhibitory effect of the tail in a concentration dependent manner. NEIL1 on the other hand contains a C-terminal tail that promotes its interaction with other BER proteins (Wiederhold et al. 2004; Das et al. 2007; Guan et al. 2007; Dou et al. 2008; Hegde et al. 2008), and acetylation of the
short C-terminal tail of hOGG1 increases its turnover rate by reducing its affinity for the product AP site (Bhakat et al. 2006).

Before DNA glycosylases actually bind damaged DNA, they must locate damaged bases within a million-fold excess of normal DNA. The Verdin lab provided insights into the search process by co-crystalizing enzyme-DNA complexes representing discrete steps during DNA-interrogation (Bruner, Norman, and Verdin 2000; Fromme and Verdin 2002). Based on these structures, Verdin and colleagues proposed that glycosylases can efficiently interrogate millions of bases by inserting a probe, in the form of an aromatic or aromatic-like side chain (e.g., Phe, Tyr, Arg, and Leu for Fpg, hOGG1 and MutY, MUG, and AlkA, respectively) into the intact DNA double helix (Banerjee, Santos, and Verdin 2006). It is unclear exactly how the probe distinguishes between damaged and undamaged bases because for example, 8-oxoG:C has only slightly weaker base pairing than A:T (Plum et al. 1995) and no discernible effect on the conformation of duplex DNA (Oda et al. 1991; Lipscomb et al. 1995). Perhaps 8-oxoG:C behaves differently upon interrogation by the Fpg or hOGG1 probes, so this would be interesting for future study. Of note, it seems that there is a strong negative selection against extruding normal DNA bases from the helix because the lesion search process is Brownian, and extrusion of every base would be highly energetically unfavorable (Banerjee, Santos, and Verdin 2006). Another feature found in hNTH1 that has been proposed to facilitate the search process involves an [4Fe-4S] cluster (Boal et al. 2009). Barton and colleagues observed higher non-specific DNA binding affinity by *E. coli* Nth in the oxidized state (Gorodetsky, Boal, and Barton 2006). Given that the presence of mismatched or oxidized bases reduces the efficiency of DNA charge transport, they proposed that oxidation of Nth upon binding DNA in regions that hinder the reduction of
Nth via charge transport (i.e. in the vicinity of oxidized bases), could increase the lifetime of Nth association. To test this hypothesis, Barton and colleagues individually knocked out genes for Nth and MutY in *E. coli*, and observed increased G to A transitions in the *mutY* strain (expected with an Nth deficiency) and an increase in G to T transversions in the *nth* strain (expected with a MutY deficiency), suggesting that the two proteins cooperate with each other. The apparent cooperativity between these two enzymes was also abolished by a mutation that renders Nth deficient in DNA-mediated charge transfer. Based on these results, Barton and colleagues proposed that Nth and MutY cooperatively take advantage of the ability of undamaged duplex DNA to transport electrons in order to efficiently scan genomic DNA for mismatches and oxidized bases.

hOGG1, hNTH1, and NEIL1 have been extensively characterized biochemically, cellularly, and *in vivo*. The other human Nei-like proteins, NEIL2 and NEIL3, are less well understood, so their cellular functions will be interesting for future investigation.

### 3.2. The Role of APE in BER

After a DNA glycosylase has excised a damaged base, the subsequent reaction step depends on the chemical moieties that the glycosylase left behind. The glycosylases hNTH1 and hOGG1 first cleave the bond between the base and sugar, then the phosphodiester bond 3’ of the resulting AP site, generating a 3’-phospho-α,β unsaturated aldehyde (PUA) and a 5’-phosphate (Figure 4, middle pathway). NEIL1 likewise first cleaves the N-glycosylic bond between the sugar and base, but then, through β,δ elimination, it cleaves the phosphodiester backbone and removes the 3’-deoxyribose, leaving a 3’-phosphate (Figure 4, left-hand pathway). Both the 3’-PUA and 3’-phosphate block extension by DNA polymerases, so they must be removed before resynthesis. The 3’-PUA is removed by the phosphodiesterase activity
of APE and the 3’-phosphate is removed by a different BER protein, polynucleotide kinase (PNK), in NEIL1-dependent repair (Chen, Herman, and Demple 1991; Izumi et al. 2000; Wiederhold et al. 2004).

In addition to the 3’ blocking groups left by hOGG1, hNTH1 and NEIL1, ROS can also attack the deoxyribose in DNA to generate strand breaks with a variety of other 3’ blocking groups, which include 3’ phosphate, 3’ phosphoglycolaldehyde, or 3’ phosphoglycolate (Breen and Murphy 1995). In addition to removing the 3’-PUA left by hOGG1 and hNTH1, APE1 also removes each of these 3’ blocking groups to generate a 3’-OH that is suitable for extension by a DNA polymerase.

APE initiates a subpathway of BER called abasic site repair, which begins with excision of an AP site, rather than the removal of a base by a glycosylase (Figure 4, right-hand pathway). AP sites are either generated directly by spontaneous depurination or by reaction of ROS with the deoxyribose at C1’ (for review, see (Mitra et al. 2007)). AP sites are also created by monofunctional glycosylases such as UNG or by abrogation of the lyase activity of a bifunctional glycosylase by APE1, as was observed in vitro for hNTH1 (Marenstein et al. 2003). APE1 then cleaves the phosphodiester backbone 5’ to the AP site, yielding a 3’-OH on the upstream nucleotide, but also leaving a 5’-deoxyribose phosphate (5’-dRP) on the downstream nucleotide, which must be removed for efficient repair. Originally it was proposed that the intrinsic lability of the 5’-dRP was sufficient for repair (Bailly and Verly 1989), but later on it was discovered that the lyase activity associated with either a glycosylase or polymerase is needed to remove this moiety (Matsumoto and Kim 1995; Jiang et al. 1997; Bebenek et al. 2001).
Apart from NEIL1-initiated BER, APE1 constitutes the first common enzyme in BER, and its endonuclease activity is essential for survival. Mice with homozygous disruption of the APE1 gene die during early embryonic development at day 5.5 (Xanthoudakis et al. 1996). Initially, it was unclear whether this result was due to loss of APE1’s redox activity or its DNA repair activity. However, two later studies demonstrated that apoptosis of mouse embryonic fibroblasts (MEFs) triggered by APE1 inactivation could be prevented by ectopic expression of either a repair competent allele of APE1 or S. cerevisiae Apn1, which has no redox activity (Fung and Demple 2005; Izumi et al. 2005). By contrast, expression of a repair defective APE1 allele failed to suppress apoptosis. These studies indicated that the DNA repair activity of APE1 is essential for survival.

Unlike S. cerevisiae and E. coli (Mitra et al. 2007), mammals express just one active APE, which has been found by immunohistochemistry in the nucleus, mitochondria, and cytosol (Pinz and Bogenhagen 1998; Tell et al. 2001). Upon translocation into mitochondria, the N-terminal 33 residues of APE1 (containing an NLS) are cleaved by a mitochondria associated N-terminal peptidase (Chattopadhyay et al. 2006). Oxidative stress increases the overall levels of APE1 as well as its levels in both the nucleus and mitochondria (Ramana et al. 1998). This is accomplished in part by transient movement of cytosolic APE1 into nucleus and mitochondria. Interestingly, treatment with Leptomycin B, a specific inhibitor of nuclear export, was observed to enhance the nuclear accumulation of APE1, suggesting that oxidative stress-induced nuclear targeting of APE1 may be due to the inhibition of nuclear export rather than enhanced nuclear import (Jackson et al. 2005).
The structure of APE1 includes features common to DNA glycosylases (Mol et al. 2000). Like the glycosylases, APE1 primarily binds the lesion-containing strand and extrudes the AP site from the double helix such that it enters into an extrahelical pocket. However, the APE1 pocket is specific to AP sites and excludes DNA bases. Like NEIL1, APE1 has a rigid, preformed surface that bends DNA 35°, which facilitates extrusion of the AP site. Abasic site flipping also occurs in a structurally unrelated AP endonuclease, Endonuclease IV, suggesting that abasic site flipping may be a conserved property of AP endonucleases (Hosfield et al. 1999). Unlike the DNA glycosylases, APE1 requires a divalent metal ion to stabilize the transition state and the leaving group of the phosphodiester cleavage reaction.

3.3. The Role of DNA Polymerase β in BER. DNA Polymerase beta (Pol β) consists of an N-terminal 8 kDa lyase domain and a C-terminal 31 kDa polymerase domain, making it the smallest eukaryotic DNA polymerase. The polymerase domain contains three subdomains, the fingers, palm, and thumb, which function in DNA binding, catalysis, and nascent base pair binding, respectively. By generating a single nucleotide gap containing a 3'-OH and 5'-phosphate, APE1 provides Pol β its most preferred substrate (Prasad, Beard, and Wilson 1994). The overall catalytic mechanism of Pol β proceeds as follows (for review, see (Yamtich and Sweasy 2010)). Pol β first binds the DNA substrate in the “open” (catalytically inactive) conformation and induces a 90° bend of the template DNA opposite the gap. Pol β then binds an incoming deoxynucleoside triphosphate (dNTP), preferentially binding to the correct dNTP that maintains hydrogen bonding with the templating base. Upon correct dNTP binding, the enzyme undergoes a conformational change such that the fingers subdomain rotates ~30° around the α-helix M, which positions the active site for catalysis. Smaller side chain rearrangements also
occur, yielding the “closed” conformation of the enzyme. Using a two-metal-ion catalytic mechanism and three catalytic aspartates, Pol β transfers the deoxynucleoside from the dNTP to the 3’ end of the DNA strand. Afterwards, a second conformational change likely occurs, in which subdomain opening allows release of the pyrophosphate and dissociation of the enzyme from DNA.

Pol β, the first polymerase identified to function in BER in vivo (Sobol et al. 1996), is a member of family X polymerases that function in DNA repair rather than replication. In addition to the above-described polymerization activity, family X polymerases contain accessory activities that aid in repair. For example, Pol β contains a dRP lyase activity that critically removes the 5’-dRP left by APE1 (Sobol et al. 2000). Interestingly, the lyase domain of Pol β contains a Helix-hairpin-Helix (HhH) motif that is also found in DNA glycosylases such as hNTH, suggesting a similar mechanism between glycosylase and polymerase catalyzed 5’-dRP excision. Other polymerases have been proposed to contribute to BER as either back-up to Pol β or providing some independent function. Pol λ, for example, another family X polymerase, contributes to BER in cell extracts, but much less so than Pol β (Braithwaite et al. 2005). Pol β−/−Pol λ−/− MEFs were observed to be hypersensitive to both alkylating and oxidizing agents over either single gene deletion alone, which suggests an independent role for Pol λ in BER (Braithwaite et al. 2010). A member of the RAD30 family of DNA polymerases, Pol ι, has also been implicated in BER by the presence of dRPase activity and its ability to substitute for Pol β in vitro (Bebenek et al. 2001). Finally, Pol γ, as the sole DNA polymerase in mitochondria, contains 5’-dRP lyase activity and can substitute for Pol β in vitro, strongly suggesting it
mediates gap-filling in mitochondrial BER (Longley et al. 1998; Pinz and Bogenhagen 1998; Kaguni 2004).

Pol β is constitutively expressed in most tissues although the level of Pol β mRNA has been observed to increase before and during DNA replication (Suzuki et al. 1991; Menegazzi et al. 1992). Increased expression of Pol β can also be induced by treatment with DNA alkylating agents such as MNNG (Narayan, He, and Wilson 1996; He et al. 2003). Possibly, cells have evolved to limit expression of Pol β because overexpression increases the frequency of frameshift mutations (Chan et al. 2007). On the other hand, expression of an inactive Pol β mutant can also drive DNA mutagenesis and is sufficient to induce cellular transformation (Lang et al. 2007). Pol β also seems to play an essential role in neural development since mice harboring homozygous Pol β deletions die immediately after birth from respiratory failure as a result of massive neuronal apoptosis (Sugo et al. 2000). Therefore, the activity and expression of Pol β are finely tuned to the requirements of individual cells.

Pol β contains three loops with important functions. Loop II (residues 240-253) is important for polymerase fidelity (Kosa and Sweasy 1999; Dalal, Kosa, and Sweasy 2004; Hamid and Eckert 2005; Lin, Jaeger, and Sweasy 2007; Lin et al. 2009) and Loop III (residues 301-316) mediates the interaction between Pol β and XRCC1 (Gryk et al. 2002). In addition to binding XRCC1, Pol β interacts with other proteins during BER, including APE1, PCNA, FEN1, PARP1, WRN, HMGB1, and APC; these proteins regulate the overall activity, strand-displacement synthesis, and dRP lyase activity of Pol β (Dantzer et al. 2000; Prasad et al. 2000; Kedar et al. 2002; Dianova et al. 2004; Wong
and Demple 2004; Balusu et al. 2007; Prasad et al. 2007). PT modifications of Pol β also
modulate its activity. Methylation by PRMT1 at residue Arg137 inhibits interactions
between Pol β and PCNA and methylation by PRMT6 at residues Arg83 and Arg152
increases the DNA binding and processivity of Pol β (El-Andaloussi et al. 2006; El-
Andaloussi et al. 2007). Acetylation of Pol β by p300 reduces its dRP lyase activity
(Hasan et al. 2002). All together, many factors regulate not just the expression levels of
Pol β, but its activities and interactions as well.

3.4. The Role of DNA Ligase III and XRCC1 in BER. The final step in BER is the
formation of a phosphodiester bond between the 3'-'OH of the newly added nucleotide
and the downstream 5'-'phosphate. All human DNA ligases use the energy of ATP to
catalyze phosphodiester bond formation in three steps (for review, see (Ellenberger and
Tomkinson 2008)). First, adenosine monophosphate (AMP) is transferred from ATP to
an active site lysine on the DNA ligase. The AMP is then transferred from the lysine to
the 5'-'phosphate of the downstream DNA. And finally, the presence of the AMP
activates the 5'-'phosphate for nucleophilic attack by the 3'-'OH on the upstream DNA,
which displaces the AMP and covalently joins the two DNA strands. Each of these steps
is highly energetically favorable, effectively making them irreversible and also having two
important consequences. First, DNA ligases are pre-adenylated on their active site
lysine before binding DNA. Second, if the 5'-'AMP-DNA adduct in the second step forms
with a DNA end unsuitable for ligation, such as those with modified 3' termini generated
by ROS or APE1 (cf. 1.3.2), DNA ligase cannot complete the ligation. Instead, such an
event leaves a “dirty” DNA break whose 5'-'AMP must be removed by a
phosphodiesterase that specifically acts on dead-end DNA ligation products (Ahel et al.
2006).
There are three DNA ligases in mammals, encoded by the genes LIG1, LIG3, and LIG4. LIG3 codes for protein products that participate in BER, as well as a subpathway of NER (Moser et al. 2007). Through germ-cell specific alternative splicing, LIG3 gives rise to two distinct mRNA’s that are translated to form DNA ligase IIIα (LigIIIα) and DNA ligase IIIβ (LigIIIβ) (Mackey et al. 1997; Perez-Jannotti, Klein, and Bogenhagen 2001). The alternative splicing replaces the C-terminal BRCT domain in LigIIIα with an NLS in LigIIIβ. From each mRNA, alternative translation initiation creates protein products that contain or lack an N-terminal mitochondrial localization signal, thereby directing the enzymes into both the nucleus and mitochondria (Lakshmipathy and Campbell 1999; Perez-Jannotti, Klein, and Bogenhagen 2001). In sum, the LIG3 gene yields four distinct protein products, two sets of which are expressed in either germ-line or somatic cells, with each set containing one that localizes to the nucleus and one to the mitochondria.

The BRCT domain of LigIIIα interacts with the BRCT II domain of XRCC1 to form a stable complex in the nucleus (Caldecott et al. 1994). XRCC1 has no known enzymatic activity, but is thought to function as a scaffold for BER (Kubota et al. 1996) because it interacts with many of the BER proteins (Caldecott et al. 1994; Taylor et al. 2000; Vidal et al. 2001; Whitehouse et al. 2001; Gryk et al. 2002; Caldecott 2003; Marsin et al. 2003; Campalans et al. 2005). However, XRCC1 is not found in mitochondria, where LigIIIα instead interacts through its catalytic region with Pol γ (De and Campbell 2007).

Presumably this interaction incorporates LigIIIα into mitochondrial replication and repair because the presence of XRCC1 in complex with LigIIIα is required for its stabilization in the nucleus. Mice with homozygous deletions of either the LIG3 or XRCC1 genes die during early embryogenesis (Tebbs et al. 1999; Puebla-Osorio et al. 2006). However,
because LigIII and XRCC1 are both required for a stable ligase complex, it isn’t clear which of the proteins is essential. Experiments using RNAi to knock-down LigIII transcripts found increased single-strand nicks in mitochondrial DNA (mtDNA) and diminished capacity to restore mtDNA after exposure to \( \gamma \)-irradiation, suggesting that the early lethality phenotype of \( \text{Lig}^{3/-} \) mice could have been caused by mitochondrial dysfunction (Lakshmipathy and Campbell 2001). As for XRCC1, Taylor et al. inhibited the interaction between LigIII and XRCC1 by mutating the BRCT II domain of XRCC1, and assessed single strand break repair (SSBR) capacity during different phases of the cell cycle (Taylor et al. 2000). SSBR was abolished during G1, but not during S phase; and they also found that wild type XRCC1 colocalizes with Rad51 after treatment with the alkylating agent ethylmethane sulfonate (EMS), suggesting an S-phase specific, LigIII-independent role for XRCC1 in homologous recombination (HR). However, when and how XRCC1 interacts with different repair pathways is still not completely understood.

DNA ligases contain a minimal catalytic subunit that consists of a nucleotidyltransferase (NTase) domain and OB-fold domain (OBD) (Ellenberger and Tomkinson 2008). All human DNA ligases also contain a DNA binding domain (DBD) on the N-terminal side of the catalytic core. These three protein segments are flexible and allow the DNA ligase to open and close around the DNA. The crystal structures of DNA ligase I and III exhibit common structural features, despite their primary sequence divergence outside the catalytic core (Pascal et al. 2004; Cotner-Gohara et al. 2010). In both enzymes, the DBD, OBD, and NTase domain completely encircle the DNA substrate. The DBD interacts with the minor groove of both strands of DNA up and downstream of the nick. The OBD binds the minor groove of the DNA downstream of the nick and alters the
curvature of the DNA backbone, enforcing an underwound conformation that widens the major and minor grooves. It was hypothesized that this allows the DNA ligase to discriminate against downstream RNA:DNA heteroduplexes by forbidding its shape and/or flexibility (Pascal et al. 2004). DNA Ligase III is unique in that it also has an N-terminal PARP-like Zinc finger domain (ZnF), which is critical for its nick-sensing activity. In conjunction with the adjacent DBD, the ZnF of LigIII cooperatively binds to nicks and gaps in duplex DNA (Cotner-Gohara et al. 2008). Then, the OBD and NTase domains encircle the substrate, displace the ZnF, and proceed with catalysis.

3.5. Long-patch Base Excision Repair. The principal pathway for repair of oxidative DNA damage is the above-described short-patch BER (SP-BER), in which one nucleotide gap is filled by Pol β. However, repair patches in human cell extracts are heterogeneous in length, with a minority of gap-filling events extending 2-6 nucleotides (Klungland and Lindahl 1997). These repair patches reflect an alternative repair pathway, called long-patch BER (LP-BER) that depends on two additional proteins, Proliferating Cell Nuclear Antigen (PCNA) and Flap Endonuclease 1 (FEN1). APE1 initiates LP-BER by catalyzing a nick 5’ to an AP site, as described above. Then Pol β displaces the downstream DNA while it synthesizes a tract of 2-6 nucleotides. The strand-displacement synthesis by Pol β generates a single stranded 5’-DNA flap, which is refractory to ligation. FEN1 is required to remove the 5’ flap (Klungland and Lindahl 1997; Kim, Biade, and Matsumoto 1998) and Lig I or III completes repair. LP-BER was inhibited when PCNA was depleted from cell extracts with a polyclonal antibody, suggesting that it too is required for LP-BER (Frosina et al. 1996). In mitochondria, Pol γ functions in place of Pol β during LP-BER, and Pol δ can substitute for Pol β in LP-BER in vitro (Klungland and Lindahl 1997; Longley et al. 1998).
The selection to proceed by short- versus long-patch BER is not well understood. *In vivo*, a range of modified AP sites are generated by reaction of ROS with DNA. One hypothesis is that initiation of LP-BER depends on the 5’ adduct left by APE1 when it cleaves a modified AP site. For example, when APE1 nicks a reduced AP site, it leaves a reduced 5’-dRP. Pol β is unable to excise the reduced 5’-dRP and therefore does not provide a suitable substrate for ligation. Indeed, when human lymphoblastoid cell extracts were incubated with regular and reduced AP sites, SP-BER was observed to repair regular AP sites and LP-BER for reduced ones (Klungland and Lindahl 1997). Notably, in their first enzymatic step DNA glycosylases generate regular AP sites, so they primarily initiate SN-BER.


4.1. Oxidative DNA Damage in the Nucleus. In metabolically active cells DNA is fully hydrated and is vulnerable to depurination by hydrolysis of the N-glycosyl bond at a rate similar to that in solution (Lindahl 1993). Based on the rate of base loss over a range of temperatures at physiologic pH and ionic strength, the rate of depurination of the human genome at 37°C has been estimated to be ~14,400 purines lost per day (Lindahl and Nyberg 1972). However, this estimate does not take into account the rate of hydrolysis induced by ROS, the production of oxidized bases, or any protective effect due to the packaging of DNA into chromatin. The most reactive ROS, the hydroxyl radical, preferentially cleaves at the minor groove of DNA, similar to DNase I (Tullius et al. 1987). Footprinting experiments by Hayes et al. (Hayes, Tullius, and Wolfe 1990) show that bases of DNA located where the minor groove faces away from the histone octamer
are more susceptible to hydrolysis by hydroxyl radicals. On the other hand, when McGhee and Felsenfeld incubated nucleosomes with dimethyl sulfate, a methylating agent, they found that nucleosomes provided very little protection of the N7 of guanine and did not detect any periodic modulation of reactivity (McGhee and Felsenfeld 1979). In accord with these results, Enright et al. (Enright et al. 1996) reported no indication of targeting of oxidative DNA damage to internucleosomal regions following the incubation of polynucleosomes with iron-EDTA in the presence of H₂O₂ and ascorbate (which catalyzes the production of hydroxyl radicals). Together, these results indicate that the susceptibility of nucleosomal DNA to hydroxyl radicals is highest when the minor groove faces away from the histone octamer, but also that the nucleosome itself provides little protection from damage induced by free radicals. However, other studies suggest that histones themselves may act as a sink for ROS. Ljungman and Hanawalt showed that histones can quench the generation of oxygen radical inflicted DNA damage and found 3-fold fewer breaks in more condensed chromatin (Ljungman and Hanawalt 1992). In sum, while chromatin provides some protection of DNA from oxidative damage by acting as a sink for ROS, nucleosomal DNA is far from immune to the effects of cellular oxidants.

4.2. BER on Nucleosomes in vitro. To test whether BER enzymes can act on oxidative lesions in chromatin, a number of groups have attempted to recapitulate the entire reaction in vitro using defined nucleosomal substrates (Nilsen, Lindahl, and Verreault 2002; Beard, Wilson, and Smerdon 2003; Menoni et al. 2007). Nilsen et al. in 2002 reconstituted nucleosomes with a segment of DNA containing the *L. variegatus* 5S rDNA gene and histone octamers purified from chicken erythrocytes (Nilsen, Lindahl, and Verreault 2002). Intrinsic properties of the 5S rDNA nucleotide sequence result in a
nucleosome particle with one major translational position, as well as a number of minor
positions which differ from the major position by multiples of 10 base pairs (Lu, Steege,
and Stafford 1980; Simpson and Stafford 1983; Dong, Hansen, and van Holde 1990;
Flaus et al. 1996). As a result, bases within the 5S rDNA segment adopt a rotationally
discrete orientation relative to the histone octamer in virtually all nucleosome particles,
and in most nucleosomes they occupy a translationally discrete position as well. Using
this phenomenon, Nilsen et al. placed single uracils opposite adenine (U:A) within the
DNA sequence at either -22, -51, or -54 nucleotides from the dyad axis, in order to
investigate the influence of rotational and translational positioning on BER. Incubating
these uracil-containing nucleosomes with the monofunctional DNA glycosylases, UNG2
or SMUG1, revealed a 3- to 9-fold reduction in efficiency of base excision when
compared with naked DNA; the levels of inhibition by the nucleosome did not change
with either the distance of the uracil from the dyad or its rotational setting. Nilsen et al.
(Nilsen, Lindahl, and Verreault 2002) next investigated the entire BER reaction by
incubating uracil-containing nucleosomes with UNG2 and APE alone, or together with
either Pol β, Pol β and XRCC1, or Pol β, XRCC1 and DNA ligase III. With the addition of
each enzyme they observed the corresponding repair intermediate and complete repair
in reactions containing all of the BER enzymes, suggesting that nucleosomes do not
completely inhibit any step of the repair process. They also observed no nucleosome
disruption or change in translational positioning during BER.

Soon thereafter, Smerdon and colleagues published an investigation of BER on
nucleosomes assembled using a different DNA sequence and a different method of
assembly, known as octamer transfer. Specifically, Beard et al. (2003) mixed purified
chicken erythrocyte core particles (CECP’s) with DNA containing a “Tg-motif”
nucleosome positioning sequence flanking an 18 bp glucocorticoid hormone receptor response element (GRE) (Beard, Wilson, and Smerdon 2003). They embedded into the DNA single uracils opposite guanine residues (U:G) directly at the dyad axis or four nucleotides away from the dyad. Incubating these nucleosomes with UNG2 revealed ~10-fold reduction in efficiency of uracil excision relative to that of naked DNA. However, unlike Nilsen et al., Beard et al. reported a 2- to 3-fold higher activity when the minor groove of the uracil faced away from the histone octamer, indicating that the rotational orientation of the uracil is an important factor for UNG2 excision activity. In stark contrast to Nilsen et al., Smerdon and colleagues did not observe extension by Pol β on nucleosome substrates, suggesting that nucleosome remodeling or disruption is required for the last two steps of repair to occur.

Other groups have investigated the activities of single or subsets of BER proteins on nucleosomes. Menoni et al. in 2007 reconstituted nucleosomes with octamers assembled with recombinant histones purified from E. coli and DNA containing the 601 nucleosome positioning sequence (Menoni et al. 2007), a synthetic DNA segment that was selected for its capacity to form a highly stable, positioned nucleosome (Thastrom et al. 1999). Instead of using a uracil as a model for repair, Menoni et al. measured the capacity of a bifunctional DNA glycosylase (mouse OGG1) to excise 8-oxoG lesions located 10 nucleotides away from the dyad axis. As in earlier studies, they found reduced glycosylase activity on nucleosomes substrates, as well as reduced APE1 and Pol β activities. This study was unique in that they added the SWI/SNF ATP-dependent nucleosome remodeling complex (cf. Section 1.1.4.) to their reactions and observed similar excision rates by mouse OGG1 and APE1 on nucleosomes and naked DNA. Importantly, incorporation of the histone variant H2A.Bbd into nucleosomes in place of
canonical H2A abrogated the increased activity induced by SWI/SNF. Together these data suggest that nucleosome remodeling can facilitate BER and the rate of BER may be modulated by the presence of histone variants. However, to date there is no in vivo evidence that ATP-dependent nucleosome remodeling by SWI/SNF occurs at sites of BER.

Hayes and colleagues have investigated the activities of human DNA Ligase I and FEN1 on nucleosomes (Chafin et al. 2000; Huggins et al. 2002). These enzymes catalyze the last two steps of LP-BER and are involved in Okazaki fragment processing. Chafin et al. reconstituted nucleosomes with DNA containing the Xenopus borealis 5S rDNA gene and octamers purified from chicken erythrocytes (Chafin et al. 2000). The DNA contained three nicks, one at the dyad axis, one at the edge of the nucleosome, and one in the adjacent linker DNA. Notably, the presence of a nick in the DNA did not change the translational positioning, reconstitution efficiency, or electrophoretic mobility of the nucleosome. The addition of Lig I to the nick-containing nucleosomes resulted in some ligation, albeit with a 10-fold reduced efficiency compared to naked DNA, similar to the above-described results for other BER enzymes. However, when they reconstituted nucleosomes with a shorter segment of the same DNA sequence, but lacking linker DNA (154 bp instead of 218 bp), they observed a 10,000-fold inhibition of Lig I. It has been shown that histone tails bind to different regions of nucleosomal DNA in the absence of linker DNA (Usachenko et al. 1994; Lee and Hayes 1998), which they hypothesized was responsible for the increased inhibition of Lig I with the shorter DNA substrate. To test if this was the case, Chafin et al. removed the tails by light trypsin digestion and incubated the tailless, nick-containing nucleosomes with Lig I. They observed a similar ligation rate
of the tailless 154 bp nucleosomes and 218 bp nucleosomes containing wild type histones, suggesting that Lig I is inhibited by histone tails in certain contexts.

In a subsequent study by Hayes and colleagues, the authors placed a 5 nucleotide DNA flap at the dyad of a nucleosome assembled using the same 154 bp nucleosome positioning sequence as Chafin et al (Huggins et al. 2002). In contrast to the partial inhibition of BER proteins, FEN1 cleaved the flap-containing nucleosomes 7-fold faster than flap-containing naked DNA, suggesting that FEN1 actually prefers a nucleosome substrate. Structural studies led Chapados et al. to propose that a hydrophobic wedge in FEN1 creates a kink in the phosphodiester backbone opposite the flap junction, and that this facilitates 3’ and 5’ flap recognition (Chapados et al. 2004). Perhaps nucleosome-induced DNA bending positions the flap in a favorable orientation for FEN1 binding and DNA bending. Measurement of the activity by FEN1 on flaps in different rotational orientations relative to the histone octamer would help this hypothesis. Chapados et al. also observed that FEN1 kinks the DNA to a final angle of 90-100° (Chapados et al. 2004), and noted the parallel between the FEN1 kink and the 90° kink induced by Pol β when it is bound to nick or gap containing DNA (Sawaya et al. 1997). Consequently, structural and nucleosome studies with FEN1 imply that DNA kinking by Pol β alone should not preclude it from functioning on nucleosomes.

In Prasad et al., 2007, Pederson and colleagues proposed a mechanism that enables BER of lesions in nucleosomes. They reconstituted nucleosomes by octamer transfer from purified CECPs to DNA containing the L. variegatus 5S rDNA nucleosome positioning sequence (Prasad, Wallace, and Pederson 2007). The authors placed within the DNA a single Tg at -22, -26, -46, or -51 bp from the dyad axis. When the Tg was
positioned so that its minor groove faced outward from the histone octamer, hNTH1 was able to excise the Tg from nucleosomes at a rate similar to that from naked DNA, without requiring or inducing nucleosome disruption. Placing the Tg in the opposite orientation, such that its minor groove faced toward the histone octamer, substantially reduced the efficiency of excision. These results were in accord with the observations by Smerdon and colleagues that the rotational orientation of the substrate affects the ability of the glycosylase to excise the base (Beard, Wilson, and Smerdon 2003). Interestingly, Prasad et al. also found that increasing the concentration of hNTH1 to near physiological levels resulted in substantially higher rates of excision of inward facing Tg residues. This and other observations led the authors to propose that hNTH1 can access occluded lesions during transient periods when nucleosomal DNA partially unwraps from the histone octamer. This proposal was consistent with studies by Li and Widom of target site binding of LexA to sites within nucleosomes (cf. 1.1.5) (Li and Widom 2004).

The studies of BER of lesions in nucleosomes summarized above have provided key insights into the factors that affect the rate of repair. However, important questions remain. For example, the rotational position of the base is a critical factor in the activity of DNA glycosylases, suggesting that lesion recognition may be a rate-limiting step in BER on nucleosomes (Beard, Wilson, and Smerdon 2003; Prasad, Wallace, and Pederson 2007). However, it is not clear if rotational orientation affects the subsequent enzymatic steps in the same fashion. Second, certain DNA glycosylases such as NEIL1 can excise damaged bases from single strand DNA and also exhibit increased expression levels during S phase, suggesting they act in conjunction with DNA replication (Otterlei et al. 1999; Dou, Mitra, and Hazra 2003). If this hypothesis is correct, these glycosylases may function in a quasi-nucleosome free environment making it of
interest to determine if they differ in their activity on nucleosomes compared to glycosylases that are constitutively expressed throughout the cell cycle. Finally, in light of the differences in activity reported for Pol β on nucleosomes, it should be determined if the nucleosome positioning sequence itself affects the rate of repair. The following chapters address these questions and their implications. Chapter 2 describes experiments that compare the cellular concentrations and activities of the constitutively expressed DNA glycosylase, hNTH1, with the replication-associated DNA glycosylase, NEIL1. Chapter 3 describes experiments with nucleosomes containing substrates for each of the steps in BER, oriented either away or towards the histone octamer. Chapter 3 also describes studies comparing two distinct nucleosome positioning sequences to examine the effects of sequence content, as well as studies that examine possible synergy among BER enzymes during the repair of lesions in nucleosomes. Finally, Chapter 4 describes conclusions from the work described herein and studies that would be of interest to pursue in the future.
5. Figure Legends

*Figure 1. Canonical core histones and their variants, from (Sarma and Reinberg 2005).* The major core histones contain a conserved histone-fold domain (HFD) flanked by N- and C-terminal tails that harbour sites for various post-translational modifications. For simplicity, only well-established sites for lysine methylation (red flags) and serine phosphorylation (green circles) are shown; other types of modifications, such as ubiquitylation, are not shown. The four residues in the histone H3.3 variant that differ from the major, canonical histone H3 (also known as H3.1) are highlighted in yellow. Three of these residues lie within the globular domain. The fourth residue (Ser31) resides in the N terminus and might be a potential site for phosphorylation. The centromeric histone CENPA has a unique N terminus that does not resemble the N-terminus in other core histones; this segment of CENPA contains two phosphorylation sites, one of which (Ser7) has been shown to be essential for completion of cytokinesis. The region in the globular domain that is required for targeting of CENPA to the centromere is highlighted in light blue. Histone H2A variants differ significantly from the major core H2A in their C terminus. For example, H2A.X harbours a conserved serine residue in its C-terminus (Ser139) that is phosphorylated early during the response to DNA double-strand breaks. Likewise, a short segment in the C terminus of the *Drosophila melanogaster* H2A.Z is essential for viability. MacroH2A has an extended C-terminal “macro domain,” the function of which is unknown. Finally, H2A.Bbd is the smallest of the H2A variants, containing a distinct N-terminus that lacks all of the conserved modification sites that are present in the canonical H2A, and a truncated C terminal segment. The primary sequences of histones H4 and H2B are also shown, along with their known methylation and phosphorylation sites. The proposed functions of the variants are listed.
Figure 2. Summary of measured equilibrium constants for site exposure, from (Anderson and Widom 2000). “Means and standard deviations are plotted as a function of location in the nucleosome, for the high affinity sequence 601.2 (dark shading, tied to the corresponding nucleosomal locations below the plot) and for the reference 5S sequences (light shading, tied to their corresponding nucleosomal locations above the plot). The data for the 5S reference sequences, including the corresponding nucleosome mapping studies, are from our earlier study.”

Figure 3. A model for the functions of chromatin remodeling complexes at DNA double-strand breaks in budding yeast, from (Bao and Shen 2007). Following DNA double-strand break formation, the Mre11–Rad50–Xrs2 (MRX) complex (MRN complex in higher eukaryotes) and the Ku70–Ku80 heterodimer are recruited to the DNA ends. Shortly thereafter, the Tel1 and Mec1 protein kinases (homologous to mammalian ATM and ATR) are recruited and phosphorylate Ser129 in H2A (Ser139 in mammalian H2A.X) over a ~50 kb region. NuA4 HAT complex is also recruited and acetylates lysine residues in the N-terminal tails of histones H2A and H4. The remodeling complex RSC is recruited to DSBs through its interaction with Mre11. In the homologous recombination pathway, RSC can remodel chromatin near DSBs to promote loading of cohesin, which holds the sister chromatids together, and thereby facilitates strand invasion and Holliday junction formation. RSC is also required at a later postsynaptic step of homologous recombination repair. INO80 is recruited through its interaction with phosphorylated H2A, and promotes formation of the presynaptic filament by controlling the loading of Rad51 onto recipient DNA. The histone chaperone SWR1 complex may be recruited to DSBs in concert with NuA4, to exchange γ-H2A.X (i.e. phosphorylated H2A.X) with H2A.Z. This
may further alter the local chromatin structure and facilitate the process of DNA repair. The SWI/SNF remodeling complex is also recruited to DSBs and promotes synapsis between donor and recipient DNA by removing nucleosomes from donor sequences. RSC, INO80 and probably SWR1 may also be involved in non-homologous end-joining, although their exact roles in that process are unknown.

**Figure 4. BER subpathways for repair of oxidative DNA lesions, adapted from (Lindahl 2000).** Two of the three BER subpathways depicted begin with the recognition of an oxidized base by NEIL1 NEIL2, or NEIL3 (left pathway), or by hNTH1 or hOGG1 (middle pathway). The 3′-phosphate left by NEIL1 is removed by PNK, whereas the 3′-PUA left by hNTH1 and hOGG1 is removed by APE1. Alternatively, the DNA glycosylase can pass an AP site to APE1 after it removes the oxidized base, at which point APE1 cleaves the phosphodiester backbone 5′ to the AP site (right pathway). All three pathways leave a gap that must be filled by Pol β. In cases where the APE1 endonuclease acted at the preceding step, Pol β also must remove a dRP residue. In the final step of BER, LigIIIα in complex with XRCC1 seals the nick left by Pol β.
Chapter 1-Table 1. Different Classes of Modifications Identified on Histones (Kouzarides 2007).

<table>
<thead>
<tr>
<th>Chromatin Modifications</th>
<th>Residues Modified</th>
<th>Functions Regulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylation</td>
<td>K-ac</td>
<td>Transcription, Repair, Replication, Condensation</td>
</tr>
<tr>
<td>Methylation (lysines)</td>
<td>K-me1 K-me2 K-me3</td>
<td>Transcription, Repair</td>
</tr>
<tr>
<td>Methylation (arginines)</td>
<td>R-me1 R-me2a R-me2s</td>
<td>Transcription</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td>S-ph T-ph</td>
<td>Transcription, Repair, Condensation</td>
</tr>
<tr>
<td>Ubiquitylation</td>
<td>K-ub</td>
<td>Transcription, Repair</td>
</tr>
<tr>
<td>Sumoylation</td>
<td>K-su</td>
<td>Transcription</td>
</tr>
<tr>
<td>ADP ribosylation</td>
<td>E-ar</td>
<td>Transcription</td>
</tr>
<tr>
<td>Deimination</td>
<td>R -&gt; Cit</td>
<td>Transcription</td>
</tr>
<tr>
<td>Proline Isomerization</td>
<td>P-cis &gt; P-trans</td>
<td>Transcription</td>
</tr>
</tbody>
</table>

Chapter 1-Table 2. Functions of ATP-dependent Chromatin Remodeling Families (Fan, Narlikar, and Kingston 2004).

<table>
<thead>
<tr>
<th>Family</th>
<th>Motor proteins</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWI/SNF</td>
<td>hBRG1, hBRM, dBRM, ySh1p, ySwi2p/Snf2p</td>
<td>Transcription, DNA replication, Recombination, Higher-order chromatin structure?</td>
</tr>
<tr>
<td>ISWI</td>
<td>hSNF2h, hSNF21, dISWI, xISWI, yISwi1p, yISw2p</td>
<td>Chromatin assembly, Transcription, Higher-order chromatin structure, DNA replication, Nucleotide excision repair</td>
</tr>
<tr>
<td>Mi-2</td>
<td>Mi2α/CHD3, Mi2β/CHD4, Chd1p, Hrp1, Hrp3</td>
<td>Transcription, histone deacetylation</td>
</tr>
<tr>
<td>INO80</td>
<td>yino80p, hiINO80</td>
<td>Transcription, DNA repair</td>
</tr>
<tr>
<td>SWR1</td>
<td>ySwr1p, hSCRAP, human p400, dDomino</td>
<td>Transcription, Histone exchange, DNA repair</td>
</tr>
<tr>
<td>CSB*</td>
<td>CSB/ERCC6, yRad26p</td>
<td>Transcription-coupled DNA repair</td>
</tr>
<tr>
<td>Rad54*</td>
<td>hRad54, hRad54B, dORK, yRad54p, hATRX, ARIP4, DRD1</td>
<td>Recombination, Transcription, DNA methylation</td>
</tr>
<tr>
<td>DDM1</td>
<td>DDM1, LSH1</td>
<td>DNA methylation</td>
</tr>
</tbody>
</table>
## Chapter 1-Table 3. Activity of BER enzymes on nucleosomal substrates (Jagannathan, Cole, and Hayes 2006).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Effect of nucleosome on activity</th>
<th>Tail effect on activity</th>
<th>Interacting factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNG2</td>
<td>Nilsen et al. (2002)—initial rate 30-fold slower; 3-fold slower at later time points, 10–30-fold slower depending on the position</td>
<td>No effect in either case</td>
<td>RPA, PCNA, APE</td>
</tr>
<tr>
<td>SMUG1</td>
<td>Nilsen et al. (2002)—9-fold slower activity</td>
<td>No effect</td>
<td>NA</td>
</tr>
<tr>
<td>TDG</td>
<td>NA</td>
<td>NA</td>
<td>Tini et al. (2002)—CBP/p300, APE</td>
</tr>
<tr>
<td>hOgg1</td>
<td>NA</td>
<td>NA</td>
<td>Tuo et al. (2002a,b)—CSB</td>
</tr>
<tr>
<td>APE1</td>
<td>Nilsen et al. (2002)—no effect</td>
<td>No effect</td>
<td>NA</td>
</tr>
<tr>
<td>Pol β</td>
<td>Nilsen et al. (2002)—reduced efficiency, completely inhibited</td>
<td>No effect</td>
<td>HAT-p300, XRCC1, WRN</td>
</tr>
<tr>
<td>FEN1</td>
<td>Huggins et al. (2002)—1.3–7-fold faster at low concentration, 2-fold reduced at high concentration</td>
<td>Yes, tails favour preferential cleavage of nucleosome substrates</td>
<td>PCNA, p300</td>
</tr>
<tr>
<td>DNA ligase III</td>
<td>Nilsen et al. (2002)—efficient on strand extended by pol β</td>
<td></td>
<td>XRCC1</td>
</tr>
<tr>
<td>DNA ligase I</td>
<td>Chafin et al. (2001)—10-fold reduced</td>
<td>Yes, stronger inhibition directed by inappropriately positioned tails</td>
<td>NA</td>
</tr>
<tr>
<td>MAG1</td>
<td>Li &amp; Smerdon (2002)—reduced activity in vivo on repair of NMPs</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Chapter 1-Figure 1. Canonical core histones and their variants.

<table>
<thead>
<tr>
<th>Histone</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>Canonical core histone</td>
</tr>
<tr>
<td>H3.3</td>
<td>Transcriptional activation</td>
</tr>
<tr>
<td>CENPA</td>
<td>Kinetochores assembly</td>
</tr>
<tr>
<td>H2A</td>
<td>Canonical core histone</td>
</tr>
<tr>
<td>H2AX</td>
<td>DNA repair and recombination, major core histone in yeast</td>
</tr>
<tr>
<td>H2AZ</td>
<td>Gene expression, chromosomal segregation</td>
</tr>
<tr>
<td>macroH2A</td>
<td>X chromosome inactivation, transcriptional expression</td>
</tr>
<tr>
<td>H2ABBD</td>
<td>Transcriptional activation?</td>
</tr>
<tr>
<td>H4</td>
<td>Canonical core histone</td>
</tr>
<tr>
<td>H2B</td>
<td>Canonical core histone</td>
</tr>
</tbody>
</table>

Chapter 1-Figure 2. Summary of equilibrium constants for site exposure.

[Diagram showing position (base pairs) and equilibrium constants for site exposure]
Chapter 1-Figure 3. A model for the functions of chromatin remodeling complexes at DNA double-strand breaks in budding yeast.
Chapter 1-Figure 4. hNTH1 and NEIL1-initiated Base Excision Repair.
CHAPTER 2: NON-SPECIFIC DNA BINDING INTERFERES WITH THE EFFICIENT EXCISION OF OXIDATIVE LESIONS FROM CHROMATIN BY THE HUMAN DNA GLYCOSYLASE, NEIL1.

Excision of lesions from nucleosomes by hNTH1 and NEIL1.

Ian D. Odell, Kheng Newick, Nicholas Heintz, Susan S. Wallace & David S. Pederson
**Abstract:** Although DNA in eukaryotes is packaged in nucleosomes, it remains vulnerable to oxidative damage that can result from normal cellular metabolism, ionizing radiation, and various chemical agents. Oxidatively damaged DNA is repaired in a stepwise fashion via the base excision repair (BER) pathway, which begins with the excision of damaged bases by DNA glycosylases. We reported recently that the human DNA glycosylase hNTH1 (human Endonuclease III), a member of the HhH GpG superfamily of glycosylases, can excise thymine glycol lesions from nucleosomes without requiring or inducing nucleosome disruption; optimally oriented lesions are excised with an efficiency approaching that seen for naked DNA (Prasad, Wallace, and Pederson 2007). To determine if this property is shared by human DNA glycoylases in the Fpg/Nei family, we investigated the activity of NEIL1 on defined nucleosome substrates. We report here that the cellular concentrations and apparent $k_{cat}/K_m$ ratios for hNTH1 and NEIL1 are similar. Additionally, after adjustment for non-specific DNA binding, hNTH1 and NEIL1 proved to have similar intrinsic activities towards nucleosome substrates. However, NEIL1 and hNTH1 differ in that NEIL1 binds undamaged DNA far more avidly than hNTH1. As a result, hNTH1 is able to excise both accessible and sterically occluded lesions from nucleosomes at physiological concentrations, while the high non-specific DNA affinity of NEIL1 would likely hinder its ability to process sterically occluded lesions in cells. These results suggest that, *in vivo*, NEIL1 functions either at nucleosome-free regions (such as those near replication forks) or with cofactors that limit its non-specific binding to DNA.
Keywords
Base excision repair, Chromatin, Nucleosome, DNA glycosylase, Human endonuclease III, Endonuclease VIII-like I, Mesothelioma

Abbreviations
APE, AP endonuclease; BER, base excision repair; BSA, bovine serum albumin; FBS, fetal bovine serum; Fpg, formamidopyrimidine DNA glycosylase; Gh, guanidinoxydantoin; Nei, endonuclease VIII; PBS, phosphate buffered saline; PNK, Polynucleotide kinase; PVDF, polyvinylidene fluoride; rDNA, ribosomal DNA; SDS-PAGE, SDS polyacrylamide gel electrophoresis; Tg, thymine glycol.
1. Introduction.

Oxidative damage to DNA occurs as a consequence of normal cellular metabolism, ionizing radiation such as that used in cancer therapy, and various chemical agents (Lindahl and Wood 1999; Klaunig and Kamendulis 2004; Engel and Evens 2006; Hada and Georgakilas 2008). Some of the resulting lesions are mutagenic, while others are cytotoxic. Most oxidative lesions are repaired via the base excision repair (BER) pathway which, in its simplest form (known as “short patch BER”), consists of four enzymatic steps (for reviews see (Wallace et al. 2003; Wilson, Sofinowski, and McNeill 2003; Almeida and Sobol 2007; David, O’Shea, and Kundu 2007; Hegde, Hazra, and Mitra 2008)). The first step is the recognition and excision of a damaged base by either a mono- or bi-functional DNA glycosylase. This is followed by cleavage of the DNA backbone at the resulting apurinic site, either by the lyase activity associated with bifunctional DNA glycosylases or by AP Endonuclease (APE). Then, either APE or polynucleotide kinase (PNK) removes inhibitory moieties at the incision site, leaving a single base gap that is filled by Polymerase β and sealed by Ligase IIIα.

BER enzymes have been extensively characterized (for structure reviews, see (Fromme, Banerjee, and Verdine 2004; Hitomi, Iwai, and Tainer 2007), see also (Imamura, Wallace, and Doublie 2009)), and the entire BER pathway reconstituted in vitro with naked DNA substrates (e.g. (Harrison, Hatahet, and Wallace 1999; Wiederhold et al. 2004)). However, much less is known about how BER enzymes function in the context of the chromatin that packages DNA in eukaryotes. The basic subunit of chromatin is the nucleosome, which consists of 147 base pairs of DNA, wrapped in a left-handed toroidal helix around a histone octamer (Luger et al. 1997). Histone octamers create a steric
impediment to many of the factors and enzymes that act on DNA. The binding of such factors may also be influenced by the bending of DNA around the octamer, which alternately compresses and expands its major and minor grooves. Several groups have reported considerable variation in the capacity of selected BER enzymes to act on lesions in nucleosomes reconstituted in vitro (Nilsen, Lindahl, and Verreault 2002; Beard, Wilson, and Smerdon 2003; Menoni et al. 2007; Prasad, Wallace, and Pederson 2007). Some of this variation can be attributed to the position of the lesion relative to the underlying histone octamer. For example, the efficiency with which the human, bifunctional DNA glycosylase hNTH1 excises lesions from nucleosomes varies with the helical orientation of the lesion and its distance from the dyad axis (i.e. center) of the nucleosome (Prasad, Wallace, and Pederson 2007). The extent to which these structural variables affect lesion processing depends as well on enzyme concentration. Specifically, at high concentrations, hNTH1 is able to capture and process sterically-occluded lesions during episodes of transient, partial unwrapping of lesion-containing DNA from the histone octamer (Prasad, Wallace, and Pederson 2007).

To further elucidate determinants that influence BER of oxidative lesions in chromatin, we have extended our studies to include the human DNA glycosylase NEIL1, a member of the Fpg/Nei family (hNTH1 is a member of the HhH GpG superfamily of glycosylases). While both NEIL1 and hNTH1 recognize and excise thymine glycol (Tg) from double-stranded DNA, several observations suggest that the two enzymes act in different cellular contexts. First, only NEIL1 is able to process lesions in single strand DNA and bubble substrates (Dou, Mitra, and Hazra 2003), a property that might make NEIL1 especially suited to removal of DNA polymerase blocking lesions (such as Tg) at replication forks (Ide, Kow, and Wallace 1985; Clark and Beardsley 1986; Hayes and
LeClerc 1986; McNulty et al. 1998). Second, the inhibitory moiety left by hNTH1 is removed by APE1 while that left by NEIL1 is removed by PNK (Wiederhold et al. 2004), and coimmunoprecipitation experiments indicate that NEIL1 interacts with PCNA and FEN1, factors that act in both DNA replication and long-patch BER (Dou et al. 2008; Hegde et al. 2008). Thus, the two glycosylases channel their substrates into different BER sub-pathways. Third, the abundance of hNTH1 does not change during the cell cycle, while that of NEIL1 increases during S phase (Hazra et al. 2002). Taken together, these observations led to the hypothesis that NEIL1 acts on lesions at replication forks while hNTH1 acts more globally and independently of DNA replication (Hazra et al. 2007).

The possibility that NEIL1 functions at replication forks, and with a different set of protein partners than does hNTH1, led us to ask if NEIL1 and hNTH1 differ in their capacity to process lesions in nucleosomes. We report here that cellular concentrations of hNTH1 and NEIL1 are similar, as are their catalytic rate constants. Additionally, the two enzymes exhibit similar intrinsic activity towards Tg lesions in nucleosomes and are similarly sensitive to the helical orientation of the lesion. However, the two enzymes differ in that NEIL1 binds undamaged double-stranded DNA far more tightly than does hNTH1. Our measurements of the in vivo abundance of NEIL1 and its relatively high affinity for lesion-free DNA suggest that NEIL1 would be severely restricted in its capacity to recognize and act on lesions in chromatin without the aid of protein partners that either recruit NEIL1 to sites of damage or reduce its non-specific interactions with DNA. By contrast, hNTH1 is more likely to be able to recognize lesions in chromatin without the aid of accessory factors.
2. Experimental Procedures.

2.1 In vivo concentrations of hNTH1 and NEIL1. The human mesothelial cell line LP9, and the human mesothelioma cell lines Gates, Mills, and Mont were seeded at 50,000 cells/ml in 60-mM dishes and grown overnight in CMRL 1066 medium with 10% fetal bovine serum (FBS; HyClone), 100 U of penicillin/ml, and 100 μg of streptomycin/ml at 37°C and in 5% CO₂. At harvest, the cells were washed once with cold PBS, pH 7.4, and lysed by the addition of 100 μl lysis buffer (Laemmli buffer containing 100 mM DTT instead of β-mercaptoethanol, plus Roche Protease Inhibitor Cocktail) per plate. Lysates were collected by scraping with a rubber policeman, and the insoluble fraction was removed by centrifugation in a microfuge for 5 min. The protein concentration of the soluble fraction was determined using a Lowry protein assay (Lowry et al. 1951). Cell extracts and recombinant hNTH1 and NEIL1 were fractionated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes with the aid of a semi-dry electroblotting system (The WEP Company). Membranes were blocked with 1% bovine serum albumin (BSA) in TBS containing 0.1% Tween prior to addition of antibodies to either hNTH1 (Novus Biologicals NB100-302SS), NEIL1 (Abcam ab21337) or Actin (Chemicon MAB1501). Immunoreactive bands were visualized by using the appropriate secondary antibodies and an ECL detection kit (USB Rodeo ECL).

2.2 DNA and nucleosome substrates. For enzyme kinetic and non-specific DNA binding studies, a 35 nt DNA oligomer containing a single thymine glycol (Tg) residue (5′-TGCAATAGCAATTgGGAAGATCAATCGTGCAGTCT-3′), and a complementary oligomer containing dA opposite the Tg position, were purchased from Midland Certified Reagent Co. (Midland, Texas) and gel purified. Appropriate amounts of the lesion
containing oligomer were end-labeled using [$\gamma$-32P]-ATP and T4 polynucleotide kinase (New England Biolabs), and annealed to equimolar amounts of the complementary oligomer in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl and 1 mM EDTA. A 14 nt DNA oligomer containing a single guanidino-hydantoin (Gh) residue (5’-GCGTCCA\textit{Gh}GTCTAC-3’, kindly provided by Cynthia Burrows (Univ. Utah; (Cairns 2009)), was end-labeled and annealed to a complementary oligomer containing dC opposite the Gh position in the same fashion. Tg-containing 184 bp DNA substrates and nucleosomes were prepared as previously described (Prasad, Wallace, and Pederson 2007). Briefly, DNA oligomers containing discretely positioned Tg residues (either 30 or 35 nucleotides from the 5' end, for “Tg-out” and “Tg-in” containing nucleosomes, respectively), were end-labeled with [$\gamma$-32P] ATP, annealed to a single strand DNA template containing the \textit{Lytechinus variegatus} 5S ribosomal DNA (rDNA) nucleosome positioning sequence, and extended with (exo-) Klenow enzyme (New England Biolabs). The resulting double-stranded 184 bp DNA was gel purified, quantified, and assembled into nucleosomes, using high salt mediated transfer of histone octamers from chicken erythrocyte donor chromatin prepared as described (Prasad, Wallace, and Pederson 2007). The efficiency of reconstitution (typically 85-95%) was assessed by electrophoresis of nucleosomes through a 5% polyacrylamide gel in 50 mM Tris base, 50 mM borate, and 1 mM H$_4$EDTA.

### 2.3 Expression and purification of NEIL1 and hNTH1.

To prepare NEIL1, BL21 cells were co-transformed with pRARE2 plasmid (Novagen) and a pET30a vector containing a C-terminal, His-tagged version of NEIL1. Cells were grown to saturation at 20°C for 48-60 hours in 1.5 L Terrific Broth (Fisher Scientific) containing 0.5% glycerol, 0.05% glucose, and 0.2% α-lactose monohydrate, to induce transcription by auto-induction
Cells were harvested and lysed in buffer A (50 mM sodium phosphate buffer, pH 8.0, 10 mM imidazole, 300 mM NaCl, and 5 mM β-mercaptoethanol) containing freshly added 1 mM PMSF and 10 mM benzamidine. Cell debris was removed by centrifugation at 26,000 g for 15 min at 4°C. The supernatant was brought to 30% (NH₄)₂SO₄ and centrifuged at 26,000 g for 20 min at 4°C. The supernatant was transferred to a clean tube, brought to 45% (NH₄)₂SO₄ and centrifuged once again. The resulting NEIL1-containing pellet was suspended in buffer A containing fresh PMSF and benzamidine as before, and dialyzed against two changes of buffer A. The dialysate was loaded onto a 5 mL HiTrap Chelating HP column (GE Lifesciences) that had been charged with 2.5 mL of 100 mM NiSO₄, and NEIL1 was eluted using a 100 mL linear gradient of 10 to 500 mM imidazole, pH 8.0 in buffer A. Fractions containing NEIL1 were identified by SDS-PAGE, pooled and dialyzed against buffer B (25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10% glycerol, and 5 mM β-mercaptoethanol). The dialysate was loaded onto a 1 mL HiTrap Heparin HP column (GE Lifesciences), and NEIL1 was eluted using a 20 mL linear gradient from 0.3 to 2 M NaCl in buffer B. Fractions containing NEIL1 were identified by SDS-PAGE, pooled, dialyzed back to 300 mM NaCl in buffer B, divided into aliquots, flash frozen in liquid nitrogen, and stored at -80°C until use. The 56 amino acid C-terminal NEIL1 truncation mutant was expressed and purified as previously described (Bandaru et al. 2004; Doublie et al. 2004).

To prepare hNTH1, an N-terminal GST-fusion construct was expressed in BL21 Star (DE3) cells using an auto-induction protocol identical to that described for NEIL1. Cells were lysed in buffer C500 (50 mM Tris-HCl, pH 8.0, and 5 mM β-mercaptoethanol containing 500 mM NaCl) containing freshly added 1 mM PMSF. Cell debris was removed by centrifugation at 26,000 g for 15 min, and the supernatant was loaded onto
a 5 mL GSTrap FF column (GE Lifesciences), pre-equilibrated with buffer C500. Protein was eluted with a linear gradient of 0-10 mM reduced glutathione in buffer C500. Fractions containing hNTH1 were identified by SDS-PAGE, pooled and dialyzed against buffer C200. The GST moiety was then removed by the addition of 5 units Precision Protease (Amersham Pharmacia Biotech) for every mg of recombinant hNTH1. After an overnight incubation at 4° C, protein was loaded onto a second 5 mL GSTrap FF column (GE Lifesciences) as above, in order to separate the cleaved and uncleaved protein fractions. Flow-through fractions containing hNTH1 were pooled, dialyzed against buffer D (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, and 5 mM β-mercaptoethanol), loaded onto a 5 mL SPFF column (GE Lifesciences) equilibrated in buffer D, and eluted using a linear gradient of 100-800 mM NaCl in buffer D. Fractions containing hNTH1 were identified by SDS-PAGE, pooled, dialyzed against 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.005% Triton-X, and 50% glycerol, and stored at -20°C. The 55 amino acid N-terminal hNTH1 truncation mutant was expressed and purified in the same manner.

2.4 Enzyme assays. All enzyme concentrations reported in the text and figures refer to active enzyme concentrations, determined as described by Blaisdell and Wallace (2007) (Blaisdell and Wallace 2007). Enzyme stocks were diluted into ice cold reaction buffer (25 mM NaHEPES NaOH, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl) containing 0.1 mg/mL BSA (New England Biolabs) immediately prior to use. Nucleosome and nucleosome length DNA control reactions were conducted in reaction buffer containing 0.05% NP-40 and 0.02 mg/mL donor chromatin. Reactions were stopped by the addition of one volume formamide containing 0.1% bromophenol blue and 0.1% xylene cyanol. Samples were subsequently made 0.1 N in NaOH by addition of 1/10 volume 1 N NaOH,
boiled for 3 min, and fractionated on 8% sequencing gels; reaction products from reactions with 14 and 35 bp substrates were fractionated on 12% sequencing gels. In all cases, reaction products were visualized and quantified by phosphorimagery. Glycosylase activity toward nucleosomes was determined as described in Prasad et al (Prasad, Wallace, and Pederson 2007), which adjusts for small amounts of contaminating naked DNA substrate present in the nucleosome preparation.

2.5 Determination of non-specific binding affinity. To compare NEIL1 and hNTH1 under conditions that were equivalent with respect to fraction of lesion bound by each enzyme, it was necessary to compensate for differences between the two enzymes in their affinity for Tg lesions and for non-specific binding to DNA. As diagrammed in Schemes 1 and 2, these DNA binding events can be represented as simple association reactions (where [S] and [NS] are the concentrations of specific and non-specific binding sites in DNA). In pseudo-steady-state conditions, the distribution of enzyme between specific and non-specific substrates will be dominated by $k_1$ and $k_1\_I$, allowing us to ignore $k_\text{cat}$, and model both the specific and non-specific DNA binding of the two enzymes as one would for DNA sequence specific binding proteins (von Hippel et al. 1974), (Ptashne 2004). This enabled us to derive Equation 1, which describes the effect of both specific and non-specific binding constants ($K_D$ and $K_{NS}$, respectively) on fraction of lesion in an enzyme-lesion complex (i.e. ES/S_T) under a specified set of reaction conditions.

$$\frac{[ES]}{[S]_T} = 1 - \frac{[S]_E}{[S]_T} = 1 - \frac{K_D}{K_D + \frac{[E]_T K_{NS}}{K_{NS} + [NS]}}$$

(1)

To solve Equation 1 for NEIL1, we first measured its affinity for Tg ($K_D$) through a series of single-turnover reactions containing a fixed amount of a $^{32}$P end-labeled, Tg-containing DNA substrate (42 pM) and varying amounts of NEIL1. Reaction rates were
determined by fitting data to a one-phase association using GraphPad Prism 5.0. The reaction rates thus obtained were plotted against the NEIL1 concentration and fitted to a one site binding (hyperbola) equation. $k_{\text{cat}}$ and $K_M$ values were obtained from the resulting curves as described (Porello, Leyes, and David 1998). To measure the non-specific DNA binding affinity of NEIL1 ($K_{\text{NS}}$) we conducted a series of reactions in which a fixed amount of Tg- or Gh-containing oligomeric substrate (25 nM) was added to a fixed amount of enzyme (25 nM NEIL1, 17 nM hNTH1) that had been pre-incubated with varying amounts of non-specific competitor (either donor chromatin or DNA purified from the donor chromatin). The fraction of specific complexes formed in each reaction was inferred from the relative fraction of Tg-containing DNA cleaved in 45 seconds. The data were fit using a one-site competitive inhibition model on GraphPad Prism 5.0, which enabled us to extract $K_{\text{NS}}$. Parallel measurements indicated that the non-specific DNA binding constant $K_{\text{NS}}$ for hNTH1 is much greater than the concentration of non-specific binding sites in our standard assay (~61 $\mu$M). Under these conditions, Equation 1 simplifies to Equation 2, which allowed us to estimate $ES/S_T$ for hNTH1 knowing just its $K_D$ for Tg.

$$\frac{[ES]}{[S]_T} = 1 - \frac{K_D}{K_D + [E]_T}$$

hNTH1 undergoes a monomer to dimer transition that affects its activity (Liu, Choudhury, and Roy 2003), making it difficult to obtain kinetic constants as precise as those for NEIL1. Therefore, in separate studies, we measured approximate $K_D$ values for interactions between selected lesions and a hNTH1 N-terminal truncation mutant. This mutation does not detectably affect the DNA binding or catalytic activity of hNTH1 but reduced dimer formation enough for us to estimate the $K_D$ for hNTH1 binding to Tg as 6.7 +/- 2.1 nM (Table 1; Prasad, Barbour, Wallace and Pederson, submitted).
3. Results.

3.1 Cellular abundance of NEIL1 and hNTH1. To compare NEIL1 and hNTH1 at physiologically meaningful concentrations, we first measured the amount of each enzyme in a mesothelial cell line (LP9) that is normal in most respects except for having been immortalized by constitutive expression of the telomerase catalytic subunit, hTERT (Dickson et al. 2000). Lanes 1-3 in Figure 1A show the antibody signal from serially diluted, recombinant full-length hNTH1 as well as that from a serially diluted, recombinant hNTH1 truncation mutant. The intensity of the antibody signal from hNTH1 in 40 µg of whole cell extract (lane 4 in Figure 1A) is similar to that generated by 0.5 ng of the full-length recombinant protein. In lanes 5-7, serial dilutions of the recombinant hNTH1 truncation protein were mixed with whole cell extract prior to electrophoresis. The intensity of the antibody signals from the full-length endogenous protein lies between those corresponding to 0.5 and 1.5 ng of the truncated enzymes. Since the antibody signal generated by full-length enzyme is 2-3 fold higher than the signal from the truncated enzyme (c.f. lanes 1-3), these results also indicate that cells contain about 0.5 ng of hNTH1 per 40 µg total protein, or roughly 0.5 fg per cell. Virtually all of the cellular hNTH1 is confined to the nucleus (Ikeda et al. 2002), which we estimated has a volume between one and three fl. Knowing that hNTH1 has a molecular weight of 34 kDa, we calculated that the nuclear concentration of hNTH1 lies between 25 and 80 nM.

The hNTH1 concentration calculated above is far lower than that reported earlier, in a study using HeLa cell nuclei (Liu, Choudhury, and Roy 2003). This led us to ask if the abundance of hNTH1 in oncogenically transformed cells is higher than normal. Figure
1B shows that the hNTH1 abundance in three independently derived mesothelioma cell lines is similar to those in the relatively normal LP9 cell line.

To measure the cellular concentration of NEIL1, we conducted western blot studies similar to those described above for hNTH1. Lanes 1-3 of Figure 1C show the antibody signal from serially diluted, recombinant full-length NEIL1 as well as a serially diluted, recombinant NEIL1 truncation mutant. The intensity of the antibody signal from 40 μg of whole cell extract (lane 4 in Figure 1C) lies between the signals from 0.5 and 1.5 ng of the full-length recombinant protein. In lanes 5-7, serial dilutions of the recombinant NEIL1 truncation protein were mixed with 40 μg whole cell extract as described for hNTH1. The antibody signal from the full-length endogenous protein lies between that of 0.5 and 1.5 ng of the truncated enzyme. These results indicated that cells contain about 0.7 ng NEIL1 per 40 μg total protein, or 0.7 fg per cell. In situ localization studies and the phenotype of the NEIL1 knockout mouse suggest that NEIL1 is present in mitochondria as well as nuclei (Shinmura et al. 2004; Vartanian et al. 2006). However, given that most of the DNA in cells is nuclear, we expect that most of the NEIL1 enzyme is nuclear. Given this assumption, and that NEIL1 has a molecular weight of 44 kDa, we estimate that its nuclear concentration ranges between 25 and 80 nM, approximately the same as that measured for hNTH1.

3.2 Impact of non-specific DNA binding on the capacity of hNTH1 and NEIL1 to excise oxidative lesions from naked DNA. Based on the cellular abundance of hNTH1 and NEIL1, we decided initially to compare the activity of hNTH1 on selected substrates with that of 25 nM NEIL1. To compensate for $k_{cat}/K_M$ differences between the two enzymes, we first determined (empirically) the hNTH1 concentration that would give an
initial reaction velocity on naked DNA equivalent to that produced by 25 nM NEIL1.

**Figure 2A** shows initial reaction velocities for 25 nM NEIL1 and an array of hNTH1 concentrations. Inspection of these data indicated that, for Tg-containing substrates, 17 nM hNTH1 best matched that of 25 nM NEIL1. Thus, hNTH1 and NEIL1 are not only similar in cellular abundance but they also exhibit similar activity toward Tg lesions in naked double-stranded DNA.

The concentration of undamaged DNA in our standard chromatin repair assay is approximately 61 μM, about 35-fold higher than in the above-described reactions with double-stranded DNA oligomers. This made it important to determine if non-specific binding of NEIL1 and hNTH1 to DNA or chromatin would affect their relative activity. We therefore conducted a series of reactions with the same amount of enzyme as before (i.e. 25 nM NEIL1 and 17 nM hNTH1) but in the presence of increasing amounts of either soluble, lesion-free chromatin or naked DNA isolated from the same preparation of chromatin. Apart from the non-specific competitor, reaction conditions were identical to those in Figure 2A, where the rate of product formation by either hNTH1 or NEIL1 was approximately constant during the first 60 sec. Therefore, we reasoned that the amount of product generated during the first 45 sec in the competition reactions would appropriately reflect the fraction of total substrate bound by enzyme. Lanes 1-7 in **Figure 2B** show that the activity of hNTH1 was relatively unaffected by addition of either a ~30, ~300, or ~3000 fold molar excess of undamaged DNA or chromatin. Subsequent experiments revealed moderate inhibition of hNTH1 when the fold-excess of non-specific competitor DNA was increased to ~10,000 (Figure 2D and data not shown).

In stark contrast, inhibition of the activity of NEIL1 toward Tg was evident in reactions
with as little as a ~100-fold molar excess of non-specific DNA competitor (Figure 2D and lanes 8-14 in Figure 2B). Although NEIL1 excises Tg lesions with reasonably high efficiency, it is far more active toward hydantoin lesions that form as oxidation products of 7,8-dihydro-8-oxoguanine (8-oxoG) (Krishnamurthy et al. 2008). If the higher activity of NEIL1 toward lesions such as Gh (guanidino hydantoin) were due to more avid binding, we would expect that a non-specific DNA competitor would not have as great an impact as it did in reactions with Tg substrates. On the other hand, if the higher activity of NEIL1 toward Gh were due to a higher rate of catalysis, we would expect that a non-specific DNA competitor would suppress the activity of NEIL1 toward Gh to the same degree as it did for Tg. To test these predictions, we conducted competition experiments with Gh-containing substrates. The results in Figure 2C support the latter prediction, and are consistent with the recent finding that the catalytic constants for NEIL1 acting on hydantoin lesions are far higher than for Tg lesions (Krishnamurthy et al. 2008). The results in Figure 2C also support a qualitative observation by the same authors, that addition of a small quantity of lesion-free DNA (25 nM) helped stabilize hNEIL1 during stopped-flow reaction conditions but that larger amounts suppressed the activity of hNEIL1.

The median length of the DNA competitor used in these studies was no more than ~400 bp, making it important to rule out the possible inhibition of NEIL1 due to non-specific binding to DNA ends. We therefore repeated the above study using full-length phage lambda DNA as a non-specific DNA competitor. At equivalent concentrations, the ~48,000 bp phage DNA proved to be an equally or slightly more effective competitor than DNA isolated from soluble chromatin, despite a >100-fold lower concentration of DNA ends (data not shown). Thus, binding to DNA ends has little or no impact on the
non-specific affinity of NEIL1 for DNA. These results collectively indicated that the specific-to-nonspecific DNA binding ratio for hNTH1 is far higher than that for NEIL1, and that it would be necessary to further adjust the relative concentrations of hNTH1 and NEIL1 to ensure equivalent amounts of free enzyme in reactions with chromatin substrates.

To estimate the relative amounts of NEIL1 and hNTH1 needed to mitigate the differential impact of non-specific DNA binding, we first quantified the non-specific DNA binding data, as shown in Figure 2D. We next determined the equilibrium dissociation constants (K_D) for NEIL1 and hNTH1 binding to Tg, as outlined in the Methods and tabulated in Table 1. We then were able to calculate non-specific DNA binding affinities (K_NS) of approximately 29 nM for NEIL1 and 30 μM for hNTH1. The non-specific affinity of NEIL1 for lambda DNA was slightly higher (K_NS ~9 nM), although the likelihood that this difference is statistically significant is only about ~85%. As a non-specific competitor, undamaged chromatin was not as effective as DNA isolated from the chromatin (K_NS ~930 nM). Indeed, the difference between the naked DNA and chromatin competitors is statistically significant and consistent with the idea that, through steric occlusion, nucleosomes reduce the impact of non-specific DNA binding on the activity of certain enzymes in chromatin.

Having measured the non-specific affinity of NEIL1 for DNA, we were able to calculate (using the equations described in the Methods) that 100 nM NEIL1 in our standard chromatin assay would exhibit about the same activity on naked DNA as would hNTH1 in the range of 1-5 nM. To further refine this estimate, we compared the activity of 100 nM NEIL1 in a reaction with a Tg-containing naked DNA substrate and 61 μM non-lesion
containing chromatin to results from a series of parallel reactions containing 0.5 to 5.0 nM hNTH1. Figure 2E shows the rate of Tg excision by 100 nM NEIL1 was similar to that exhibited in reactions with 1 to 2 nM hNTH1.

3.3 Comparison of hNTH1 and NEIL1 on nucleosome substrates. Having determined conditions that would compensate for differences between hNTH1 and NEIL1 in their non-specific DNA binding, we next measured the relative activity of the two enzymes toward Tg-containing nucleosomes. Both hNTH1 and NEIL1 require the oxidized base to flip out of the DNA helix via the minor groove (Hollis, Ichikawa, and Ellenberger 2000; Zharkov et al. 2002). Hence, a Tg positioned such that the minor groove faces away from the histone octamer (Tg-out) is more accessible than a Tg residue at which the minor groove faces toward the histone octamer (Tg-in), as illustrated in Figure 3A (Beard, Wilson, and Smerdon 2003; Prasad, Wallace, and Pederson 2007). As shown in Figure 3B, both 2 nM hNTH1 and 100 nM NEIL1 were able to excise lesions from Tg-out nucleosomes with a relatively high efficiency. For hNTH1, this result is in accord with earlier studies (Prasad, Wallace, and Pederson 2007) and indicates that hNTH1 can efficiently process sterically accessible lesions in nucleosomes at enzyme concentrations equivalent to or below those in cells (though somewhat higher than those required for naked DNA substrates). For NEIL1, efficient excision of thymine glycol residues occurred only when NEIL1 was used at concentrations equal to or higher than those in cells.

Previous studies had indicated that hNTH1 forms a ternary complex with lesion-containing nucleosomes and can remove damaged bases without inducing or requiring nucleosome disruption (Prasad, Wallace, and Pederson 2007). To determine if this was
the case for NEIL1 as well, and to rule out the possibility that NEIL1 disrupts lesion-containing nucleosomes, we gel-fractionated Tg-out nucleosomes in the absence and presence of hNTH1 and NEIL1. Lanes 1-3 in Figure 4 show that both hNTH1 and NEIL1 alter the migration of naked, nucleosome-length Tg-containing DNA. Lanes 5-8 in Figure 4 show the effect of both low and high concentrations of hNTH1 and NEIL1 on the mobility of lesion-containing nucleosomes. The failure of hNTH1 and NEIL to increase the amount of naked DNA in lanes 5-8 indicated that neither enzyme disrupted nucleosomes. Lanes 5-8 in Figure 4 also show that both enzymes form ternary complexes with Tg-containing nucleosomes.

The above described mobility gel shift study indicated that NEIL1 can excise lesions from Tg-out nucleosomes without inducing nucleosome disruption, but it was necessary as well to rule out the possibility that NEIL1 had altered the translational position of DNA relative to the underlying octamer, thereby moving the Tg lesion into a nucleosome-free segment of DNA. To map the predominant translational position of Tg-out nucleosomes, we conducted quantitative restriction enzyme cleavage analyses in the absence and presence of hNTH1 and NEIL1. Nucleosomes and naked DNA controls were incubated with either Bam HI or Eco RV, which cleave at sites immediately adjacent to either edge of the nucleosome in its dominant translational position, or with Psi I or Dra I, whose cognate sites lie within the nucleosome (c.f. Figure 5A). Figures 5A and 5B show patterns of restriction site exposure and protection for lesion-containing nucleosomes identical to those described previously (Prasad, Wallace, and Pederson 2007), indicating that most of the histone octamers resided at a single common position. Minor positional variants do exist but, as described previously (Prasad, Wallace, and Pederson 2007), do not appear to expose the Tg lesion. Figures 5A and 5B also show that neither NEIL1 nor
hNTH1 altered the restriction enzyme cleavage pattern. Thus, as with hNTH1, NEIL1 can excise sterically accessible lesions from nucleosomes without altering nucleosome positions.

Earlier studies indicated that the histone octamer substantially blocks access to Tg lesions at sites where the minor groove of the lesion faces toward the histone octamer. However, at high concentrations hNTH1 was able to process such lesions in a relatively efficient manner, probably because spontaneous, transient partial unwrapping of DNA from the histone octamer enables hNTH1 to capture lesions in the unwrapped DNA population (Prasad, Wallace, and Pederson 2007). Figure 3C shows that at an active enzyme concentration of 100 nM, NEIL1 was unable to excise more than 10% of the lesions from Tg-in nucleosomes in a 10 min interval. At an active enzyme concentration of 2 nM, the activity of hNTH1 on Tg-in nucleosomes was equally poor. The key difference, however, is that 100 nM NEIL1 equals or slightly exceeds our estimate of its concentration in vivo while the 2 nM hNTH1 used for comparative purposes is far below its in vivo concentration. In reactions conducted with hNTH1 amounts comparable to those in vivo, the efficiency with which hNTH1 excises occluded lesions rises dramatically, as shown in Figure 3C. (Because of aggregation of NEIL1 at high concentrations it was not possible to examine its activity at very high concentrations.) In summary, hNTH1 is able to function efficiently towards both accessible and occluded lesions in chromatin due to its high substrate specificity and low non-specific DNA binding. On the other hand, NEIL1 (which has an even higher affinity toward Tg lesions in naked DNA) may be unable to efficiently process lesions in canonical nucleosomes without the aid other proteins that either suppress its non-specific DNA binding or actively recruit it to sites of oxidative damage.
4. Discussion.

In this study we have determined that the \textit{in vivo} concentrations of the DNA glycosylases hNTH1 and NEIL1 are similar to one another, and that both enzymes are able to excise thymine glycol lesions from nucleosomes without inducing nucleosome movement or disruption. Previous studies that compared the capacity of selected DNA glycosylases to function on chromatin substrates have not (explicitly) accounted for enzymatic variables that could influence results, such as differences in $k_{\text{cat}}$ or $K_m$ or in non-specific DNA binding affinity. By adjusting enzyme concentrations to compensate for such variables, this study provides a standard template for future comparisons of enzyme action on chromatin substrates.

The key difference between NEIL1 and hNTH1, which bears on their likely roles \textit{in vivo}, is that NEIL1 has a much higher affinity for undamaged DNA than hNTH1. The likely \textit{in vivo} concentration of hNTH1 (~25 to ~80 nM) is sufficiently high to imagine that the enzyme finds oxidative lesions in two steps, beginning with a diffusion-driven, three-dimensional search for DNA, that results in non-specific DNA binding. Once bound to DNA the enzyme would engage in a highly efficient ‘one-dimensional’ search along the DNA for oxidative lesions. This model is predicated on classic studies of lac repressor (notably by Riggs and von Hippell and their colleagues (Riggs, Bourgeois, and Cohn 1970; von Hippel et al. 1974)). In the case of hNTH1, the efficiency of lesion discovery may be further enhanced through a charge-transport mechanism proposed by Barton and colleagues (Lusser and Kadonaga 2004; Hizume et al. 2009). Specifically, hNTH1, like its prokaryotic counterpart, contains an iron-sulfur cluster that may become oxidized upon DNA binding (Simpson and Stafford 1983). If hNTH1 binds more avidly to DNA in
its oxidized [4Fe4S]$^{3+}$ state, its dissociation rate would be enhanced by reduction of the iron-sulfur cluster, which could occur by electron transport from an electron donor through the DNA base pair stack. DNA lesions that interfere with base stacking would likely interfere with this charge transport and thereby increase the dwell-time of hNTH1 near sites of DNA damage. Thus, a combination of diffusion-driven and electron transport mediated search mechanisms may permit highly efficient lesion discovery.

In sharp contrast to hNTH1, NEIL1 exhibited a relatively high non-specific DNA binding affinity. This would make it virtually impossible for NEIL1 to efficiently discover oxidative lesions in nuclear DNA via the mechanism suggested for hNTH1 (assuming that NEIL1 is homogenously distributed within the nucleus at a concentration ranging between ~25 and ~80 nM). There are several potential evolutionary solutions to this conundrum that are consistent with known properties of NEIL1. Specifically, NEIL1 is found in both mitochondria and nuclei, and its abundance increases during S phase (Hazra et al. 2002; Takao, Kanno, Kobayashi, et al. 2002; Shinmura et al. 2004; Hu et al. 2005). Given the relatively small size of the mitochondrial genome, NEIL1 might be able to discover oxidative lesions in mitochondria via the simple diffusion-driven search mechanism like that described for hNTH1. In nuclei, an increase in the concentration of NEIL1 during S phase would increase the efficiency of lesion discovery. Additionally, our non-specific competition studies indicate that NEIL1 has a higher affinity towards undamaged naked DNA than nucleosomal DNA. Thus, the packaging of DNA into chromatin likely reduces the fraction of NEIL1 that is lost to non-specific interactions with DNA. It is unlikely, however, that these factors, even in combination, would enable NEIL1 to discover lesions at an adequate rate without the additional involvement of proteins that either recruit NEIL1 to sites of oxidative damage or bind NEIL1 in such a
way as to reduce its non-specific DNA affinity. Relevant to this point, Mitra and colleagues have reported that NEIL1 interacts with such DNA replication and repair factors as PCNA and FEN-1, which prompted them to hypothesize that the primary role of NEIL1 is in replication associated repair (Dou, Mitra, and Hazra 2003; Hazra et al. 2007; Dou et al. 2008; Hegde et al. 2008). It remains to be determined if these or other NEIL1-interacting proteins influence its non-specific interactions with DNA.

Conflict of Interest Statement
The above authors have no financial interests.

Acknowledgements. We thank Dr. Cynthia J. Burrows for supplying the Gh-containing oligomers, Lauren Harvey and April Averill for the expression and purification of enzymes used in this study and Dr. Viswanath Bandaru for providing the GST-hNTH1 expression vector. We thank Dr. Amalthiya Prasad and Joy-El Barbour for guidance in the preparation and assay of lesion-containing nucleosomes, and for sharing kinetic data on hNTH1Δ55. We thank Drs. Scott Morrical, Jeffrey Bond, and Susan Robey-Bond for helpful discussions of enzyme kinetics. The research was supported in part by an NSF grant MCB-0821941 to D.S.P., and NIH grant P01-CA098993 awarded by the National Cancer Institute.
5. References.


6. Figure Legends.

**Figure 1. In vivo concentrations of hNTH1 and NEIL1.**

(A) Upper panel: hNTH1 western blot showing, in lanes 1-3, serially diluted, full length, recombinant hNTH1 (“wt”) and a 55 amino acid N-terminal truncation mutant (hNTH1Δ55). In lanes 4-7, 0, 5, 1.5, and 0.5 ng of hNTH1Δ55 were added to 40 µg of LP9 whole cell extract prior to electrophoresis. Lower panel: hNTH1 blots were stripped and incubated with an anti-actin antibody as a loading control. (B) Upper panel: western blot of hNTH1 amounts in LP9 whole cell extracts compared to those in three mesothelioma cell lines (Gates, Mills, and Mont). Extracts were mixed with 1.5 or 0.5 ng of hNTH1Δ55 prior to electrophoresis, to facilitate quantification. Lower panel: actin control as above. (C) Upper panel: NEIL1 western blot showing, in lanes 1-3, serially diluted, recombinant full length NEIL1 (“wt”) and a 56 amino acid C-terminal truncation mutant of NEIL1 (NEIL1Δ56). In lanes 4-7, 0, 5, 1.5, and 0.5 ng of NEIL1Δ56 were added to 40 µg LP9 whole cell extract prior to electrophoresis. Lower panel: actin control as above.

**Figure 2. Adjustment of hNTH1 and NEIL1 concentrations to compensate for differences in enzyme efficiency and non-specific DNA binding affinity.**

(A) Graph showing initial rates of excision of Tg lesions by 25 nM NEIL1 (■, solid line) and varying concentrations of hNTH1 (▲, dotted lines for reactions containing 10, 11, 13, 15, 17 and 19 nM hNTH1), assayed as described in the Methods. Data from the single-turnover portion of each reaction curve were fit by linear regression. Note that the rate of excision by 17 nM hNTH1 closely matches that observed with 25 nM NEIL1. (B) Non-specific DNA binding assay. A 35 bp, end-labeled, Tg-containing DNA substrate
was added to reaction tubes containing varying amounts of undamaged competitor DNA or chromatin and either 17 nM hNTH1 (Lanes 1-7) or 25 nM NEIL1 (lanes 8-14). Reaction extents were determined after 45 seconds at 37°C. Lanes 1 and 8, 2 and 9, 3 and 10 and 4 and 11 contained, respectively, 0, 0.76, 7.6 and 76 μM DNA. Lanes 5 and 12, 6 and 13, and 7 and 14 contained, respectively, 0.76, 7.6 and 76 μM chromatin. (C) 25 nM NEIL1 was incubated with a 14 bp double-stranded oligomer containing Gh. Lanes 1, 2, 3, and 4 contained, respectively, 0, 2.4, 24, and 240 μM undamaged DNA and lanes 5, 6, and 7 contained, respectively, 2.4, 24, and 240 μM chromatin. (D) The amounts of substrate cleaved in the reactions shown in figures 2B and 2C, were quantified by phosphorimagery. These and data from additional, independent competition experiments were plotted against the logarithm of the concentration of lesion-free DNA. The upper panel depicts the activities of NEIL1 (■) and hNTH1 (▲) towards Tg when incubated with 25 nM substrate alone, or together with the indicated amounts of lesion-free DNA. The lower panel depicts the activity of NEIL1 (●) towards Gh when incubated with the same concentrations of lesion-free DNA competitor. The vertical dashed line in the upper panel indicates the concentration of lesion-free chromatin in our standard chromatin assay (61 μM). (E) Graph showing reaction curves for 100 nM NEIL1 in the presence of 61 μM chromatin (■, solid line) and varying concentrations of hNTH1 (▲, dotted lines for reactions containing 0.5, 1, 2 and 5 nM hNTH1). Note that, in the presence of 61 μM chromatin, the rate of excision by 2 nM hNTH1 most closely matches that observed with 100 nM NEIL1.

*Figure 3. NEIL1 and hNTH1 exhibit similar intrinsic activities toward nucleosomes with ‘outward-facing’ Tg lesions but the capacity of NEIL1 to excise lesions from*
sterically occluded sites in nucleosomes is limited by its high non-specific DNA binding.

(A) Depiction of the helical orientation of thymine glycol lesions in Tg-in and Tg-out nucleosomes, based on a 1.9 Å resolution nucleosome crystal structure (PDB 1KX5 (Davey et al. 2002)). Arrows indicate direction of Tg flipping needed to enter the active site of either hNTH1 or NEIL1. (B) Reaction curves for 100 nM NEIL1 and 2 nM hNTH1 with Tg-out containing nucleosomes (solid lines) and naked DNA (dashed lines). (C) Reaction curves obtained with 100 nM NEIL1 and varying concentrations of hNTH1 with Tg-in containing nucleosomes (solid lines) and naked DNA controls (dotted lines). Note that 100 nM NEIL1 matches or slightly exceeds its estimated concentration in vivo. The activity of NEIL1 at concentrations above 100 nM (active enzyme) was similar or only slightly higher than the activity shown for 100 nM (not shown). Note that while the histone octamer substantially inhibits hNTH1 at low concentrations, hNTH1 is increasingly able to process the sterically occluded lesion in Tg-in nucleosomes when its concentration is increased from 2 nM to 17, 68, and 272 nM hNTH1. The 68 nM reaction curve closely matches the estimated in vivo concentration of hNTH1.

Figure 4. Both hNTH1 and NEIL1 remove sterically accessible lesions from nucleosomes without requiring or inducing nucleosome disruption. Electrophoretic mobility shift assay of hNTH1 and NEIL1 bound to Tg-containing naked DNA and Tg-out nucleosomes. Note that both hNTH1 and NEIL1 form ternary complexes with nucleosomes, indicated by rectangles in lanes 5 and 6 and 7 and 8. Note that neither enzyme increases the amount of material that co-migrates with naked DNA, indicating that neither enzyme irreversibly disrupts the nucleosome during lesion processing.
Figure 5. Both hNTH1 and NEIL1 remove sterically accessible lesions from nucleosomes without detectably altering nucleosome position. 

(A) Diagram that depicts the predominant translational setting of the model, Tg-containing nucleosomes used in this study, and the restriction enzyme sites used to monitor nucleosome position. The extent of cleavage of restriction sites in nucleosomes, assessed as described below in Figure 5B, is expressed in the histogram as a fraction of the cleavage obtained at the corresponding sites in naked DNA. The open, solid, and grey bars indicate, respectively, the relative double-stranded DNA cleavage by the indicated restriction enzyme when incubated alone, with 100 nM NEIL1, and with 17 nM hNTH1. Note that both the Bam H1 and Eco RV sites, located immediately adjacent to either side of the nucleosome, remain accessible following the addition of either hNTH1 or NEIL1, suggesting that neither enzyme alters the dominant translational position of the nucleosome. 

(B) Restriction enzyme cleavage analyses of Tg-containing nucleosomes in the absence and presence of either 100 nM NEIL1 or 17 nM hNTH1. Naked DNA (“D”) and nucleosomes (“N”) were incubated with a 50-fold unit excess of the indicated restriction enzyme for 10 min at 37°C. The resulting DNA products were separated on non-denaturing gels, and quantified by phosphorimagery to assess the extent of cleavage at selected restriction sites. Note that single strand DNA cleavage by either glycosylase slightly retards the migration of the double-stranded DNA, which accounts for the minor bands, such as those seen in lanes 13 and 14. Lanes 1 and 10 contain uncleaved naked DNA controls.
**Chapter 2-Table 1.** Kinetic constants for NEIL1 and hNTH1.

<table>
<thead>
<tr>
<th></th>
<th>$k_{\text{cat}}$ (sec$^{-1}$)</th>
<th>$K_M$ (nM)</th>
<th>$k_{\text{cat}}/K_M$ (sec$^{-1}$ nM$^{-1}$)</th>
<th>$K_{NS}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEIL1</td>
<td>0.13</td>
<td>0.25</td>
<td>0.52</td>
<td>0.029</td>
</tr>
<tr>
<td>hNTH1</td>
<td>0.073</td>
<td>6.7</td>
<td>0.01</td>
<td>30</td>
</tr>
</tbody>
</table>

Chapter 2-Scheme 1.

\[
E_F + S_F \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} [ES] \overset{k_{\text{cat}}}{\rightarrow} E + P, \text{ where } K_D = k_{-1}/k_1
\]

Chapter 2-Scheme 2.

\[
E_F + NS \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} [E-NS], \text{ where } K_{NS} = k_{-2}/k_2
\]
Chapter 2-Figure 1. *In vivo* concentrations of hNTH1 and NEIL1.

(A) WCE: - - - + + + + +
    wt (ng): 5 1.5 0.5 - - - - -
    Δ55 (ng): 5 1.5 0.5 - 5 1.5 0.5

(B) WCE:
    Δ55 (ng): 1.5 0.5 1.5 0.5 1.5 0.5 1.5 0.5

(C) WCE: - - - + + + + +
    wt (ng): 5 1.5 0.5 - - - - -
    Δ56 (ng): 5 1.5 0.5 - 5 1.5 0.5

Actin:
Chapter 2-Figure 2. Adjustment of hNTH1 and NEIL1 concentrations to compensate for differences in enzyme efficiency and non-specific DNA binding affinity.
Chapter 2-Figure 3. NEIL1 and hNTH1 exhibit similar intrinsic activities toward nucleosomes with ‘outward-facing’ Tg lesions, but the capacity of NEIL1 to excise lesions from sterically occluded sites in nucleosomes is limited by its high non-specific DNA binding.
Chapter 2-Figure 4. Both hNTH1 and NEIL1 remove stERICALLY accessible lesions from nucleosomes without requiring or inducing nucleosome disruption.

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th></th>
<th>Nucleosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>nM hNTH1:</td>
<td>-</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>nM NEIL1:</td>
<td>-</td>
<td>-</td>
<td>25 25 100</td>
</tr>
</tbody>
</table>

1  2  3  4  5  6  7  8
Chapter 2-Figure 5. Both hNTH1 and NEIL1 remove sterically accessible lesions from nucleosomes without detectably altering nucleosome position.
Erratum to “Non-specific DNA binding interferes with the efficient excision of oxidative lesions from chromatin by the human DNA glycosylase, NEIL1,” by Ian D. Odell, Kheng Newick, Nicholas H. Heintz, Susan S. Wallace and David S. Pederson, 2010, DNA Repair 9, 134-143.

Shortly after this paper was published, we discovered an error in the amount of protein per cell that was used to calculate the nuclear concentrations of NEIL1 and hNTH1. The correct concentrations for both enzymes lies between 250 and 800 nM, ten-fold higher than reported. While we regret this error, it did not affect any of the other values reported in the paper. Additionally, the principal conclusions of the paper remain valid, namely that the relatively high affinity of NEIL1 for undamaged DNA likely prevents its acting on oxidative lesions in the absence of accessory factors (even at concentrations up to 800 nM). On the other hand, the corrected values for the concentration of hNTH1, together with its far lower affinity for lesion-free DNA, makes it even more likely that it can discover and process oxidative lesions in vivo, without the aid of accessory factors.
CHAPTER 3: SUBSTRATE HAND-OFF DURING BASE EXCISION REPAIR OF OXIDATIVE LESIONS IN NUCLEOSOMES

Base Excision Repair in Nucleosomes.

Ian D. Odell, Joy-El Barbour, Alan Tomkinson, Susan S. Wallace, David S. Pederson
Abstract: The packaging of eukaryotic DNA into nucleosomes restricts the binding of many factors that act on DNA. Accordingly, chromatin remodeling is a key step in many nuclear processes, including transcription, DNA replication, and most DNA repair pathways. Here, however, we show that base excision repair (BER) of oxidative lesions can occur on nucleosome substrates without nucleosome translocation or irreversible disruption. Individual BER enzymes form a succession of ternary complexes with lesion-containing nucleosomes, and the rates of substrate processing during the first three steps of BER (catalyzed by a DNA glycosylase, AP endonuclease, and DNA polymerase β) vary with the helical orientation of the substrate relative to the underlying histone octamer. In contrast, helical orientation does not influence the activity of DNA Ligase III-α, which carries out the final step of BER. This result is consistent with a model in which DNA ligase III can encircle its DNA substrate only when the substrate is exposed through transient, partial unwrapping of DNA from the histone octamer. We also report a synergistic interaction between DNA polymerase β and the DNA Ligase III-α XRCC1 complex that enhances the repair of lesions in nucleosomes.
Keywords
Base excision repair, Chromatin, Nucleosome, DNA glycosylase, Human endonuclease III, AP endonuclease, DNA Polymerase beta, DNA Ligase III-alpha, XRCC1

Abbreviations
BER, base excision repair; hNTH1, human endonuclease III, also NTHL1, NTH1; APE, human AP endonuclease, also APE1, REF1, APEX1; Pol β, human DNA polymerase beta, also β Pol; LigIIIα, human DNA Ligase III-α; XRCC1, X-ray cross-complementing factor 1; dNTP, deoxynucleoside triphosphate; dTTP, deoxythymidine triphosphate; BSA, bovine serum albumin; rDNA, ribosomal DNA; SDS-PAGE, SDS polyacrylamide gel electrophoresis; TBE, tris-borate-EDTA buffer; Tg, thymine glycol; THF, tetrahydrofuran, furan
1. Introduction.

Oxidative DNA damage results from exposure of DNA to reactive oxygen species (ROS), generated as byproducts of aerobic cellular metabolism, and also through exposure to environmental agents such as γ-irradiation. The most reactive ROS is the hydroxyl radical, which reacts with DNA to yield apurinic/apyrimidinic (AP) sites, single strand breaks, and a plethora of oxidative base damages (Breen and Murphy 1995). Base Excision Repair (BER) enzymes recognize these damages, and replace them with the corresponding undamaged bases in an error-free fashion. In its simplest form, BER consists of four enzymatic steps (Wallace et al. 2003; Wilson, Sofinowski, and McNeill 2003; Almeida and Sobol 2007; David, O'Shea, and Kundu 2007; Hegde, Hazra, and Mitra 2008). The first step is the recognition and excision of a damaged base by either a mono- or bi-functional DNA glycosylase. Oxidized bases in particular are mostly excised by bifunctional DNA glycosylases in either the HhH-GPD family or Fpg/Nei family. HhH-GPD family glycosylases, such as hNTH1, first cleave the bond between the base and sugar, and then the phosphodiester bond 3' of the resulting AP site. The 3’-blocking group that remains is removed by the phosphodiesterase activity of APE, which generates a single nucleotide gap suitable for extension by Pol β. In the final step of BER, DNA Ligase III-α (LigIIIα) catalyzes formation of a phosphodiester bond between the 3’-OH of the newly synthesized nucleotide and the downstream 5’-phosphate.

The information in a human genome is encoded within ~2 m of DNA, which is compacted between 1,000 and 10,000-fold by an array of proteins that, together with the DNA, is called chromatin. The fundamental unit of chromatin is the nucleosome, a repeating unit made up of a core that contains two copies each of histones H2A, H2B,
H3, and H4, around which 147 bp of DNA is wrapped in 1.65 left-handed superhelical turns (Luger et al. 1997; Richmond and Davey 2003). The nucleosome compacts DNA about 5-fold, sterically occludes access to the DNA on the inner surface of the superhelix and constrains the overall DNA conformational flexibility. Consequently, nucleosomes limit the accessibility of DNA to nuclear processes, such as transcription, replication, recombination, and repair.

Nucleosomes do not appear to provide significant protection to DNA from ROS. Enright et al. found no preferential targeting of oxidative DNA damage to internucleosomal regions when they incubated polynucleosomes with hydroxyl radicals generated through use of iron-EDTA compounds (Enright et al. 1996). As well, nucleosomes provide little protection from the methylating agent, dimethyl sulfate (McGhee and Felsenfeld 1979). However, bases located where the minor groove faces away from the histone octamer are more susceptible to hydrolysis by hydroxyl radicals (Hayes, Tullius, and Wolfe 1990). It is noteworthy that histones themselves may act as a sink for ROS, thereby reducing the frequency of free radical inflicted DNA damage (Ljungman and Hanawalt 1992). In sum, the most vulnerable nucleosomal DNA is located where the minor groove faces outward from the nucleosome, but none of the DNA is completely protected from oxidation.

To test whether BER can occur in chromatin, a number of labs have attempted to assemble the entire reaction in vitro using mononucleosome substrates (Nilsen, Lindahl, and Verreault 2002; Beard, Wilson, and Smerdon 2003; Menoni et al. 2007). Nilsen et al. first showed that the entire reaction proceeds on nucleosome substrates without nucleosome disruption or altered DNA translational positioning (Nilsen, Lindahl, and
Verreault 2002). Beard et al. later showed that lesion orientation is a major factor that determines the rate of base excision by the DNA glycosylase, UNG2 (Beard, 2003 #30). However, in contrast to the earlier report by Nilsen et al., they observed no activity of Pol β on nucleosome substrates (Beard, Wilson, and Smerdon 2003). Hayes and colleagues have analyzed the rates of DNA Ligase I and FEN1 on nucleosome core particles (Chafin et al. 2000; Huggins et al. 2002). They observed ~10-fold inhibition of DNA Ligase I, consistent with the extent of glycosylase inhibition observed by Beard et al. but, surprisingly, they observed higher endonuclease activity by FEN1 on nucleosomes than naked DNA.

Our lab has previously investigated the effects of nucleosome structure on the rate of thymine glycol (Tg) excision by hNTH1, a bifunctional DNA glycosylase that excises oxidized pyrimidines (Prasad, Wallace, and Pederson 2007; Odell et al. 2010). Consistent with the study by Beard et al. (2003), we observed reduced hNTH1 efficiency when the minor groove of the damaged base was occluded by the histone octamer core. However, hNTH1 was able to overcome these steric impediments in a concentration-dependent manner, likely as a result of excising its substrate during episodes of spontaneous partial unwrapping of DNA from the octamer core (Prasad, Wallace, and Pederson 2007). To continue our investigations of the effects of substrate rotational position on BER, we reconstituted nucleosomes with each BER intermediate positioned such that the minor groove is accessible or occluded by the histone octamer. We discovered that like UNG2, hNTH1 and NEIL1, the activities of APE and Pol β also highly depend on the rotational position of their substrates. In contrast, LigIIIα in complex with XRCC1 (LigIIIα/XRCC1) shows similar ligation efficiency that is independent of the orientation of the minor groove. Additionally, nucleosome binding by LigIIIα/XRCC1
enhances the extension of occluded gaps by Pol β, suggesting that the interaction between Pol β and XRCC1 plays an important role in the activity of Pol β on nucleosomes in vivo.

2. Experimental Procedures.

2.1. Construction of DNA containing BER lesions and intermediates. The 147 bp core of the 601 nucleosome positioning sequence and 184 bp DNA containing the Lytechinus variegatus 5S rDNA (Lv5S) nucleosome positioning sequence, each containing a single, discretely positioned thymine glycol (Tg) residue, were prepared as previously described (Prasad, Wallace, and Pederson 2007). Briefly, synthetic oligomers Tg-out, Tg-in, or Tg-in(601) (Table 1) were end-labeled with [γ-32P] ATP, annealed to their respective template, and extended with (exo-) Klenow enzyme (New England Biolabs). The resulting double-strand DNA was gel purified and assembled into nucleosomes as described below. Nucleosome length Lv5S DNA containing a furan was prepared in the same manner, but with oligomers F-out or F-in, which contain tetrahydrofuran (THF) in place of the Tg base. To prepare gap or nick-containing DNA fragments, the DNA oligomers Out (3') and In (3') (Table 1) were 5'-end labeled with [γ-32P] ATP, annealed to equimolar amounts of 5slv template, and extended with (exo-) Klenow enzyme (New England Biolabs) to create 154 and 149 nucleotide-long DNA segments. The extension reactions were stopped with one volume 25x NET (400 mM NaOAc, 25 mM H4EDTA, 100 mM Tris base), then mixed with an equimolar amount of the appropriate 32P 5'-end labeled upstream oligomers (Gap-out, Gap-in, Nick-out, or Nick-in) in 12.5X NET, and annealed to create a full-length DNA fragment containing a single, discretely positioned gap or nick.
3'-phospho-α,β-unsaturated aldehyde (3’-PUA) containing DNA was prepared by incubating Tg containing naked DNA with excess hNTH1 for 30 minutes at 37°C. hNTH1 was removed by phenol/chloroform extraction, and the DNA was ethanol precipitated and suspended in the appropriate volume for nucleosome reconstitution.

DNA containing a 5'-deoxyribose phosphate (5’-dRP) has a much shorter half life than the time it takes to reconstitute nucleosomes (Bailly and Verly 1989). Therefore, to assess the activity of Pol β on gaps containing a 5’-dRP, we pre-incubated F-out or F-in nucleosomes with 5 or 50 nM APE for 1.5 or 30 minutes at 37°C, respectively. In this manner, we were able to generate nucleosomes containing a gap with a 5’-dRP immediately before addition of Pol β.

hNTH1 and APE assays on naked DNA were measured using a double strand 35 bp DNA fragment containing either a single Tg or THF residue at position 14 (X35), as previously described (Odell et al. 2010). A 3’-PUA on this DNA was generated as described above.

2.2. Nucleosome Reconstitution. Histones H2A, H2B, H3, and H4 were expressed in *Escherichia coli* and assembled into octamers as described by Luger et al. and Dyer et al. (Luger, Rechsteiner, and Richmond 1999; Dyer et al. 2004). To assemble nucleosomes, we mixed 0.15 pmoles of end-labeled substrate containing DNA and 1.35 pmoles of an unlabeled 195 bp Lv5S DNA fragment with purified octamer in a 1.25:1 molar ratio in a final volume of 20 μl of HED2000+NP-40 (25 mM NaHEPES NaOH, pH 8.0, 1 mM Na-EDTA, 1 mM DTT, 2 M NaCl and 0.05% Nonidet P-40). The octamer plus DNA solution was introduced into a button dialysis chamber constructed from a PCR
tube, as described by Thastrom et al. (Thastrom, Lowary, and Widom 2004). The button
dialysis chambers were placed in a 1.7 ml/cm 6-8 kDa MWCO Spectra/Por dialysis tube
containing 5 ml HED2000+NP-40 and incubated at 37° C for 30 minutes in 100 ml of
pre-warmed HED2000+NP-40. After 30 minutes, the outer buffer was replaced with 100
ml 37°C HED0+NP-40 (25 mM NaHEPES NaOH, pH 8.0, 1 mM Na-EDTA, 1 mM DTT,
and 0.05% Nonidet P-40) and the dialysis was continued with slow stirring at room
temperature for 2 hours. After 2 hours, the outer buffer was replaced with 100 ml of room
temperature HED0+NP-40 buffer and dialysis continued with slow stirring at 4° C
overnight. The resulting nucleosomes were visualized by electrophoresis through a 5%
native polyacrylamide gel in ¼ x TBE buffer, followed by either autoradiography or
phosphoimaging, and the efficiency of reconstitution was determined as described
(Prasad, Wallace, and Pederson 2007).

2.3. Expression and Purification of BER enzymes. hNTH1, Pol β, and DNA Ligase III-
α in complex with XRCC1 were expressed and purified as previously described (Murphy
et al. 2008; Odell et al. 2010). The gene for human APE was obtained from a human
cDNA library, inserted into a pTYB2 vector, and together with the plasmid pRARE2
(Novagen), transformed into BL21 Star (DE3) cells (USB). The resulting transformants
were grown to saturation at 20°C for 48–60 h in 1.5 l Terrific Broth (Fisher Scientific)
containing 0.5% glycerol, 0.05% glucose, and 0.2% α-lactose monohydrate, to induce
transcription by auto-induction (Studier 2005). Cells were harvested and lysed in chitin
buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, pH 8.0) containing freshly
added 20 μM PMSF and 1 mM β-mercaptoethanol. Cells were lysed by sonication and
debris was removed by centrifugation at 26,000×g for 1 hour at 4°C. The cleared lysate
was loaded at 1 ml/min onto a 25 ml chitin column that had been equilibrated with 10
column volumes of chitin buffer. The column was washed with 10 additional column volumes of chitin buffer at 2 ml/min, and then with 3 column volumes of chitin buffer containing freshly added 1 mM DTT at 5 ml/min. Following an overnight incubation at 4°C, protein was eluted with chitin buffer at 2 ml/min. Protein containing fractions were identified by incubating 10 μl of each 2 ml fraction with 80 μl H₂O and 20 μl Protein Assay Dye Reagent Concentrate (Bio-Rad) in a microtiter plate, pooled, and dialysed first against SP buffer (20 mM NaHEPES NaOH, pH 7.0, 10% glycerol, 5 mM β-mercaptoethanol) containing 300 mM NaCl for 3 hours at 4°C, and then overnight against SP buffer containing 150 mM NaCl. Precipitated protein in the dialysate was removed by centrifugation at 26,000×g for 15 min at 4°C, and the supernatant was loaded onto a 5 ml HiTrap SP FF column (GE Lifesciences) that had been equilibrated with SP buffer containing 150 mM NaCl. APE was eluted using a 100 ml linear gradient of 150–800 mM NaCl in SP buffer. Fractions containing APE were identified by SDS-PAGE, pooled, dialyzed against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, and 50% glycerol, and stored at -20°C.

2.4. Enzyme Assays. All enzyme concentrations reported in the text and figures refer to total protein concentration, except in the case of hNTH1, where the active fraction was determined as described by Blaisdell and Wallace (Blaisdell and Wallace 2007). The final substrate concentration was always 4 nM with 36 nM unlabeled non-lesion containing nucleosomes. Enzyme stocks were freshly diluted into ice cold BER reaction buffer (25 mM NaHEPES NaOH, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 0.2 mM NaEDTA, 1 mM DTT, and 0.1 mg/ml BSA) containing 20 μM dTTP unless otherwise indicated in the figure legend, and added to reactions as indicated in the text and figure legends. The dilution and reaction buffers with LigIIIα/XRCC1 included 1 mM ATP. To monitor enzyme
activity, aliquots from BER reactions were stopped by the addition of 4 volumes 0.1 N NaOH, 90% formamide, and 0.1% bromophenol blue and 0.1% xylene cyanol or, in the case of hNTH1 reactions, the same buffer minus the 0.1 N NaOH. Reaction products were resolved on 12% or 15% sequencing gels. To assess the fate and integrity of lesion-containing nucleosomes, aliquots from BER reactions were resolved on a 5% native polyacrylamide gel in ½ x TBE buffer.

3. Results.

3.1. Reconstitution of nucleosome substrates containing oxidative lesions. To examine each step in the BER pathway in detail, we assembled nucleosomes with either of two different DNA fragments, shown previously to form well-positioned nucleosomes (Simpson and Stafford 1983; Dong, Hansen, and van Holde 1990; Pennings, Meersseman, and Bradbury 1991; Flaus et al. 1996; Thastrom et al. 1999; Schalch et al. 2005; Prasad, Wallace, and Pederson 2007). The first of these contained the *L. variegatus* 5S rDNA gene (Lv5S) and a single thymine glycol (Tg) positioned in one of two discrete locations. The nucleosomes we refer to as Tg-out(5S) contain a Tg whose minor groove faces outward from the histone octamer, whereas Tg-in(5S) nucleosomes contain a Tg whose minor groove faces toward the histone octamer. The second nucleosome substrate consisted of the 147 bp core of the 601 nucleosome positioning sequence (a synthetic DNA segment that was selected for its capacity to form a highly stable, positioned nucleosome (Thastrom et al. 1999; Schalch et al. 2005)) with a Tg located 47 nucleotides from the dyad axis. Thus the Tg in the resulting nucleosome, dubbed Tg-in(601), is located in approximately the same region as the Tg in Tg-in(5S).

The enzyme-DNA structures shown in Figure 1A indicate that Nth family DNA glycosylases and human APE interact primarily with the lesion-containing strand of DNA. Additionally, they bind without severely distorting the DNA, which led us to predict that
they could act on Tg-out nucleosomes. This proved to be true for both hNTH1 and NEIL1 DNA glycosylases (Prasad, Wallace, and Pederson 2007; Odell et al. 2010). By contrast, we predicted that the oxidative lesion in Tg-in nucleosomes would be inaccessible, although even these lesions proved to be accessible at enzyme concentrations high enough to permit capture of lesions when exposed during transient unwrapping of DNA from the histone octamer. Inspection of Figure 1A shows that Pol β induces large perturbations in the DNA and that LigIIIα fully encircles its DNA substrate. Thus, at the outset it was unclear if either of these enzymes would be able to bind substrates in nucleosomes, regardless of their helical orientation, without a prior translational shift or disruption of the nucleosome.

3.2. Reconstitution of complete base excision repair reactions with model nucleosomes. Figures 1C, 1D, 1E, 1F, and 1G show DNA products formed at each step in a reaction in which hNTH1, APE, Pol β, and LigIIIα/XRCC1 were added in a sequential fashion to Tg-out(5S) and Tg-in(601) naked DNA, and Tg-out(5S), Tg-in(5S), and Tg-in(601) nucleosomes. These products correspond to the expected products in the reaction scheme depicted in Figure 1B. hNTH1-initiated BER begins with the cleavage of the N-glycosylic bond between the damaged base (in this case, thymidine glycol) and its associated sugar residue. This is followed by cleavage of the phosphodiester bond 3' to the AP site, generating a 3'-phospho-α,β unsaturated aldehyde (PUA) and 5'-phosphate (lanes 2 in Figures 1C-1G). Subsequently, APE removes the 3'-PUA, generating a faster migrating primer with a 3'-OH evident in lanes 3 of Figures 1C-1G. As shown in lanes 4 of Figures 1C-1G, Pol β is then able to extend this primer by 1 or more nucleotides. Finally DNA LigIIIα is able to seal the single nucleotide extension product to generate an intact repaired DNA product with the same
mobility as the starting DNA (lanes 5 in Figures 1C-1G). Comparison of reactions conducted with 5S nucleosomes and the corresponding naked DNA (Figures 1D and 1E vs. Figure 1C) or with the 601 nucleosomes and their corresponding naked DNA (Figure 1F vs. Figure 1G) indicate that each step of BER can proceed on nucleosomes. To achieve a level of repair in nucleosomes comparable to that in DNA, we had to increase the concentrations of enzymes and incubation times accordingly as we progressed from naked DNA to Tg-out nucleosomes to Tg-in nucleosomes (cf. Table 2). The product of enzyme concentration and time (E×t) are listed in subcolumns 3, 6 and 9 of Table 2 as a metric to compare how efficiently each substrate is repaired. Compared to naked DNA, repair of Tg-out(5S) nucleosomes required between 2.5- and 5-fold higher E×t, suggesting that nucleosomes inhibit BER even for accessible lesions. Moreover, Tg-in(5S) nucleosomes required between 120- and 165-fold higher E×t for hNTH1, APE and Pol β, suggesting that they are each much less efficient in acting on inward facing substrates. LigIIIα/XRCC1, however, did not seem to improve beyond a 7.5-fold increase in E×t for ligation of inward facing nicks. Interestingly, hNTH1 excised Tg from Tg-in(601) naked DNA with reduced efficiency compared to both Tg-out(5S) and Tg-in(5S) naked DNA, suggesting that the location of the lesion on the inner side of 601 DNA curvature is sufficient to reduce the apparent activity of hNTH1. Otherwise, we observed a similar level of BER on both naked DNA and nucleosomes reconstituted with the Lv5S and 601 DNA sequences, indicating that the level of repair for the Lv5S sequence is not sequence specific and that repair cannot be attributed to minor translational variants that might result in exposure of the lesion in a subpopulation of nucleosomes.

3.3. Lesion orientation plays a major role in the efficiency of the first three steps of hNTH1-initiated BER, but not the final step. The requirement for higher enzyme
concentrations and longer incubation times to repair Tg-in nucleosomes suggested to us that APE, Pol β, and LigⅡα/XRCC1 each processed their substrates less efficiently where the minor groove faces inward towards the octamer core. To test the effects of lesion orientation on each enzyme in more detail, we reconstituted nucleosomes with Lv5S DNA containing each of the substrates formed during hNTH1-initiated BER: Tg, 3’-PUA, single nucleotide gap, and nicked DNA (cf. Methods). We placed the substrates at positions corresponding to those in the Tg-out(5S) and Tg-in(5S) nucleosomes. Consistent with our earlier studies, hNTH1 excised lesions from Tg-out(5S) nucleosomes more efficiently than from Tg-in(5S) nucleosomes; as well, we observed a slower lyase step after base excision in both orientations (filled-in vs. open triangles in Figure 2A). To assess the removal of 3’-PUA generated by hNTH1, we incubated 3’-PUA-out(5S) and 3’-PUA-in(5S) nucleosomes with APE, as shown in Figure 2B. As with hNTH1, APE proved faster at cleaving the outward facing substrate than the inward facing substrate. We also generated inward facing 3’-PUA sites by incubating Tg-in(5S) nucleosomes with hNTH1 prior to the addition of APE (filled-in squares in Figure 2B). Here APE cleaved the inward facing 3’-PUA less efficiently than nucleosomes reconstituted with 3’-PUA DNA. These results suggest that the orientation of the 3’-PUA affects APE in a manner similar to the effect of lesion orientation for hNTH1, and that initiation of repair with hNTH1 only modestly affects the rate of 3’-PUA excision by APE. Once APE has removed the 3’ blocking group, the DNA is ready for extension by Pol β. We incubated Gap-out(5S) and Gap-in(5S) nucleosomes with Pol β, resolved the extension products on a 12% sequencing gel, and plotted the results in Figure 2C. Surprisingly, Pol β extended the outward facing gap with similar efficiency to the excision reactions by hNTH1 and APE. Likewise, its activity was inhibited by positioning the gap on the inside of the DNA superhelix.
The final step of BER involves phosphodiester bond formation between the 3'-OH of the newly synthesized nucleotide and the downstream 5'-phosphate by LigIIIα. To test the activity of LigIIIα, we incubated Nick-out(5S) and Nick-in(5S) nucleosomes with LigIIIα in complex with XRCC1 (LigIIIα/XRCC1). Because the stability of LigIIIα depends on the accessory factor XRCC1, they were co-expressed in Sf9 cells and purified as a complex. Figure 2D shows the rate of ligation for nicks in each orientation. Unlike the previous three steps in the pathway, there was no difference in ligation efficiency when the nick was facing away from or towards the histone octamer. When we increased the concentration of LigIIIα/XRCC1 by 10-fold, the rate of ligation was also enhanced (Supplementary Figure 1), as we observed previously for hNTH1 excision of Tg-in nucleosomes. These results suggest that LigIIIα requires dissociation of DNA from the octamer in order to permit it to encircle the DNA, and complete catalysis on nucleosomes.

3.4. Investigation of abasic site repair on nucleosomes. Because hNTH1 is a bifunctional DNA glycosylase, the 3' and 5' end moieties during repair are distinct from repair that involves cleavage of an AP-site by APE, which occurs in BER initiated by a monofunctional DNA glycosylase or by APE during abasic site repair. To investigate the effects of the differences in end chemistries during BER on nucleosomes, we studied abasic site repair, a subpathway of BER initiated by phosphodiester bond cleavage 3' of an AP-site by APE. In the second step, two enzymatic activities of Pol β are required: extension of the primer and removal of the 5'-dRP on the downstream DNA. After Pol β extension and lyase activities, the nick is again sealed by LigIIIα/XRCC1.
In order to compare the endonuclease and phosphodiesterase activities of APE on nucleosomes, we first compared them on naked DNA. By treating a short sequence of DNA containing a Tg with excess hNTH1, we were able to generate the 3’-PUA. To measure the endonuclease activity of APE, we used the same double strand oligodeoxynucleotide sequence, but containing a single tetrahydrofuran (THF), an AP-site analog against which APE has been extensively studied (Wilson et al. 1995; Chou and Cheng 2003; Maher and Bloom 2007; Mundle et al. 2009). When we incubated these naked DNA substrates with APE, we observed faster excision of the 3’-PUA than cleavage of the AP-site by APE (Figures 3A and 3B). Previous studies in whole cell extracts have compared the phosphodiesterase activities of APE on two different 3’ blocking groups (3’-phosphoglycolate and 3’-phosphoglycolaldehyde) to its endonuclease activity on THF (Chen, Herman, and Demple 1991; Suh, Wilson, and Povirk 1997). In both studies they found the opposite substrate preference, such that APE cleaved AP sites faster than it excised 3’ blocking groups. Taken together these results suggest that APE has evolved to remove the 3’-PUA generated by hNTH1 over other 3’ blocking lesions.

To test the endonuclease activity of APE on nucleosomes, we reconstituted them with DNA containing a single THF in the same locations as Tg-out and Tg-in. Figure 3C shows the results of incubating THF-out(5S) and THF-in(5S) nucleosomes with the same concentration of APE that we used on 3’-PUA(5S) nucleosomes (Figure 2B). Comparatively, the rate of THF-in cleavage by APE was similar to that of 3’-PUA-in (solid lines of Figure 3C vs. Figure 2B). However, APE cleaved THF-out more efficiently than the outward facing 3’-PUA, showing a reversal in substrate preference from naked DNA (dotted lines of Figure 3C vs. Figure 2B). The reversal could be for a number of
reasons, perhaps that the nucleosomal THF substrate is oriented in a more favorable position for APE binding and recognition than the 3'-PUA. However, APE was consistent for both substrates in that it cleaved both 3'-PUA-out and THF-out more efficiently than each of their inward facing counterparts.

After APE cleaves an AP site, it leaves a 5'-dRP, which must be removed by the lyase domain of Pol β. Due to the lability of the 5'-dRP moiety, nucleosomes would lose it during the course of reconstitution (Bailly and Verly 1989). Therefore, we tested the extension activity of Pol β by reconstituting nucleosomes with THF-out(5S) or THF-in(5S) DNA, cleaving the THF with excess APE, and then adding the appropriate concentration of Pol β. Comparison of the solid line in Figure 3D to the solid line in Figure 2C shows a similar extension rate by Pol β of the inward facing gap containing a 5'-dRP as the gap lacking a 5'-dRP. In contrast, extension of the outward facing gap was slower in the presence of the 5'-dRP than without it (dotted lines in Figure 3D vs. Figure 2C), suggesting that positioning of the lyase domain of Pol β on the 5'-dRP inhibits its ability to extend a gap on nucleosomes.

3.5. Fate of lesion-containing nucleosomes during BER. To determine if nucleosomes were disrupted at any point during BER, aliquots from completed BER reactions were run on 5% native polyacrylamide gels. Figure 4A shows naked DNA and nucleosome templates before the BER reactions, (lanes 1, 3, 4, 6, and 7), and immediately after the BER reactions (lanes 2, 5, and 8). In lanes 5 and 8, the complete absence of a band with the mobility of naked DNA and the presence of a major band with the mobility of an intact nucleosome indicates that neither Tg-out nor Tg-in nucleosomes suffered irreversible disruption during BER. In addition to the nucleosome
band evident in lane 5 of Figure 4A there is also a supershift band that, as described below, likely reflects a residual ternary complex that forms between nucleosomes and BER enzymes.

To further investigate the state of nucleosomes during each step of BER, we conducted a series of single step enzyme reactions using nucleosomes containing substrates matched to the particular enzyme of interest. It was previously reported that hNTH1 lesion processing entails the formation of a nucleosome-hNTH1 ternary complex, which contains DNA processing intermediates (Prasad, Wallace, and Pederson 2007). The ternary complex could be visualized in a gel mobility shift assay by virtue of the fact that hNTH1 has a very slow turnover rate. This explanation predicts that glycosylases that exhibit high turnover rates would fail to form detectable ternary complexes. To test this prediction, we incubated Tg-out(5S) nucleosomes with hNTH1 mutants lacking the N-terminal 55 or 72 amino acids. Removal of these tails has been shown to increase hNTH1 turnover without disrupting enzymatic activity (Liu, Choudhury, and Roy 2003). Figure 4B shows that only full-length hNTH1 forms a detectable ternary complex with Tg-containing nucleosomes. Additionally, trapping of the hNTH1 Schiff base intermediate with NaCNBH$_3$ increased the amount of supershifted nucleosomes, further corroborating that it represents a bona fide BER complex (Figure 4C). Two groups have proposed a substrate hand-off mechanism in which APE displaces DNA glycosylases following excision of a damaged base (Parikh et al. 1999; Wilson and Kunkel 2000). To determine if this occurs during repair of lesions in nucleosomes, we added APE to the hNTH1-nucleosome ternary complexes. This resulted in displacement of hNTH1, as shown in Figure 4D. Despite reports that APE also remains bound to its product (Parikh et al. 1998; Waters et al. 1999), we were unable to reliably detect a supershifted
nucleosome particle in the presence of APE. We were, however, able to isolate nucleosome-Pol β complexes, both before and after nucleotide extension. Incubating Pol β with Gap-in(5S) nucleosomes in the absence of dNTPs led to formation of the supershifted particle shown in lane 3 of Figure 4E. We also initiated repair of Tg-out(5S) nucleosomes with hNTH1, followed by sequential addition of APE and Pol β in the presence of dTTP. After virtually quantitative action by hNTH1 and APE, extension by Pol β (quantified separately on a denaturing gel) resulted in a supershifted particle with the same mobility as that of Pol β bound nucleosomes (lane 3 of Figure 4F). Thus, it appears that like hNTH1, Pol β also remains bound to its product on nucleosomes.

Ordinarily, LigIIIα/XRCC1 binds to nicks that remain after the filling of gaps by Pol β. Because LigIIIα/XRCC1 contains bound ATP it was not possible to examine complex formation in the absence of ATP. However, LigIIIα contains an N-terminal PARP-like Zinc finger domain (ZnF), which enables it to bind gapped as well as nicked DNA (Cotner-Gohara et al. 2010). This provided the opportunity to examine the binding of LigIIIα/XRCC1 to gap containing nucleosomes. Incubating LigIIIα/XRCC1 with Gap-out(5S) and Nick-out(5S) nucleosomes led to formation of the supershifted particles shown in lanes 2 and 3 of Figure 4G. Additionally, incubation of LigIIIα/XRCC1 with Gap-in(5S) nucleosomes generates a similar shift (lane 4 of Figure 4E), suggesting that the ZnF also enables LigIIIα to bind occluded gaps. Taken together, our results suggest that each BER enzyme remains bound to its product, only then to be displaced by the subsequent BER enzyme.
3.6. Nucleosome binding by LigIIIα/XRCC1 enhances the rate of nucleotide extension by Pol β. Based on finding a succession of nucleosome ternary complexes with BER enzymes, we hypothesized that protein-protein interactions could increase the rate of BER by efficiently handing off the substrate from one enzyme to the next, as described for BER enzymes in general (Wilson and Kunkel 2000). To test this, we incubated different combinations of BER enzymes with either the preceding or subsequent enzyme prior to adding them to nucleosomes containing the appropriate substrate. Only one combination had a large effect on the rate of repair. When we pre-incubated Pol β with LigIIIα/XRCC1 before adding it to the reaction with nucleosomes, we observed a higher rate of Pol β extension. The left panel of Figure 5A shows the extension of Gap-in(5S) nucleosomes by Pol β alone, whereas the middle panel shows single nucleotide extension and ligation products of Gap-in(5S) nucleosomes generated by premixing Pol β with LigIIIα/XRCC1 before addition to the reaction. Enhanced extension was already evident at 30 seconds, by comparing lanes 2 in each panel. As expected, no extension or ligation products were observed when we incubated LigIIIα/XRCC1 alone with Gap-in(5S) nucleosomes, shown in the right panel of Figure 5A. Consequently, the ligation products were dependent on extension of the gap by Pol β, so we considered them equivalent to extension products in our quantification of Pol β extension shown in Figure 5B.

Because LigIIIα/XRCC1 binds gapped DNA on nucleosomes (Figure 4G), we hypothesized that LigIIIα/XRCC1 could facilitate recruitment of Pol β to occluded gaps in nucleosomes. To test this hypothesis, we incubated Gap-in(5S) nucleosomes for 5 minutes with Pol β alone, LigIIIα/XRCC1 alone, or Pol β pre-incubated with
LigIIIα/XRCC1, then loaded the reactions directly onto a 5% native polyacrylamide gel (Figure 4E). The reactions did not contain any dNTPs in order to avoid extension by Pol β and completion of BER by LigIIIα/XRCC1. Lane 1 of Figure 4E shows the mobility of Gap-in(5S) naked DNA and lane 2 shows the mobility of Gap-in(5S) nucleosomes. When incubated with Pol β alone, there is a supershift of the nucleosome observed in lane 3, suggesting a Pol β bound nucleosome particle. We observed a much slower mobility supershift when Gap-in(5S) nucleosomes were incubated with LigIIIα/XRCC1, shown in lane 4, which highly suggests that LigIIIα/XRCC1 binds an inward facing gap in nucleosomes. When we then incubated Gap-in(5S) nucleosomes with Pol β pre-incubated with LigIIIα/XRCC1, we observed a nucleosome supershift slightly slower than the one we observed with LigIIIα/XRCC1 alone, indicating that LigIIIα/XRCC1 is bound to the nucleosome before Pol β has extended the primer. These results suggest that nucleosome binding by LigIIIαXRCC1, either directly or via Pol β, enhances the extension of occluded gaps by Pol β.

4. Discussion.

To further our understanding of how Base Excision Repair (BER) occurs in cells, we have investigated the influence of nucleosomes on the efficiency of each step in short patch BER. BER is initiated by DNA glycosylases and APE, which possess similar DNA binding motifs and substrate recognition mechanisms; they each bind the substrate containing strand of DNA and flip the damaged residue out of the DNA duplex into an active site in the enzyme. We discovered that, as with DNA glycosylases, lesion orientation is a key factor for efficient APE activity, such that outward facing substrates are cleaved more efficiently than inward facing ones. The similarities in DNA binding and substrate recognition between APE and DNA glycosylases suggest more generally that
proteins that bind DNA asymmetrically, (e.g. the substrate-containing strand of DNA by DNA glycosylases and APE) will similarly be affected by rotational orientation of DNA relative to the histone octamer. This is consistent with our previous report that DNA glycosylases from distinct families, but with similar DNA binding and substrate recognition mechanisms, also have similar intrinsic activity on nucleosomes (Odell et al. 2010).

Pol β bends the DNA template opposite a gap by 90°, so it was surprising to find high rates of nucleotide extension by Pol β on nucleosome substrates. Nevertheless, this finding is consistent with the discovery that FEN1, which bends the DNA opposite a flap by 90-100°, apparently prefers nucleosomal substrates over naked DNA (Huggins et al. 2002). Additionally, our results are consistent with the report by Nilsen et al., in which they observed efficient extension by Pol β on nucleosomes (Nilsen, Lindahl, and Verreault 2002). However, it is not completely clear why Beard et al. did not observe extension by Pol β (Beard, Wilson, and Smerdon 2003). We discovered that extension by Pol β is less efficient when it is required to cleave a 5'-dRP (comparing Figures 2C and 3D), so initiation of BER with UNG2 may have reduced the extension activity by Pol β for Beard et al. Additionally, lesion distance from the dyad axis correlates with Pol β extension activity. Like Nilsen et al., we placed lesions in the outer wrap of the nucleosome and observed good Pol β activity, whereas Beard et al. and Menoni et al. both placed lesions within 10 nucleotides of the dyad axis and both observed little to no Pol β activity without the help of nucleosome remodeling enzymes (Nilsen, Lindahl, and Verreault 2002; Beard, Wilson, and Smerdon 2003; Menoni et al. 2007). The correlation between Pol β activity and substrate distance from the dyad axis could be a result of
assistance by spontaneous partial unwrapping of nucleosomal DNA, which decreases exponentially with proximity to the dyad (Anderson and Widom 2000). Notably, sequence context and altered translational positioning do not explain the differences observed in Pol β activity because we observed efficient repair with both the Lv5S and 601 nucleosome positioning sequences. In sum, Pol β functions well in the outer wrap of the nucleosome, but may require nucleosome disruption or altered positioning in order to function near the dyad axis.

Given that human DNA ligases must encircle their substrates (Pascal et al. 2004; Cotner-Gohara et al. 2010), it appears that the octamer core would prevent ligase circularization and thus, catalysis of ligation. For both lesion orientations, we observed similar rates of phosphodiester bond formation by LigIIIα to that of the first three enzymes on occluded substrates, suggesting that the histone octamer inhibits LigIIIα from completing ligation for any substrate orientation. As described earlier for hNTH1, it is likely that spontaneous partial unwrapping of DNA from the histone octamer allows DNA glycosylases, APE, and Pol β to access occluded substrates. If this is true, it is likely that spontaneous partial unwrapping of nucleosomal DNA is required for ligation by LigIIIα. Interestingly, the initial rate of ligation by LigIIIα was relatively quick for about one third of the nucleosome population, and is consistent with the measurements by Hayes and colleagues of ligation by DNA Ligase I on nucleosomes (Chafin et al. 2000). We hypothesize that the quick initial rate represents ligation of the fraction of nucleosomes that exist with their DNA in the partially unwrapped state at equilibrium.
In addition to the structural attributes of BER enzymes and nucleosomes themselves, protein-protein interactions probably enhance the overall efficiency of BER in the cell. We observed formation of ternary complexes between BER enzymes and nucleosomes during repair, suggesting that BER enzymes displace one another in an ordered fashion while keeping the nucleosome intact. It has been hypothesized that the individual steps in BER involve recognition of a product-enzyme complex by the next enzyme in the pathway in order to avoid the cytotoxic or mutagenic effects of unrepaired BER intermediates (Wilson and Kunkel 2000). For hNTH1, APE, and Pol β, we did not find significant changes in the kinetics of BER of nucleosome substrates when initiated with the previous enzyme, as shown for 3'-PUA-in excision by APE (Figure 2B). Therefore, substrate-handoff appears to primarily protect DNA intermediates during the course of repair for the first three steps. However, nucleosome binding by LigIIIα/XRCC1 significantly enhanced extension of occluded gaps by Pol β. In accord, cells that express a mutant form of XRCC1 that does not interact with Pol β are more sensitive to hydrogen peroxide (Dianova et al. 2004). Therefore, the interaction between Pol β and XRCC1 may be important for efficient BER via substrate hand-off on nucleosomes in vivo.

**Conflict of Interest Statement**

The above authors have no financial interests

**Acknowledgements**
5. References


6. Figure Legends

Figure 1. *Reconstitution of complete base excision repair reactions with model nucleosomes.* (A) Crystal structures of the enzymes that catalyze each step in short-patch BER (Mol et al. 2000; Fromme and Verdine 2003; Beard et al. 2009; Cotner-Gohara et al. 2010). (B) Schematic of hNTH1 initiated BER, adapted from (Lindahl 2000). (C-G) Sequencing gels showing the reaction products after sequential addition of BER enzymes to naked DNA, Tg-out nucleosomes, Tg-in nucleosomes, Tg(601) naked DNA, and Tg(601) nucleosomes. Enzyme concentrations and incubation times are listed in Table 2.

Figure 2. *Influence of lesion orientation on the rate of each step of BER.* (A) Rates of glycosylase and lyase activity by 20 nM hNTH1 on Tg-out and Tg-in containing nucleosomes (Δ dotted line, Tg-out glycosylase; ▲ dotted line, Tg-out lyase; Δ solid line, Tg-in glycosylase; ▲ solid line, Tg-in lyase). (B) Rates of phosphodiesterase activity by 5 nM APE on 3'-PUA containing nucleosomes. (◊ dotted line, PUA-out nucleosomes; ♦ solid line, PUA-in nucleosomes, ■ solid line, PUA-in generated by 100 nM hNTH1 on Tg-in containing nucleosomes). (C) Rates of extension by 3 nM Pol β on Gap-out and Gap-in containing nucleosomes (○ dotted line, Gap-out; ● solid line, Gap-in). (D) Rates of ligation by 5 nM LigIIIα/XRCC1 on Nick-out and Nick-in containing nucleosomes. (□ dotted line, Nick-out; ■ solid line, Nick-in).

Figure 3. *Characterization of abasic site repair on nucleosomes.* (A) Endonuclease and phosphodiesterase activities of 1 nM APE on 35 bp duplex DNA oligomer containing either THF or 3'-PUA. Lanes 1-5 in each panel correspond to 0, 10, 30, 90, and 240
seconds. (B) Quantification of A. Because we needed to dilute the enzyme ~50,000 fold, the error between dilutions was more than the difference in observed activity. Therefore, the error reported in C reflects the experiment done in triplicate, but from the same enzyme dilution. In three separate experiments we observed higher relative phosphodiesterase to endonuclease activity by APE on naked DNA. (C) Rates of endonuclease activity by 5 nM APE on THF-out and THF-in nucleosomes. (◊ dotted line, F-out nucleosomes; ♦ solid line, F-in nucleosomes). (D) Quantification of extension by Pol β of nucleosomes containing a gap with 5'-dRP. (○ dotted line, Gap-out with 5'-dRP; • solid line, Gap-in with 5'-dRP).

**Figure 4. Fate of lesion-containing nucleosomes during BER.** After sequential addition of all the BER enzymes to naked DNA or nucleosome reactions, aliquots were loaded directly onto a 5% native polyacrylamide gel. (A) The migration of Tg-containing naked DNA is shown in lanes 1, 3, and 6. Tg-out and Tg-in(601) nucleosomes are in lanes 4 and 7, respectively. The mobility of repair products of Tg-out naked DNA, Tg-out nucleosomes and Tg-in(601) nucleosomes are shown in lanes 5 and 8. (B) Tg-out nucleosomes were incubated with 0, 20, 60, 100, and 400 nM hNTH1 for 2.5 mins at 22°C before resolution on 5% native polyacrylamide gel. In lanes 1-5, nucleosomes were incubated with full-length hNTH1, whereas lanes 6-10 and 11-15 were incubated with Δ55 and Δ72 N-terminal truncations of hNTH1. (C) Tg-out nucleosomes were incubated alone (lane 1), with 35 nM APE (lane 2), or with 14 nM hNTH1 and 0, 14, and 35 nM APE (lanes 3-5) for 15 mins at 21°C before resolution on a 5% native polyacrylamide gel. (D) Tg-out naked DNA and nucleosomes were cross-linked to hNTH1 by incubation with either 25 or 50 mM NaCNBH₃. Lane 1 is hNTH1 cross-linked to naked DNA, Lanes 2-4 contain Tg-out nucleosomes alone or with 16 nM hNTH1 and 25 or 50 mM
NaCNBH$_3$. (E) Nucleosomes were incubated in BER reaction buffer lacking any dNTP either alone (lane 2), with 33 nM Pol β (lane 3), with 15 nM LigIIIα/XRCC1 (lane 4), or with 33 nM Pol β premixed with 15 nM LigIIIα/XRCC1 (lane 5), before resolution on a 5% native polyacrylamide gel. Lane 1 shows the mobility of naked DNA alone. (F) Tg-out nucleosomes were incubated in BER reaction buffer containing 20 μM dTTP and no enzymes (lane 2) or sequentially added 100 nM hNTH1, 50 nM APE, and 33 nM Pol β for 30, 15, and 30 mins (lane 3), before separation on a native gel. (G) Lane 1 shows the migration of Gap-out nucleosomes. Lanes 2 and 3 show the products of incubating Gap-out and Nick-out nucleosomes with 15 nM LigIIIα/XRCC1.

**Figure 5. Nucleosome binding by LigIIIα/XRCC1 enhances the rate of nucleotide extension by Pol β.** (A) Extension and ligation products of Pol β alone, LigIIIα/XRCC1 alone or Pol β pre-incubated with LigIIIα/XRCC1 were incubated with Gap-in nucleosomes. For each panel, lanes 1-5 show time points taken at 0, 0.5, 1.5, 5, and 20 minutes. The left panel shows single nucleotide extension by Pol β alone. The middle panel shows extension and ligation products by Pol β and LigIIIα/XRCC1. The right panel shows no extension or ligation by LigIIIα/XRCC1 alone. (B) Quantification of A. (● solid line, Pol β alone, ● dotted line, Pol β alone on Gap-in with 5’ dRP; ○ solid line, Pol β pre-incubated with LigIIIα/XRCC1 on Gap-in, no 5’-dRP).

**Supplementary Figure 1. Higher concentration of LigIIIα/XRCC1 has higher ligation activity on nucleosomes.** Nick-out and Nick-in nucleosomes were incubated with 5 and 50 nM LigIIIα/XRCC1 for 0.5, 1.5, 5, and 20 mins. (□ dotted line, Nick-out with 5 nM LigIIIα/XRCC1; ■ solid line, Nick-in with 5 nM LigIIIα/XRCC1; ○ dotted line, Nick-
out with 50 nM LigIIIα/XRCC1; • solid line, Nick-in with 50 nM LigIIIα/XRCC1). Error bars reflect the standard error about the mean for the experiment done in three replicates. The error bars are within the size of the symbol if not seen.
## Chapter 3-Table 1. List of DNA sequences used to generate BER substrates.

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg-out</td>
<td>GGTACCGATCCAGGGATTTTATAAGCTGATGACGTgCATAAACATCCCTG</td>
</tr>
<tr>
<td>Tg-in</td>
<td>GGTACCGGATCCAGGGATTTTATAAGCTGATGACGTgCATAAACATCCCTG</td>
</tr>
<tr>
<td>F-out</td>
<td>GGTACCGGATCCAGGGATTTTATAAGCTGATGACGFGACGTCTAATACATCCCTG</td>
</tr>
<tr>
<td>F-in</td>
<td>GGTACCGGATCCAGGGATTTTATAAGCTGATGACGFGACGTCTAATACATCCCTG</td>
</tr>
<tr>
<td>Gap-out</td>
<td>GTACCGGATCCAGGGATTTTATAAGCTG</td>
</tr>
<tr>
<td>Gap-in</td>
<td>GTACCGGATCCAGGGATTTTATAAGCTG</td>
</tr>
<tr>
<td>Nick-out</td>
<td>GTACCGGATCCAGGGATTTTATAAGCTG</td>
</tr>
<tr>
<td>Nick-in</td>
<td>GTACCGGATCCAGGGATTTTATAAGCTG</td>
</tr>
<tr>
<td>Out (3')</td>
<td>Phos-GACGTCATAACATCCCTGACCCTTTTTAAATAGCTTAACATTTTGATCAAGCAA</td>
</tr>
<tr>
<td>In (3')</td>
<td>Phos-CATAACATCCCTGACCCTTTTTAAATAGCTTAACATTTTGATCAAGCAA GAGCC</td>
</tr>
<tr>
<td>5sslv Template</td>
<td>TCGAAGCTACGGACGTCAGGTGAGACGTATATAGCTGCGGATATAGCAAC TGAAGGCGACGTATATAGCTGGAAGGCTATTCTATTGTAGATCATGCAAGCAA GAGCC</td>
</tr>
<tr>
<td>Tg-in (601)</td>
<td>CTGGAGAATCCCGGTGGAGGCCGCGTGCAATTGGTCGTAGACAGCTCTA GACACCGTAAACG</td>
</tr>
<tr>
<td>601 Template</td>
<td>ACAGGATGTATATATCTGACGCGTGGAGACTAGGGAGTAATCCCCCTCGTACCGGGCAAC GTCGGTGAAGCTGTAATAGCTGAGTCAGTCAAAAGGAGCTATTCTATTGTGCAAGCAA GAGCC</td>
</tr>
<tr>
<td>X35</td>
<td>TGTCAATAGCAAGGAGAAGTGCTAGTAAACG</td>
</tr>
<tr>
<td>X35-RC</td>
<td>AGACTCAGTTGACTTCTCCACATTGACTATTGACA</td>
</tr>
</tbody>
</table>

## Chapter 3-Table 2. Enzyme concentrations and incubation times for repair of naked DNA, Tg-out, Tg-in, and Tg(601) nucleosomes.

<table>
<thead>
<tr>
<th></th>
<th>5sslv Naked DNA</th>
<th>5sslv Tg-out Nucleosomes</th>
<th>5sslv Tg-in Nucleosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>hNTH1</td>
<td>5</td>
<td>5</td>
<td>1x</td>
</tr>
<tr>
<td>APE</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Pol β</td>
<td>3</td>
<td>2</td>
<td>1x</td>
</tr>
<tr>
<td>LigIIIα/XRCC1</td>
<td>10</td>
<td>2</td>
<td>1x</td>
</tr>
<tr>
<td></td>
<td>601 Naked DNA</td>
<td>601 Tg-in Nucleosomes</td>
<td></td>
</tr>
<tr>
<td>hNTH1</td>
<td>20</td>
<td>10</td>
<td>1x</td>
</tr>
<tr>
<td>APE</td>
<td>5</td>
<td>1</td>
<td>1x</td>
</tr>
<tr>
<td>Pol β</td>
<td>3</td>
<td>2</td>
<td>1x</td>
</tr>
<tr>
<td>LigIIIα/XRCC1</td>
<td>10</td>
<td>2</td>
<td>1x</td>
</tr>
</tbody>
</table>
Chapter 3-Figure 1. Reconstitution of complete base excision repair reactions with model nucleosomes.
(B) OXIDATIVE LESION REPAIR

(C) Naked DNA

Lane: 1 2 3 4 5
Chapter 3-Figure 2. Influence of lesion orientation on the rate of each step of BER.
Chapter 3-Figure 3. Characterization of abasic site repair on nucleosomes.

(A) 3'-PUA(35)+APE

(B) 1 nM APE
THF(35) & 3'-PUA(35) Naked DNA
Chapter 3-Figure 4. Fate of lesion-containing nucleosomes during BER.

(A) Naked DNA  \hspace{1cm} \text{Tg-out(5S) Nucs}  \hspace{1cm} \text{Tg-in(601) Nucs}

Lane: 1 2 \hspace{1cm} 3 4 5 \hspace{1cm} 6 7 8

(B) \begin{align*}
\text{[hNTH1]}: & \quad 0 \quad \Delta55 \text{ hNTH1} \quad \Delta72 \text{ hNTH1} \\
\text{DNA} & \hspace{1cm} \text{Nuc} \hspace{1cm} \text{Nuc+hNTH1} \hspace{1cm} \text{Nuc} \hspace{1cm} \text{Nuc} \hspace{1cm} \text{Nuc} \hspace{1cm} \text{Nuc} \\
\text{Lane:} & \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8 \quad 9 \quad 10 \quad 11 \quad 12 \quad 13 \quad 14 \quad 15
\end{align*}
Chapter 3-Figure 5. Nucleosome binding by LigIIIα/XRCC1 enhances the rate of nucleotide extension by Pol β.

(A)

(B) 30 nM Pol β
Gap-in +/- dRP Nucleosomes

Fraction Extended

Time (Mins)
Chapter 3-Supplementary Figure 1. Increasing concentrations of LigIIIα/XRCC1 more efficiently ligate nick containing nucleosomes.
CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

To further our understanding of how Base Excision Repair (BER) functions in the cell, we have investigated the influence of nucleosome structure on the efficiency of repair for a number of BER enzymes. We initially examined the differences between two human DNA glycosylases that have overlapping substrate specificities. Because hNTH1 and NEIL1 both excise oxidized pyrimidines from DNA, we could compare their abilities to excise thymine glycol (Tg) from nucleosome substrates. We found that the cellular concentrations and apparent efficiency of Tg excision from naked DNA (as judged by $k_{cat}/K_m$ ratios) for hNTH1 and NEIL1 are similar. However, we also discovered that NEIL1 differs from hNTH1 in that it binds undamaged DNA far more avidly. After adjustment for non-specific DNA binding, hNTH1 and NEIL1 proved to have similar intrinsic activities towards nucleosome substrates. Collectively these results indicate that hNTH1 is able to excise both accessible and sterically occluded lesions from nucleosomes at physiological concentrations, while the high non-specific DNA affinity of NEIL1 would likely hinder its ability to process sterically occluded lesions in cells. In general, the structural differences between the two glycosylases were not sufficient to alter their intrinsic efficiency towards nucleosomes. Instead, their similar activities on nucleosomes are likely to be determined features they have in common.

The steps in BER that follow the excision of the damaged base are catalyzed by APE, Pol β, and LigIIIα in complex with XRCC1 (LigIIIα/XRCC1). DNA glycosylases and APE possess similar DNA binding motifs and substrate recognition mechanisms; they each bind the substrate containing strand of DNA and flip the damaged residue out of the DNA duplex into an active site in the enzyme. Like DNA glycosylases, APE also initiates
a subpathway of BER, suggesting that these enzymes have co-evolved to bind and extrude damaged residues in a similar manner. We wanted to find out if these similarities extended to their activity on nucleosomes, as suggested by our results with hNTH1 and NEIL1, but we also wanted to find out if BER enzymes with different DNA binding motifs could also act without nucleosome remodeling or disruption. The last two steps of BER are catalyzed by Pol β and LigIIIα, whose structures are quite different from APE and the glycosylases, so to settle the matter, we set out to reconstitute the entire BER pathway and to study individual steps in BER with nucleosomes containing the appropriate substrates. We found that like DNA glycosylases, lesion orientation is relevant to the activities of APE and Pol β, such that substrates occluded by the octamer are processed slowly, whereas substrates whose strand of DNA is accessible for enzyme binding are processed with efficiency closer to that of naked DNA. The similarities in DNA binding and substrate recognition between APE and the glycosylases suggested that this would be the case, as we had discovered for hNTH1 and NEIL1. However, Pol β bends the DNA 90° across from the gap, so it was surprising to find high rates of nucleotide extension by Pol β on nucleosome substrates. Nevertheless, this is consistent with the activity of FEN1 on nucleosome substrates, as it too bends the DNA 90-100° across from the flap, and seems to actually prefer nucleosomal substrates over naked DNA (Huggins et al. 2002). For both lesion orientations, LigIIIα catalyzed phosphodiester bond formation with similar efficiency to that of the first three enzymes on occluded substrates, suggesting that the histone octamer occludes LigIIIα from completing ligation for any substrate orientation. Given that human DNA ligases must encircle their substrates, it seems logical that the octamer would prevent ligase circularization and thus, catalysis of ligation. As described earlier for hNTH1, it is likely that dissociation of DNA off the histone octamer allows DNA glycosylases, APE, and Pol β to access
occluded substrates. If this is true, it seems that spontaneous partial unwrapping of nucleosomal DNA is required for ligation by LigIIIα, probably to permit circularization of LigIIIα around its substrate.

In addition to the structural motifs of each BER enzyme, protein-protein interactions may be an important factor for the overall efficiency of BER in the cell. While investigating Pol β and LigIIIα/XRCC1, we discovered that Pol β extends occluded gaps more efficiently in the presence of the LigIIIα/XRCC1 complex. We also discovered during our studies of NEIL1 that purified XRCC1 stimulates NEIL1 turnover on naked DNA (Appendix A). Both NEIL1 and Pol β have previously been shown to interact with XRCC1 (Gryk et al. 2002; Wiederhold et al. 2004), but it appears that XRCC1 exerts its effects by different mechanisms. Consistent with the effects of XRCC1 on PNK and its affinity for nicked DNA (Whitehouse et al. 2001; Mani et al. 2004), XRCC1 likely stimulates NEIL1 turnover by displacing it from its product, whereas the LigIIIα/XRCC1 complex probably stimulates Pol β extension by increasing its accessibility to occluded gaps on nucleosomes. Our findings suggest that mutations that alter the interaction between BER enzymes may affect the overall efficiency of the pathway in the cell. Contacts between Pol β and XRCC1 are primarily mediated by Loop III of Pol β and the N-terminal domain of XRCC1 (Gryk et al. 2002). Because mutations that abrogate the interaction between Pol β and XRCC1 have already been mapped, it would be interesting to clone Pol β variants harboring those mutations and test their effects on BER. Based on our observations with nucleosomes, we hypothesize that disruption of the interaction between Pol β and XRCC1 would result in less efficient overall repair. It would be interesting to transfect Pol β+/− MEFs with the Pol β mutant that doesn’t bind XRCC1 and
measure the effects of the mutation by examining the sensitivity of wild type, *Pol β*−/− and transfected cells to oxidative or alkylating DNA damaging agents.

In our studies of the complete BER reaction, we assembled octamers with canonical histones that we expressed and purified from *E. coli* (see Appendix D for details). We also expressed and purified the histone H3 variant H3.3 in the same manner. By substituting H3.3 for H3.1 during octamer assembly, we were able to make H3.3 containing nucleosomes and complete some preliminary studies, as described in Appendix F. Because H3.3 is enriched in actively transcribed regions of the genome (Mito, Henikoff, and Henikoff 2005; Schwartz and Ahmad 2005), we hypothesized that nucleosomes carrying it would have altered characteristics from those with H3.1, and that these differences could result in changes in the efficiency of BER. As we did for H3.3, other histone variants could be expressed and incorporated into nucleosomes in a similar manner. Their effects on BER would be interesting for future studies. For example, unstable nucleosomes might be disrupted by BER enzymes, or lesions on nucleosomes with a higher rate of spontaneous partial unwrapping of nucleosomal DNA might be repaired more efficiently. In addition to primary sequence variants, histone post-translational modifications, such as acetylation (Neumann et al. 2009), can be site-specifically added during expression in *E. coli*, and these modified histones could also be incorporated into nucleosomes and studied in a similar manner.

BER enzymes play roles in biological processes other than DNA repair. For example, many DNA repair proteins also appear to be important for apoptosis (Bernstein et al. 2002). The human DNA glycosylase NEIL3 has only recently been characterized
biochemically (Liu et al. 2010), and evidence points to it having a role outside of the canonical BER pathway. The N-terminal half of the protein contains a glycosylase domain, and the C-terminal half contains three Zinc finger motifs. NEIL3 is expressed during embryogenesis in areas known to harbor neural stem and progenitor cells, and its expression is upregulated in certain tumors, suggesting that it functions in cells with high proliferative potential (Kauffmann et al. 2008; Hildrestrand et al. 2009). NEIL3 has also been identified in a screen for host factors that are critical for HIV replication (Zhou et al. 2008). These findings hint that NEIL3 initiated BER may not exist solely to repair oxidative DNA damage that results from aerobic metabolism. Instead, it may be involved in some type of cell signaling, similar to what has been proposed for hOGG1 (Perillo et al. 2008). The biological role of NEIL3 will be very interesting for future investigation.


Muller, H. J. 1932. Further studies on the nature and causes of gene mutations.


APPENDIX A: STIMULATION OF NEIL1 BY XRCC1.

It was previously reported that XRCC1 stimulates the activity of the DNA glycosylase, hNTH1. To test if it also stimulates NEIL1, 5 nM NEIL1 was pre-incubated on ice with 0, 50 or 250 nM XRCC1, then added to a reaction with 25 nM of a 35 bp substrate containing Tg at position 14 (generated as described in Experimental Procedures in Chapter 2). Figure 1 shows the rates of Tg excision and subsequent lyase activity by NEIL1 in the presence of increasing concentrations of XRCC1. The filled-in squares correspond to NEIL1 alone, the open triangles to NEIL1 with 50 nM XRCC1, and the open squares to NEIL1 with 250 nM XRCC1. Less than 50 nM XRCC1 did not increase the activity of NEIL1, consistent with the concentrations used by Campalans et al. that reported stimulation of hNTH1 by XRCC1 (Campalans et al. 2005). Additionally, XRCC1 did not increase the activity of NEIL1 in single turnover conditions (data not shown).

These data suggest that XRCC1 stimulates the rate of product release by NEIL1, likely by displacement of NEIL1 from its product by XRCC1. This idea is consistent with the observed affinity of XRCC1 for nicked or 1 nt gap containing duplex DNA ($K_d$s are 65 and 34 nM, respectively) (Mani et al. 2004).

Appendix A-Figure 1. Stimulation of NEIL1 by XRCC1.

![Figure 1](image_url)
APPENDIX B: INHIBITION OF NEIL1 BY LAMBDA PHAGE DNA.

Digestion of nuclear DNA with micrococcal nuclease (MNase) yields double strand breaks in the linker regions of DNA between nucleosomes because nucleosomes protect DNA from single strand cleavage by MNase. Consequently, this generates a high number of free DNA ends, which if bound by NEIL1, could be an alternative explanation to its inhibition by chromatin competitor, as reported in Chapter 2. To test this possibility, NEIL1 was incubated with the same Tg containing double stranded oligomer and under the same conditions described in Chapter 2, except in the presence of increasing concentrations of λ phage DNA in place of chromatin competitor. The extent of Tg excision by NEIL1 after 45 seconds was assessed in the same manner as described in Chapter 2, and the results are plotted in Figure 1. As we observed with chromatin competitor, the activity of NEIL1 was likewise inhibited by λ phage DNA, highly suggesting that NEIL1 is inhibited by non-specific DNA binding rather than binding to DNA ends.

Appendix B-Figure 1. Inhibition of NEIL1 by λ phage DNA.
APPENDIX C: STIMULATION OF hNTH1 BY CHROMATIN COMPETITOR.

While investigating the inhibition of NEIL1 by non-specific DNA competitor, we observed increased excision of Tg by hNTH1 in the presence of chromatin competitor (Figure 1, open triangles). The competition assay was performed in the same manner as described in Chapter 2, except with increasing amounts of chromatin competitor in place of naked DNA.

It is unclear why excess chromatin competitor, but not naked DNA, would increase the activity of hNTH1. Perhaps the enhancement of hNTH1 activity by chromatin competitor reflects a difference in search mechanism by hNTH1. If it searched nucleosomes and naked DNA in a different fashion, one may promote more efficient search while the other was more inhibitory. For example, if hNTH1 hopped along or between mononucleosomes because the octamer sterically occludes the inner surface of the superhelix, but slid alone naked DNA which is fully accessible, perhaps the mononucleosomes could promote a more efficient search mechanism. These results could be an interesting starting point for future studies.

**Appendix C-Figure 1.** Stimulation of hNTH1 Tg excision by chromatin competitor.
APPENDIX D: HISTONE EXPRESSION AND PURIFICATION.

Histone Expression: Histones H2A, H2B, H3, and H4 were expressed and assembled into octamers based on the methods described by Luger et al. and Dyer et al. (Luger, Rechsteiner, and Richmond 1999; Dyer et al. 2004). Briefly, BL21 (DE3) pLysS One Shot cells were transformed with the appropriate histone containing plasmid (AmpR) and plated onto TYE agar (1% bacto-peptone, 0.5% yeast extract, 0.8% NaCl, 1.5% agar) containing 100 μg/ml Ampicillin and 25 μg/ml Chloramphenicol. From this plate, a single colony was transferred to inoculate 50 ml of 2xTY broth (1.6% bacto-peptone, 1.0% yeast extract, 0.5% NaCl, 20% glucose, 100 μg/ml ampicillin and 25 μg/ml chloramphenicol), which was incubated at 37° C, 225 rpm to an A600 ~ 0.2, then transferred to a Fernbach flask containing 600 ml 2xTY broth. The 650 ml culture was incubated at 37° C, 170 rpm until it reached an A600 ~ 0.6, at which point expression of the histone was induced with 0.2 mM IPTG for two hours. After two hours, the cells were harvested by centrifugation at 8000g at room temperature for 10 minutes. The pellet was then suspended in 50 ml wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM Benzamidine, and 1 mM β-mercaptoethanol) and centrifuged again at 8000g at room temperature for 10 minutes. The pellet was suspended in 25 ml wash buffer and stored at -80° C.

Inclusion Body Preparation: The cell pellet containing the expressed histone was thawed at 37° C in a water bath to induce cell lysis. To enhance lysis, the cells were then sonicated with a MSE Ultrasonic Power Unit 3 times for 4 minutes each at 1-1.5 amps. Between sonications, the cells were chilled to less than 10° C. The resulting cell extract was centrifuged at 12,000g for 20 minutes at 4° C and the pellet was suspended in 25 ml
wash buffer containing 1% Triton X-100. Centrifugation and suspension of the cell extract was repeated in the same manner 2 more times, except the last time was suspended in wash buffer without Triton X-100 and stored for a limited time at -20°C.

**Histone Purification:** A Sephacryl S-200 column (26 mm diameter, 40 cm height) was pre-equilibrated with at least 1.5 column volumes of SAU-1000 buffer (7M Urea, 20 mM NaOAc, pH 5.2, 1 M NaCl, 1 mM Na-EDTA, and 5 mM β-mercaptoethanol) at 0.6 ml/min at 4°C. The inclusion body pellet was thawed, 500 μl DMSO added, and incubated for 30 minutes at room temperature. The solubilized pellet was then broken up with a glass rod and incubated another hour with 4 ml unfolding buffer (6M Guanidinium-HCl, 20 mM Tris-HCl, pH 7.5, and 5 mM DTT). The pellet was then suspended by repeated pipetting and centrifuged at 23,000g for 10 minutes at room temperature. The histone-containing supernatant was set aside and the DMSO solubilization, unfolding, and centrifugation were repeated once more. The two supernatants from this procedure were pooled and loaded onto the Sephacryl S-200 column at 0.81 ml/min. After loading the histones, SAU-1000 buffer was continued over the column at 0.81 ml/min. 3 minute fractions were collected at 4°C for 2 hours. \( A_{276} \) and \( A_{260} \) were measured for each fraction to identify protein-containing fractions. Samples of these were then resolved on an 18% SDS polyacrylamide gel to identify the histone containing fractions. To refold the histone, the histone-containing fractions were pooled and dialyzed 3 times against 500 ml ddH₂O, 2 mM β-mercaptoethanol at 4°C using 6-8 kDa MWCO Spectra/Por dialysis tubing. The histone concentration was determined by \( A_{276} \) and the extinction coefficients listed in Luger et al. (Luger, Rechsteiner, and Richmond 1999), and distributed in 60 nmole aliquots, flash frozen in liquid nitrogen, and lyophilized for long-term storage at -80°C.
**Octamer Assembly and Purification:** A Superdex 200 HR column (9 mm diameter, 30 cm height) was pre-equilibrated with at least 1.5 column volumes of refolding buffer (2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM Na-EDTA, and 5 mM β-mercaptoethanol) at 0.35 ml/min. 60 nmole aliquots of each lyophilized histone were brought to ~2 mg/ml with unfolding buffer and incubated for 1 hour at room temperature. To assist dissolving the histones, each solution was passed through a 26 gauge syringe. Each histone concentration was measured by $A_{276}$ and mixed in equimolar ratios. The mixed histones were dialyzed against 3 buffer changes of 600 ml refolding buffer using 6-8 kDa MWCO Spectra/Por dialysis tubing at 4° C. The refolded octamer was concentrated to a final volume of ~200 µl with a 15 ml 10 kDa MWCO Amicon Ultra centrifugal filter by centrifugation at 4,000g for 20 minutes at 4° C. 3 mg of the concentrated octamer was loaded onto the Superdex 200 HR column by gravity feed, with refolding buffer continued after loading the octamer at 0.35 ml/min. 5 ml of flow through was collected, at which point 3 minute fractions were collected in low adhesion microfuge tubes for 105 minutes. Protein containing fractions were identified using a Bradford microtiter plate assay. These were then TCA precipitated, suspended in SDS load buffer, and resolved on 18% SDS polyacrylamide gel to identify octamer containing fractions. The purified octamers were pooled and dialyzed against refolding buffer containing 50% glycerol and stored at -20° C.
APPENDIX E: CONSTRUCTION OF DNA CONTAINING BER LESIONS OR INTERMEDIATES.

184 bp DNA containing the *Lytechinus variegatus* 5S rDNA nucleosome positioning sequence and a single, discretely positioned Tg residue was prepared as previously described (Prasad, Wallace, and Pederson 2007). Nucleosomes length, Furan-containing DNA was prepared in the same manner, but with an oligomer containing tetrahydrofuran in place of the Tg base. To prepare Gap or Nick-containing DNA fragments, the DNA oligomers Out (3') and In (3') (*Table 1*) were 5'-end labeled with [γ-\(^{32}\)P] ATP and T4 PNK, annealed to equimolar amounts of 5slv template, and extended with (exo-) Klenow enzyme (New England Biolabs) to create 154 and 149 nucleotide-long DNA segments. The extension reactions were stopped with one volume 25x NET (400 mM NaOAc, 25 mM H\(_4\)EDTA, 100 mM Tris base), and then mixed with an equimolar amount of the appropriate \(^{32}\)P 5'-end labeled upstream oligomers (Gap-out, Gap-in, Nick-out, or Nick-in) in 12.5X NET, and annealed to create a full-length DNA fragment containing a single, discretely positioned gap or nick.

3'-phospho-\(\alpha,\beta\)-unsaturated aldehyde (3'-PUA) containing DNA was prepared by incubating Tg containing naked DNA with excess hNTH1 for 30 minutes at 37°C. hNTH1 was removed by phenol/chloroform extraction, and the DNA was ethanol precipitated and suspended in the appropriate volume for nucleosome reconstitution. The concentration of 3'-PUA was calculated by scintillation counting 1% of the DNA before and after hNTH1 treatment, extraction and precipitation.
DNA containing a 5'-deoxyribose phosphate (5'-dRP) has a much shorter half life than the time it takes to reconstitute nucleosomes (Bailly and Verly 1989). Therefore, to assess the activity of Pol β on gaps containing a 5'-dRP, we pre-incubated F-out or F-in containing nucleosomes with 5 or 50 nM APE for 1.5 or 30 minutes at 37°C, respectively. In this manner, we were able to generate nucleosomes containing a gap with a 5'-dRP immediately before addition of Pol β.

hNTH1 and APE naked DNA assays were measured on a double strand 35 bp DNA fragment containing either a single thymine glycol (Tg) or Furan residue at position 14 (X35), as previously described (Odell et al. 2010). A 3'-PUA on this DNA was generated as described above.
APPENDIX F: PRELIMINARY STUDIES WITH HISTONE H3.3 CONTAINING NUCLEOSOMES.

Using the same technique for histone expression and purification as described in Appendix E and nucleosome assembly described in Chapter 3, nucleosomes containing H3.3 in place of H3.1 were reconstituted with DNA from the *L. variegatus* 5S rDNA (Lv5S) nucleosome positioning sequence. H3.3 containing nucleosomes were stable in 1x HED (25 mM NaHEPES NaOH pH 8.0, 1 mM EDTA, and 1 mM DTT) over multiple weeks at 4°C.

Jin and Felsenfeld reported that H3.3 containing nucleosomes are unusually sensitive to salt-dependent disruption (Jin and Felsenfeld 2007). To confirm this observation, we reconstituted H3.3 nucleosomes with a 195 bp segment of Lv5S DNA, and incubated these nucleosomes in 1x HED containing either 10 or 100 mM NaCl or KGlu for one hour at 37°C. The reactions were then resolved on a 5% native polyacrylamide gel with 1/4x TBE buffer and the results are shown in Figure 1. For both low and high salt buffers, we observed no nucleosome disruption of H3.3 nucleosomes, suggesting that there may be a difference in salt sensitivity between our nucleosomes and those purified by Jin and Felsenfeld. Additionally, these results indicate that H3.3 nucleosomes are stable in the BER reaction buffer used in Chapter 3 during the time it would take to complete BER, and therefore, could be investigated in a similar manner in future studies. Because Jin and Felsenfeld isolated their H3.3 nucleosomes from 6C2 cells, the salt sensitivity they observed could result from post-translational modifications that do not exist on our *E. coli* expressed histones. Alternatively, the increased salt sensitivity could have resulted from a differential sensitivity between H3.1 and H3.3 containing
nucleosomes to the C-terminal Flag- or HA-epitope tags fused to H3.1 and H3.3 in the Jin and Felsenfeld study.

To investigate nucleosome translational positioning, H3.3 nucleosomes were reconstituted with Tg-51 DNA in the same manner as reported in Chapter 3. These were then incubated with 105 μU/μl DNase I in 10 mM Tris-HCl pH 8.0, 2.5 mM MgCl₂ and 0.5 mM CaCl₂ for 30, 90, and 300 seconds (Figure 2, Lanes 3-5). Control digestions of Tg-51 naked DNA were completed with 10.5 μU/μl DNase I in the same buffer for 30 and 60 seconds (Figure 2, Lanes 2 and 6). Lane 1 contains labeled pBR322 plasmid digested with MspI and filled in with Klenow enzyme (New England Biolabs) and α³²P-dCTP. DNase I was removed by phenol/chloroform extraction and the DNA was ethanol precipitated and suspended in 1x formamide loading buffer before resolution on an 8% sequencing gel. The protection provided by H3.3 nucleosomes was not as obvious as the digestion patterns observed by Prasad et al. (Prasad, Wallace, and Pederson 2007). For purposes of comparison, the results of DNase I digestion of Tg-51 containing nucleosomes from Prasad et al. 2007 are shown in the right hand panel of Figure 2 (Prasad, Wallace, and Pederson 2007). By comparing the intensity of the bands in relation to the marker lane, it appears that the pattern of DNase I sensitivity was similar for both sets of nucleosomes, suggesting that the Lv5S DNA has similar translational positioning for both H3.3 nucleosomes and nucleosomes containing octamers originating from chicken erythrocytes (Prasad, Wallace, and Pederson 2007).

It is possible that H3.3 containing nucleosomes have a different rate of spontaneous DNA unwrapping off the histone octamer. H3.1 and H3.3 containing octamers were reconstituted with the 184 bp Tg-46 DNA construct described in Chapter 2 (Tg-in). After
confirming their reconstitution efficiencies were greater than 95%, they were separately incubated with 100 nM hNTH1 in 1x HED containing 100 mM NaCl. Different time points were taken by removing an aliquot and stopping the reaction with an equal volume formamide. The samples were separated on an 8% sequencing gel and analyzed by phosphoimaging. The rates of Tg excision by hNTH1 were plotted in Figure 3. No difference in Tg excision by hNTH1 was observed, suggesting that there is no difference in rate of spontaneous nucleosome unwrapping between H3.1 and H3.3 nucleosomes. To be certain, this experimental setup could be repeated with a lower concentration of hNTH1. Alternatively, the rate of unwrapping could be probed by digesting the nucleosomes with a restriction enzyme whose target site is buried within the nucleosome, such as Dra I for the 5S rDNA nucleosome positioning sequence.
Appendix F-Figure 1. H3.3 nucleosomes are stable for extended incubations in buffers containing 100 mM salt.
Appendix F-Figure 2. DNase I digestion of H3.3 containing nucleosomes.
Appendix F-Figure 3. Rate of Tg-46 excision by 100 nM hNTH1 with H3.1 and H3.3 containing nucleosomes.