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Structure-function analysis of human enzymes initiating nucleobase repair in DNA and RNA

Doctoral thesis
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Happy Crystals!
Trondheim, December 2007
Ottar Sundheim

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RNA base damage and repair. Feyzi E, **Sundheim O**, Westbye MP, Aas PA, Vågbø CB, Otterlei M, Slupphaug G and Krokan HE. *In press,* ***Current Pharmaceutical Biotechnology*** **(2007)**

Abbreviations

AGOG	Archaeal GO glycosylase
AGT	O^6-alkylguanine-DNA transferase
AID	Activated induced deaminase
AlkA	3-meA-DNA glycosylase II
AP	Apurinic/apyrimidinic
APE1	AP endonuclease 1
AT	Ataxia telangiectasia
ATLD	Ataxia-telangiectasia-like disease
BER	Base excision repair
CPD	Cyclobutane pyrimidine dimmer
CS	Cokayne Syndrome
CSR	Class switch recombination
C-terminus	Carboxyl terminus
DDR	Direct damage repair
DHU	Dihydrouracil
DNA	Deoxyribonucleic acid
Ds	Double strand
DSB	Double strand break
DSBH	Double stranded β-helix
DSBR	Double strand break repair
dUTPase	Deoxyuridine triphosphate hydrolase
E. coli	*Escherichia coli*
EMSA	Electrophoretic mobility shift assay
EndoIII	Endonuclease III
EndoIV	Endonuclease IV
EndoVIII	Endonuclease VIII
EYFP	Enhanced yellow fluorescent protein
FAP	Familial adenomatous polypsis
Fapy	Formamidopyrimidine
Fen1	Flap endonuclease 1
Fpg	Formamidopyrimidine-DNA glycosylase
FTO	Fat mass and obesity associated

GGR	Global genome repair
H2TH	Helix two turn helix
hABH1-8	Human AlkB homolog 1-8
HhH	Helix hairpin helix
HIGM2	Hyper immunoglobulin M2
HmU	Hydroxymethyl uracil
hMYH	Human MutY homolog
hNEIL1-3	Human Nei Like 1-3
hNth	Human Endonuclease III
hNTH1	Human Nth homolog 1
hOGG1	Human OGG1
HR	Homologous recombination
hSMUG1	Human SMUG1
HSV1	Herpes Simplex Virus type 1
hUNG	Human UNG
Ig	Immunoglobulin
kDa	Kilo Dalton
MAP	MYH-associated colon cancer
MBD4	Methyl binding domain protein 4
MED1	Methyl-CpG binding endonuclease 1
MIG	Mismatch repair glycosylase
MMR	Mismatch repair
MRN	Mre11/Rad50/Nbs1
mRNA	Messenger ribonucleic acid
Mth1	MutT homolog 1
MUG	Mismatch-specific uracil-DNA glycosylase
NBS	Nijmegen breakage syndrome
Nei	Endonuclease VIII
NEIL	Endonuclease VIII like
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
N-terminus	Amino terminus
Nth	Endonuclease III

Ogg1	8-oxoguanine-DNA glycosylase
PARP-1	Poly(ADP-ribose) polymerase-1
PCNA	Proliferating cell nuclear antigen
PNK	Polynucleotide kinase
RFC	Replication factor C
RNA	Ribonucleic acid
RPA	Replication protein A
SAM	S-adenosyl methionine
SAXS	Small angular x-ray scattering
SBR	Strand break repair
SHM	Somatic hyper mutation
SMUG1	Singel-strand-selective monofunctional uracil-DNA glycosylase
Ss	Single strand
SSBR	Single strand break repair
TAG	3-methyladenine-DNA glycosylase
TCR	Transcription coupled repair
TDG	Thymine-DNA glycosylase
Tg	Thymine glycol
tRNA	Transfer ribonucleic acid
TTD	Trichothiodystrophy
UDG	Uracil-DNA glycosylase
UNG1	Uracil-DNA *N*-glycosylase 1 (mitochondrial form)
UNG2	Uracil-DNA *N*-glycosylase 2 (nuclear form)
UV	Ultraviolet light
X. laevis	*Xenopus laevis*
XP	Xeroderma pigmentosum
XRCC1	X-ray Repair Cross-Complementing protein 1
xSMUG1	Xenopus SMUG1
εA	$1,N^6$-ethenoadenine
εC	$3,N^4$-ethenocytocine
βFU	1-(2´-deoxy-2´-fluoro-β-D-arabinofuranosyl)-uracil
1-meA	1-methyladenine

1-meG	1-methylguanine
2OG	2-oxoglutarate
3-meA	3-methyladenine
3-meC	3-methylcytosine
3-meT	3-methylthymine
5-meC	5-methylcytosine
5-OHC	5-hydroxyuracil
6-4PP	6-4 photoproducts
7-meG	7-methylguanine
8-oxoG	7,8-dihydro-8-oxoguanine

Introduction

In contrast to the general belief established by pioneering molecular biologists, the essential information-storing molecule for life (DNA) is constantly damaged. The most common type of DNA damage is base damage, which occurs at the rate of several thousand base pairs per cell per day in humans (Lindahl, 1993). Damage can stem from numerous sources: by-products of normal aerobic respiration, environmental chemicals found in cigarette smoke or chemical drugs, ultraviolet (UV) light or ionizing radiation (IR). To maintain the genetic information, cells have evolved efficient, specific means to repair DNA damage. The focus of this thesis is on the structural biochemistry of the DNA glycosylase (or glycosylase/AP lyase) step that catalyzes the key damage recognition and excision activities of the Base Excision Repair (BER) pathway and enzymes that perform Direct Damage Reversal (DDR). The following introduction is to a large extent based on the review paper entitled "DNA base damage recognition and removal: New twists and grooves" by Joy L. Huffman, Ottar Sundheim and John A. Tainer published in Mutation Research in 2005 (Huffman et al., 2005) with current updates in the field, particularly with respect to the structure-function studies on the human uracil-DNA glycosylases and the AlkB family of enzymes.

Repair pathways and single step repair

To deal with the wealth of DNA damage, cells have evolved several repair pathways that maintain the integrity of the genome. The diversity of repair mechanisms in each cell is mirrored in the number of gene products involved, e.g. in humans there are more than 150 known proteins known to be involved in repair pathways of DNA ((Friedberg et al., 2006), and "Human DNA Repair Genes" at http://www.cgal.icnet.uk/DNA_Repair_Genes.html). DNA repair pathways may be divided into 5 major pathways: Base Excision Repair (BER), Mismatch Repair (MMR), Nucleotide Excision Repair (NER), Single Strand Break Repair (SSBR), and Double Strand Break Repair (DSBR). In addition, at least three families of repair enzymes (oxidative demethylases, methyltransferases and photolyases) repair DNA damages by Direct Damage Reversal (DDR). However, the complexity of the different repair

pathways is more comprehensive, hence a more detailed division of the main groups is more explanatory and descriptive. Here, I will briefly outline the different main DNA repair pathways/step and defined subgroups thereof.

Base Excision Repair

BER occurs in two stages: an initial, damage-specific stage carried out by a monofunctional DNA glycosylases or glycosylase/AP lyases targeted to distinct base lesions, and a damage-general stage during which the resulting abasic (AP) site intermediates and 3′-termini are processed, followed by DNA repair synthesis and ligation (Fortini and Dogliotti, 2007; Hitomi et al., 2007; Krokan et al., 2000) (Figure 1). Initiation of BER is the committed step, in that each subsequent step produces another form of DNA damage, such as an AP site, until repair is completed. In the case of monofunctional DNA glycosylases, the recognized damaged base is removed. Then a separate enzyme with AP-lyase activity acts upon the AP site and cleaves the DNA backbone. Bifunctional DNA glycosylase/AP lyases combine the first two functional steps within a single enzyme. The damage-general stages of BER require a repair DNA polymerase to insert the proper deoxymononucleotide, and finally the nick in DNA is sealed by DNA ligase to complete repair of the damaged base site. All these enzymes represent essential steps in BER and are frequently conserved from *E. coli* to humans, as well as in archea. Base excision repair may, after removal of the damaged base and incision of the DNA backbone, proceed via a short patch repair pathway (replacement of damaged nucleotide exclusively) or a long patch repair pathway (replacement of 2-8 nucleotides). Removal by short patch or long patch strategies is dependent on the initial glycosylase enzyme acting upon the damage, chemical modifications of the AP sites and possibly the stage of the cell cycle (reviewed in (Krokan et al., 2000)).

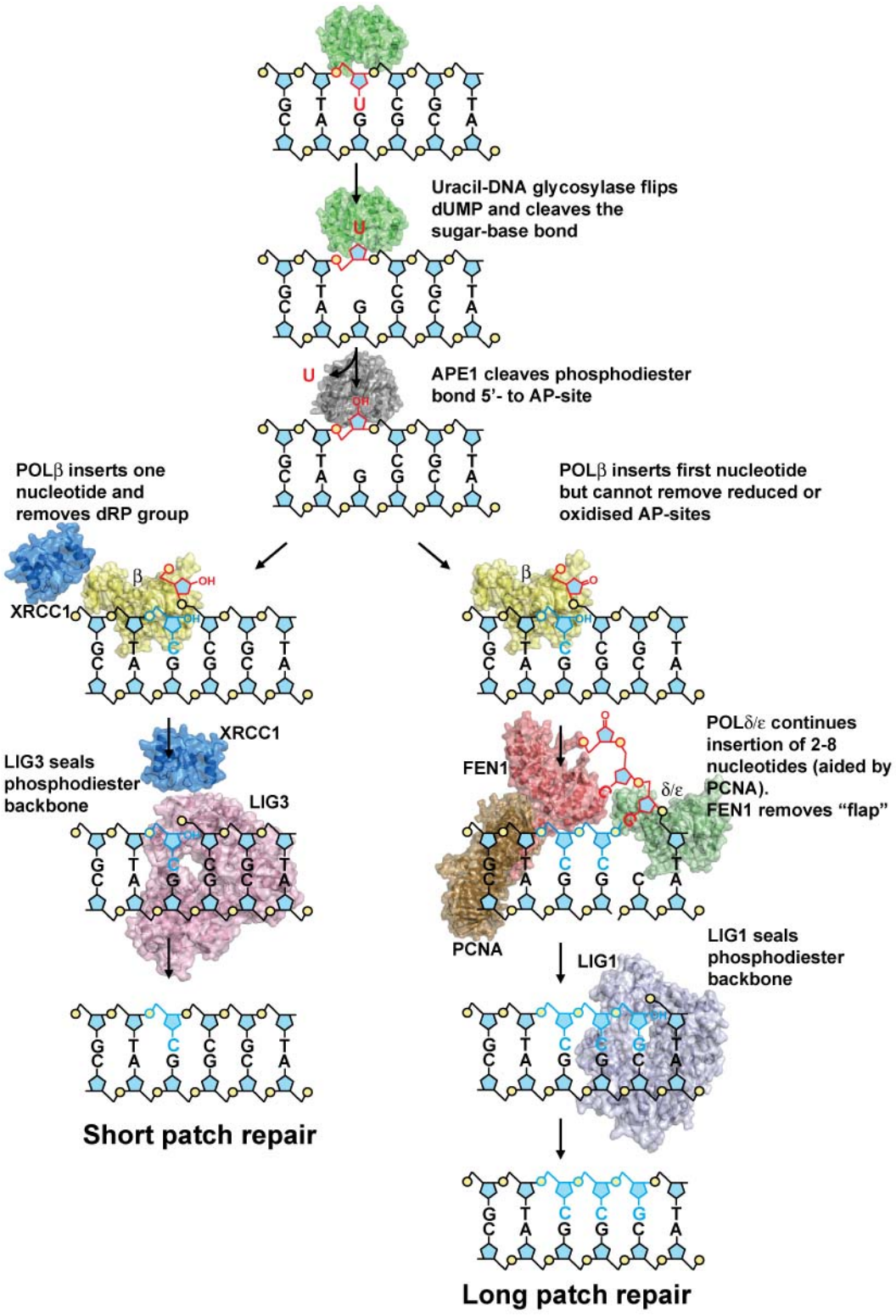

Figure 1: Schematic overview of short- and long patch repair of uracil initiated by UNG. The simplified figure illustrates the sequential enzymatic steps in the repair process and only the core factors are included. The proteins are exemplified by using the crystal structures of UNG (PDB ID 1AKZ), APE1 (PDB ID 1DEW), BRCT

domain of XRCC1 (PDB ID 1CDZ), Polβ (PDB ID 1BPX) to illustrate Polβ and Polδ/ε, Fen1/PCNA (PDB ID 1UL1), and DNA ligase 1 (PDB ID 1X9N) to illustrate DNA lig 1 and 3.

Nucleotide Excision Repair

Two NER subpathways, Global Genome Repair (GGR) and Transcription Coupled Repair (TCR), remove a variety of lesions that significantly distort the DNA helix throughout the genome and in the transcribed strand in transcriptionally active genes, respectively. These lesions are generally too large to be accommodated within the active sites of DNA glycosylases. The lesions are instead removed as a part of a larger oligonucleotide. Defects in NER are linked to three rare autosomal-recessive inherited syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD). Lesions that are repaired by NER include the UV-induced cyclobutane pyrimidine dimers (CPD) and (6-4)-photoproducts (6-4PP), in addition to lesions induced by polycyclic aromatic hydrocarbons found in e.g. tobacco smoke and DNA crosslinks introduced by agents such as the chemotherapeutic agent cisplatin (reviewed in (Leibeling et al., 2006)). NER is a multistep process involving at least 20-30 proteins. The general mechanism may be divided in 5 distinct events: 1) damage recognition; 2) strand segregation; 3) strand incision on both sides of the lesion; 4) removal of the ~28-mer single-stranded oligonucleotide; and 5) gap filling using the undamaged strand as template. The two subpathways differ only in the first step. In GGR a heterotrimeric complex of XPC, HHR23B and centrin2 recognizes the damage and recruits downstream NER factors. This heterotrimeric complex is, however, dispensable in TCR where the stalled RNA polymerase II complex seems to be the responsible sensor for attracting downstream NER components to the lesion ((de Boer and Hoeijmakers, 2000) and references within).

Mismatch Repair

Although the 3´-5´ proofreading activity of the primary DNA polymerase δ corrects at least 99% of its own errors, the remaining mispaired nucleotides as well as DNA bubble structures resulting from “slippage” at repeated sequences must be

repaired to maintain genomic stability. Thus, the mismatch repair system has been conserved through evolution to correct such replication-produced errors. The MMR pathway may be divided into four events: 1) recognition of the mismatch damage by MutS proteins; 2) recruitment of repair enzymes; 3) excision of the new-synthesized strand containing the mismatch lesion; 4) resynthesis by DNA polymerase using the parental strand as a template (Jascur and Boland, 2006). In humans, the damage is recognized by heterodimers that are homologs of *E. coli* MutS (hMSH2/6 or hMSH2/3). Recently, Modrich and colleagues reported that the human homolog of *E. coli* MutLα, the heterodimer consisting of hMLH1/hPMS2, possessed endonuclease activity that produces several nicks at both sides of the mismatch damage. The nicks 5′ of the mismatch serve as entry sites for MutS activated ExoI that removes the new synthesized daughter strand over the damage site in a 5′-to-3′hydrolytic reaction (Kadyrov et al., 2006). Inactivation the human MMR proteins MSH2, MSH6, and MLH1 are responsible for over 95% of all known HNPCC/Lynch syndrome-associated mutations. Furthermore, mutations in PMS2 are associated with Turcot syndrome and early onset colorectal cancer (Peltomaki, 2005).

Single Strand break repair

Unmodified single strand breaks (SSB) produced by DNA damaging agents are suitable for direct rejoining by DNA ligases. However, by the reaction of reactive oxygen species the 3′end of the single strand breaks may be modified and these are subsequently not substrates for direct repair by DNA ligases. In addition, ionizing radiation (IR) produces base damage in close proximity of SSBs. The major pathway in repair of modified SSBs is BER, which itself is a major source of SSBs. (Dianov and Parsons, 2007). Notably, incomplete BER in the vicinity of SBR could result in the more dangerous double strand break lesion. Poly(ADP-ribose) polymerase-1 (PARP-1) binds different types off SSBs (Parsons et al., 2005) and recruits repair proteins through its interaction with XRCC1 (X-ray Repair Cross-Complementing protein 1) (Masson et al., 1998). Depending of the nature of the SSB, the damage may be processed to generate 3′-hydroxyl and 5′-phosphate termini prior to initiating repair mechanisms. It is known that APE1, polynucleotide kinase (PNK), tyrosyl-

DNA phosphodiesterase 1 (TDP-1), and aprataxin are all important proteins in processing of modified SSBs (reviewed in (Dianov and Parsons, 2007)).

Double strand break repair

Double strand breaks (DSBs) are introduced in cells from ionizing radiation, free radicals, chemicals, and during replication at SSBs (Friedberg et al., 2006). Repair of DSBs is critical to life, as they are one of the most toxic and mutagenic DNA lesions in human cells. DSB may, if not repaired properly, give rise to chromosomal breakage. There are two main pathways in repair of DSBs, and they are usually described as non-homologous end joining (NHEJ) and homologous recombination (HR) (reviewed in (Helleday et al., 2007). The choice of preferred pathway in repair of DSBs depends on the stage of the cell-cycle. NHEJ is dominating in G1, whereas HR is more prominent in the S and G2 phases (Essers et al., 2000; Liang et al., 1998; Takata et al., 1998).

NHEJ is template independent since the enzymes involved in the repair bring the DNA termini together and join them without the need for homology. Repair by NHEJ may be divided mechanistically into 4 steps: 1) DSB detection; 2) formation of DNA-protein complex bridging the two ends; 3) processing of non-complementary or damaged ends; and finally 4) ligation (Weterings and van Gent, 2004). The central enzymes acting in NHEJ repair are the Ku70/80 heterodimer, DNA-dependent protein kinase catalytic subunit (DNA-PK_{CS}), and DNA ligase IV in complex with the co-factors XRCC4 and XLF (Helleday et al., 2007). Some DNA termini require processing prior to ligation and enzymes like PNK (Chappell et al., 2002), Artemis (Ma et al., 2002), and WRN (Perry et al., 2006) have been reported to be involved in NHEJ. In addition, several DNA polymerases, such as pol μ and pol λ that can fill in 5′ single-stranded extensions, are also suggested to be involved in NHEJ (Nick McElhinny et al., 2005). The DNA end-processing may lead to either loss or gain of nucleotides, making NHEJ a less accurate pathway than HR for repair of DSBs.

Whereas NHEJ often is error-prone, HR is “error-free” using a template, preferably in the sister chromatid, to resynthesize damaged or missing DNA. HR is initiated by 5′-3′ DNA degradation of DNA ends, leaving 3′ single-stranded overhangs of typically several hundred base pair in length. These overhangs can then

invade and displace the original strands of a homologous sequence creating a heteroduplex. The subsequent steps of homology directed repair are redirected into either synthesis-dependent strand annealing pathway, double-Holliday junction model for DSB repair, single-strand annealing in repair of two-ended DSBs, or repair of broken replication fork by one-ended double-strand break repair ((Helleday et al., 2007) and see http://web.mit.edu/engelward-lab/animations.htm for animations). The initial resection step is common for all the subpathways in HR and the Mre11/Rad50/Nbs1 (MRN) complex that localizes to DSBs may execute this process (Helleday et al., 2007). Mutations in the Nbs1 protein are responsible for the Nijmegen breakage syndrome (NBS), a rare genetic disorder that results in developmental defects, microcephaly, immune deficiency, and high incidence of cancer (The International Nijmegen Breakage Syndrome Study Group 2000; Matsuura et al., 1998; Varon et al., 1998). Furthermore, mutations in the Mre11 gene have been reported to cause ataxia-telangiectasia-like disease (ATLD), a genetic disorder with a phenotype similar to ataxia-telangiectasia (AT) and NBS (reviewed in (Petrini, 2000)). The complete biological role of the MRN complex in HR has not been completely sorted out. However, it is generally accepted that the MRN complex is bridging the two dsDNA ends, thereby preventing separation of chromosomes, as first suggested by Hopfner *et al*., (Hopfner et al., 2000a; Hopfner et al., 2000b). The 3´ ssDNA overhang is protected by RPA (replication protein factor A) (Sugiyama et al., 1997). RPA is displaced by the strand exchange protein Rad51, aided by Rad52, Rad55/57 heterodimer, Rad54, and the breast cancer susceptibility protein BRCA2, generating a nucleoprotein filament that invades homologous segments (West, 2003).

Direct Damage Reversal

DDR does not rely on cascades of enzymatic events; instead the DNA lesion is repaired in a single step by a single protein. To date, four different systems for DDR have been described in the literature: 1) light-dependent reversal of UV-induced *cis-syn* pyrimidine dimers by photolyases identified in almost all living organisms exposed to light except for placental mammals like mice and humans (Essen and Klar, 2006); 2) one-step ligation of simple single-strand breaks (described above); 3) direct reversal of O^6-alkylguanine and O^4-alkylthymine lesions in a stoichiometric suicide reaction by DNA alkyltransferases such as the human AGT (O^6-alkylguanine-DNA

alkyltransferase) (Daniels and Tainer, 2000; Pegg, 2000); and 4) enzymatic removal of the methyl group from 1-methyl adenine (1-meA) and 3-methyl cytosine (3-meC) by proteins in the AlkB family, as recently reviewed in Falnes *et al*., 2007 (Falnes et al., 2007), and demethylation of 3-methyl thymine by the FTO (fat mass and obesity associated) dioxygenase enzyme (Gerken et al., 2007).

DNA glycosylases and direct damage repair enzymes

The efficiency of BER and DDR relies upon the remarkably specific detection and removal of damaged bases in the context of an enormous background of normal DNA. The elucidation of the initial steps of BER and direct damaged base repair has provided critical insights into protein-DNA interactions and chemistry with broad and profound impacts on our understanding of biochemistry, cell biology and life itself. For example, the DNA glycosylases that initiate BER must be exquisitely specific even for sometimes single-atom modifications. Furthermore, they must flip damaged nucleotides ~180° out of the base stack into damage-specific pockets, and use unique DNA binding motifs to initiate a choreographed and perhaps coordinated handoff of damaged DNA intermediates to downstream components ((Allinson et al., 2004; Dianova et al., 2001; Levin et al., 2000; Wong and Demple, 2004) and paper 2 (Pettersen et al., 2007)). Experimental characterization of base repair processes are providing critical understanding of structural biology at escalating levels of complexity, from DNA base damage and protein-DNA complexes to dynamically assembled macromolecular machines and even to the level of understanding how single-site mutations can lead to diseases such as cancer. I will in the following sections give an overview of structure-function relationships of the known human DNA glycosylases and direct damage repair enzymes.

DNA glycosylase structural families

Crystal structures have been determined for several DNA glycosylases, allowing for classification into structural families by architectural folds: helix-hairpin-helix (HhH), helix-two-turn-helix (H2TH), and uracil-DNA glycosylases (UDGs) (Figures 2, 3, and 4). However, overall folds do not provide mechanistic details

regarding the varying specificity of these enzymes, and, hence, a full understanding of individual family members requires detailed structural and biochemical analyses, particularly of enzyme:DNA complexes. In addition, each enzyme is regulated and targeted differentially, and the structural basis for these differences must lie in subtle changes in protein surfaces and protein–DNA conformations that are not yet well understood.

The Helix-hairpin-Helix motif

The helix-hairpin-helix (HhH) motif was first discovered in *E. coli* EndoIII (Endonuclease III or Nth) (Thayer et al., 1995) as a sequence-independent DNA binding motif. The HhH motif is a major motif for sequence independent DNA binding proteins that is present in a superfamily of glycosylases, including EndoIII, AlkA (3-methyladenine-DNA glycosylase II), and Ogg1 (8-oxoguanine glycosylase), which remove a broad spectrum of oxidized and alkylated base lesions (Bruner et al., 2000; Kuo et al., 1992; Thayer et al., 1995; Yamagata et al., 1996). Structures of a number of HhH-containing DNA glycosylases have been determined (Figure 2) (Bruner et al., 2000; Drohat et al., 2002; Eichman et al., 2003; Fromme et al., 2004; Guan et al., 1998; Labahn et al., 1996; Lingaraju et al., 2005; Mol et al., 2002; Vassylyev et al., 1995; Yamagata et al., 1996). Structural studies on bacterial EndoIII, AlkA, MutY, and human Ogg1 (hOGG1) in complex with DNA have shown that their HhH motifs participate in DNA recognition through interaction with phosphate and oxygen atoms of the DNA backbone (Bruner et al., 2000; Fromme et al., 2004; Fromme et al., 2003; Hollis et al., 2000; Lau et al., 1998). The HhH motif has also been found in a number of other proteins that bind DNA in a sequence-independent manner, such as DNA polymerase β and NAD^+-dependent DNA ligase (Doherty et al., 1996).

The core fold of the HhH-containing glycosylases is comprised of two α-helical N- and C-terminal domains. The N-terminal domain typically has four α helices and the C-terminal domain has six to seven α helices. A number of these helices point their helical N-termini, and thus positively charged dipoles, toward the DNA binding site, located at the cleft between the two domains. Cocrystal structures

have revealed that the DNA is bent 60-70° at the lesion site by HhH-containing enzymes.

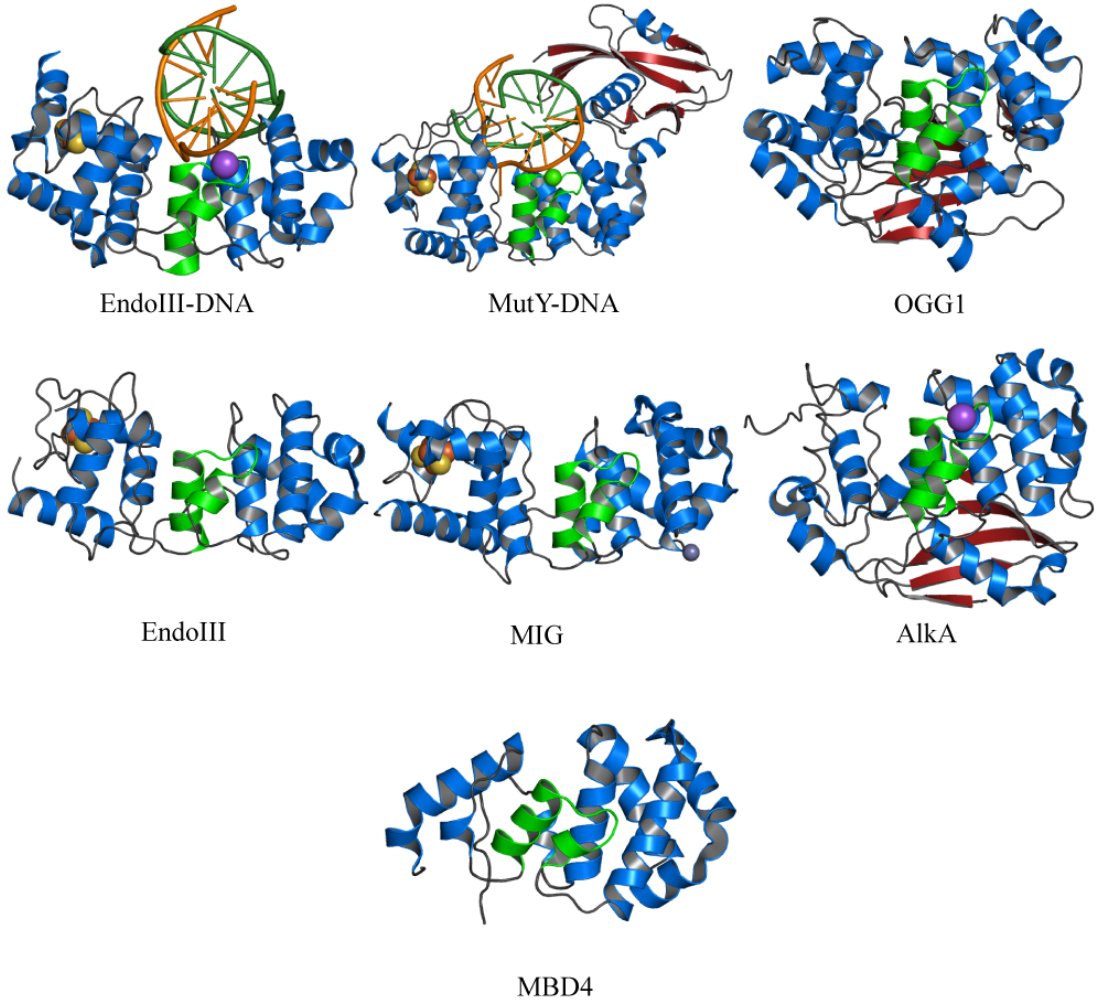

Figure 2: Representative structures of selected HhH superfamily members. HhH motifs are colored green, α helices are blue, β strands dark red, the lesion strand is orange, and the complementary strand is dark green. The DNA binding cleft is located between the N- and C-terminal domains for EndoIII (PDB ID 1P59), and DNA is almost encircled by addition of the MutT domain in MutY (PDB ID 1RRQ). The iron sulfur clusters in the DNA-bound EndoIII and MutY structures and in the apo structures of EndoIII (PDB ID 2ABK) and MIG (PDB ID 1KEA) are displayed as mustard and dark oranged colored spheres. AlkA (PDB ID 1DIZ) and OGG1 (PDB ID 1KO9) have additional β sheet containing domains. The HhH motifs of EndoIII, MutY and AlkA bind magnesium-, calcium-, and sodium-ions, respectively. MBD4 (PDB ID 1NGN) display only limited structural homology with the other HhH family members outside the HhH motif.

The HhH motif itself, also found near the cleft, is composed of two α helices that cross at a conserved angle and are linked by a type II hairpin. Specificity of base removal strongly correlates with the amino acid sequence within this motif. In most HhH-containing glycosylases of known structure, the hairpin loop shows strong sequence conservation, with consensus sequence L/F-P/K/H-G-V/I-G-K/R/T (Doherty et al., 1996). The HhH structural element is followed by a Gly/Pro-rich loop and a highly conserved aspartate (referred to a GPD element). The aspartate is proposed to activate nucleophilic attack of the scissile glycosylic bond. TAG (3-methyladenine glycosylase) and AGOG (archaeal GO glycosylase) are HhH proteins (Drohat et al., 2002; Lingaraju et al., 2005), yet this was an unexpected feature in both due to low sequence homology with other HhH enzymes of known structure.

The HhH DNA glycosylase structures show a variety of small additions, such as an [Fe_4-S_4] iron sulfur cluster in EndoIII, MutY (adenine DNA glycosylase) and MIG (thymine DNA glycosylase), a MutT-like domain in MutY, a β sheet in AlkA and hOGG1, a zinc-binding domain in TAG, and a methyl-CpG binding domain in MBD4 (methyl-CpG binding domain protein 4). The iron sulfur cluster found in EndoIII-like enzymes is involved in DNA binding (Fromme et al., 2003). The loop extending from the iron sulfur cluster protrudes into the minor groove of DNA and interacts with the HhH motif in DNA binding and damage recognition. Similar types of interactions involving different loop structures have been reported in other DNA glycosylases; i.e. the asparagine loop of hOGG1 or the leucine wedge loop of AlkA, which intercalate into the minor groove of DNA (Bruner et al., 2000; Lau et al., 1998). The MutT-like domain of MutY recognizes 8-oxoG opposite adenine to be excised (Fromme et al., 2004).

The Helix-Two Turn-Helix motif

The helix-multi turn-helix motif was first discovered in the flap endonuclease 1 (FEN1) structure (Hosfield et al., 1998), but it also occurs in several DNA glycosylases as a prevalent helix-two turn-helix (H2TH) motif. Family members to date include bacterial EndoVIII (Endonuclease VIII or Nei), MutM/Fpg, and the mammalian Nei-like proteins (NEIL1, NEIL2, and NEIL3), and representative

structures have been determined for each subgroup (Figure 3) (Doublie et al., 2004; Fromme and Verdine, 2002, 2003a; Gilboa et al., 2002; Serre et al., 2002; Sugahara et al., 2000; Zharkov et al., 2002). Interestingly, these enzymes catalyze similar mechanisms of base removal and backbone cleavage as the HhH enzymes, although they use a completely different molecular scaffold.

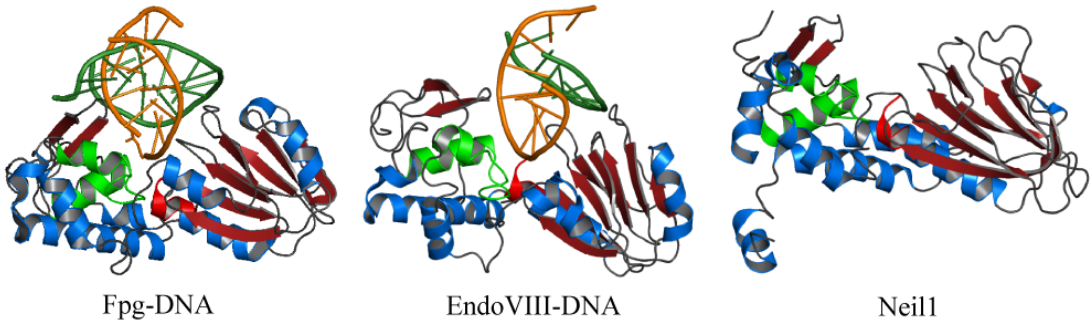

Figure 3: Structures of the H2TH family of DNA repair glycosylases, represented by the DNA bound Fpg (PDB ID 1KFV) and EndoVIII (PDB ID 1K3W) and the apo structure of Neil1 (PDB ID 1TDH). H2TH motifs are colored green, α helices are blue, β strands dark red, the lesion strand is orange, and the complementary strand is dark green. The six N-terminal residues are colored red.

The overall topology of these enzymes is conserved across the glycosylase family. Similar to the HhH proteins, N- and C-terminal proteins have conserved amino acids at positions 1-6, followed by two 4-stranded β sheets that form an antiparallel β sandwich flanked by two helices. The C-terminal domain contains the H2TH motif and is helix-rich, with the zinc finger contributing the only two β strands. The β-hairpin loop of the zinc finger motif intercalates into the minor groove of DNA. Positively charged residues line the surface of the cleft to create an electrostatically positive surface for DNA binding, rather than the helix dipoles used by the HhH enzymes. Interestingly, the structure of the catalytic core of NEIL1 revealed “a zinc-less finger” in which the structural motif was conserved despite lacking a true zinc binding site (Doublie et al., 2004); however, NEIL2 has been shown to bind zinc as observed in the other families members (Das et al., 2004). The H2TH motifs are used in a manner similar to the HhH, namely recognizing DNA through interactions with the backbone.

The Uracil-DNA Glycosylase Superfamily

The uracil-DNA glycosylases (UDGs) comprise a prominent and highly important glycosylase structural superfamily, as UDGs are the major enzymes that recognize and initiate repair of uracil resulting from both misincorporation of dUTP and cytosine deamination in DNA. Five distinct families have been identified to date with confirmed glycosylase activity (Pearl, 2000; Sartori et al., 2002). Although the families share limited sequence similarity, structures have revealed that they possess a common core fold. Family 1, typified by the uracil-DNA *N*-glycosylase (UNG) enzyme and its close orthologs are highly conserved DNA glycosylases. Family 2 enzymes, the bacterial MUG (mismatch-specific uracil-DNA glycosylase) and the eukaryotic homolog thymine-DNA glycosylase (TDG), initiate BER of G:U/T mismatches. Single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) comprises the third enzyme class in the UDG superfamily. The families 4 and 5, identified exclusively in thermophilic organisms, contain iron sulfur clusters and possess relative high sequence similarity to each other. However, family five is distinct from the other UDGs since it lacks a polar active site residue that mediates the hydrolysis of the glycosylic bond (Kosaka et al., 2007; Sartori et al., 2002).

Representatives from all five families have been structurally characterized: UNG proteins from human, Herpes Simplex Virus type 1 (HSV1), *E. coli, Deinococcus radiodurans*, Epstein-Barr vírus, and vaccinia vírus (Geoui et al., 2007; Leiros et al., 2001; Leiros et al., 2005; Mol et al., 1995; Savva et al., 1995; Schormann et al., 2007; Xiao et al., 1999); MUG/TDG from human, *E. coli,* and *Deinococcus radiodurans* (Baba et al., 2005; Barrett et al., 1998; Moe et al., 2006); SMUG1 from *Xenopus laevis* (Wibley et al., 2003); family 4 UDGa (Hoseki et al., 2003) and family 5 UDGb (Kosaka et al., 2007) from *Thermus thermophilus* HB8 (Figure 4). The topology of the common core of the UDG superfamily consists of a central four-stranded parallel twisted β sheet flanked by α helices (Mol et al., 1995). The β sheet in MUG is extended, with one extra strand oriented in an antiparallel direction at the edge of the sheet (Barrett et al., 1998). A positive DNA binding groove traverses one face of the molecule, where the C-terminal ends of the sheet form the base of the cleft. The uracil binding pocket penetrates back from the groove

into the core of the enzyme. UNG and SMUG1 have an additional small β sheet made up of two and three strands, respectively, located on the larger lobe of the DNA binding cleft (Wibley et al., 2003). MUG also lacks the coil of helices at the N-terminal side present in both UNG and SMUG1. A short helix immediately follows the β2 strand in MUG and SMUG1, and a segment of ~40 residues leading to helix 5 is unique to the SMUG1 fold. The iron sulfur clusters of family four and five are apparently not involved in DNA binding (Kosaka et al., 2007).

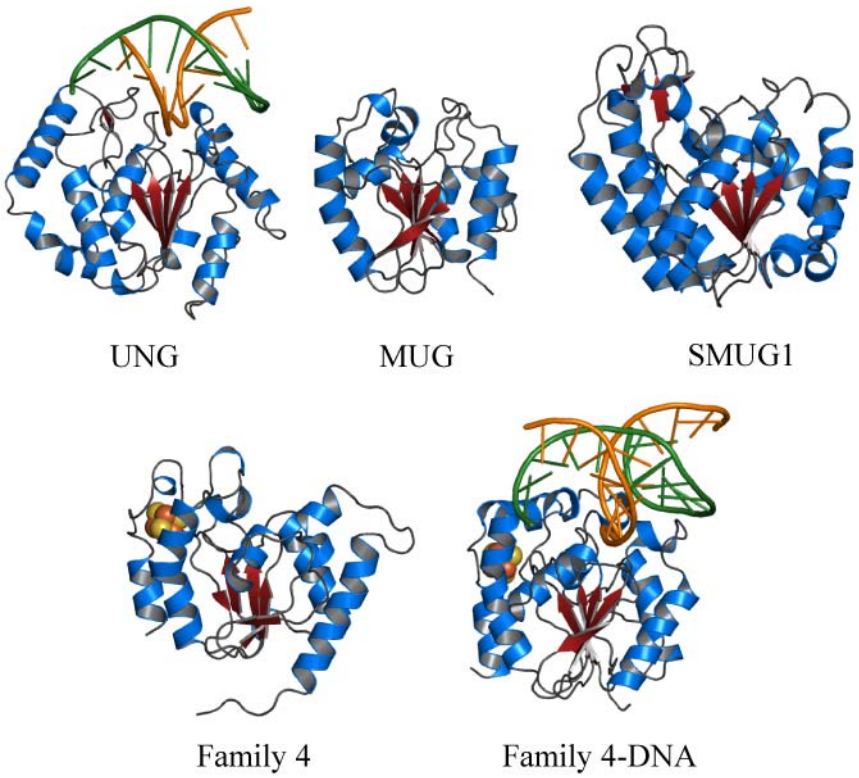

Figure 4: Overall structures of the members in the uracil-DNA glycosylase family. Secundary structure elements are colored blue and dark red for α helices and β strands, respectively. The DNA lesion strand is orange and the complementary strand is dark green in the DNA bound structures of UNG (PDB ID 1SSP) and family 5 UDG (PDB ID 2DDG). Family 2, 3, and 4 are represented by structures of *E. coli* MUG (PDB ID 1MUG), xenopus SMUG1 (PDB ID 1OE5), and thermophilic type four UDG (PDB ID 1UI0). The iron sulfur clusters in family 4 and 5 are displayed as mustard- and dark oranged-colored spheres.

Monofunctional DNA glycosylases and Bifunctional DNA glycosylases/AP lyases

A general mechanistic distinction can be drawn between monofunctional DNA glycosylases and bifunctional DNA glycosylases/AP lyases. Monofunctional DNA glycosylases cleave only the glycosidic bond between N and C1′ and then the product abasic site is acted upon by an AP endonuclease. Bifunctional DNA glycosylases process the abasic site with an AP lyase activity inherent to the glycosylase itself. In either case, the resulting strand break requires further processing by other proteins (lyases and/or nucleases) to remove the sugar-phosphate residue remaining at the 3′- or 5′-end, respectively. Repair is completed by the concerted actions of a DNA polymerase to fill the gap and a DNA ligase to seal the strand (Reviewed in (Fortini and Dogliotti, 2007; Hitomi et al., 2007; Krokan et al., 2000; Memisoglu and Samson, 2000; Seeberg et al., 1995).

Monofunctional glycosylases typically use an activated water molecule as a nucleophile in attacking the C1′ of the target nucleotide, whereas bifunctional glycosylases/AP lyases often use a lysine side chain or an N-terminal proline (Dodson et al., 1994). An intermediate step in the mechanism for AP lyase activity in the bifunctional enzymes is formation of a Schiff base between the nucleophilic lysine or proline and C1′ of the sugar. This Schiff base can be chemically reduced to form a covalently "trapped" complex resembling the Schiff base intermediate (Tchou and Grollman, 1995). This trapping reaction has been instrumental in determination of several enzyme-DNA complex crystal structures (Verdine and Norman, 2003), which have provided much insight into the mechanisms of DNA recognition and AP lyase activity for the bifunctional enzymes (Fromme et al., 2003; Fromme and Verdine, 2002, 2003b; Gilboa et al., 2002; Zharkov et al., 2002). This covalent enzyme-DNA trapping reaction is impossible with monofunctional DNA glycosylases that use an activated water as the attacking nucleophile, but has been observed in some other instances where protein residues act as nucleophiles (Williams and David, 1998).

Common themes for base damage recognition

Despite differences in protein folds and specific residues used to recognize damaged bases, unifying common themes for BER initiation have emerged. Among these, extrahelical flipping of the damaged base into a lesion-specific recognition

pocket is particularly intriguing, as it must rely on an intrinsic property of the damaged DNA. All DNA glycosylases studied to date bind the minor groove, kink DNA at the site of damage, and flip the lesion base out of the DNA major groove. Thus, an initial step in recognition evidently exploits the deformability of the DNA at a base pair destabilized by the presence of the lesion. Each glycosylase is necessarily damage-specific, so only bases that can be accommodated in a defined binding pocket upon nucleotide flipping provide the necessary contacts and orientation for base excision. The critical importance of the extrahelical base binding pocket for glycosylase specificity was first shown by Kavli *et al*., who demonstrated that the uracil pocket in human UNG (hUNG) could be mutated to allow the removal of normal cytosine and thymine bases from DNA (Kavli et al., 1996).

A second emerging theme is a twist about the 3′-phosphate during flipping of some bases, first noticed for the direct damage repair protein AGT (Daniels et al., 2004) but also present in some other enzymes that flip the lesion site extrahelically. Other protein:DNA complexes contain an extrahelical nucleotide and rotated 3′-phosphate, including the DNA glycosylases AlkA (Hollis et al., 2000), hOGG1 (Bruner et al., 2000), MUG (Barrett et al., 1998), hUNG (Parikh et al., 1998), and the endonucleases APE1 (Mol et al., 2000) and EndoIV (Hosfield et al., 1999).

A third, and as yet, incompletely understood feature of DNA glycosylases is their coordination with the enzymes that follow them in the BER pathway. Abasic sites or nicked DNA strands left unrepaired are more cytotoxic than base lesions (Lindahl, 1993), so most glycosylases remain bound to their product until "handed off" to the next enzyme in the pathway. Although the exact nature of this transfer is not clear, it is likely that protein-DNA interaction surfaces play a significant role coupled to protein-protein interactions and steric displacements.

Specific mechanisms for recognition of damage

As new enzyme:substrate DNA structures are accumulated, themes for recognition of specific types of damage as well as discriminating between canonical and non-canonical bases are expected to emerge. These systems are delicately tuned such that canonical bases are largely excluded by steric and hydrogen bonding patterns, with differences such as a single hydrogen bond playing critical roles. As will be discussed in the following sections, recognition of oxidized bases or uracil is

typically achieved by these means, and alkyl adducts are more specified by π-π and cation-π interactions resulting from the delocalized positive charge on the modified base. However, upon examination of the atomic details of base recognition, the theme quickly turns to a lack of discernable theme for recognition. For example, structures of hOGG1, MutM, MutY, and MutT in specific complexes with 8-oxoG reveal that each uses different residues and makes different discriminating contacts to the 8-oxoG base (Figure 5).

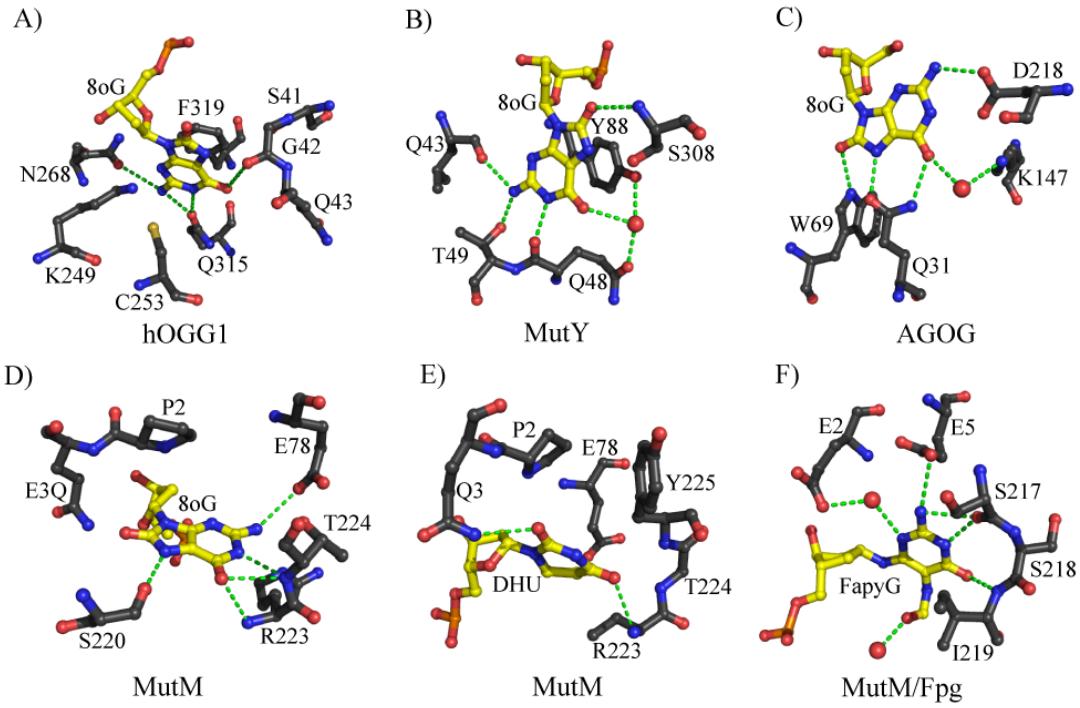

Figure 5: Specific recognition of oxidized bases presented as balls and sticks models. The lesion is colored yellow, amino acids are in grey, and hydrogen bonds are presented as green dashes. A) Recognition of 8oxoG by hOGG1 (PDB ID 1NC3). B) 8oxoG specific binding in MutY (PDB ID 1RRQ). C) 8oxoG recognition in AGOG (PDB ID 1XQP). D)-F) MutM specifically recognizes 8oxoG (D, PDB ID 1R2Y), DHU (E, PDB ID 1R2Z), and FapyG (F, PDB ID 1TDZ).

Oxdation damage

Oxidation of cellular macromolecules occurs at significant frequencies in aerobic organisms due to by-products of normal metabolism and the immune response. Furthermore, the oxidation of both mitochondrial and nuclear DNA has been implicated in human disease and aging. Strand breaks, abasic sites, and oxidized base residues, with 7,8-dihydro-8-oxoguanine (8-oxoG) and 5-hydroxycytosine (5-OHC) representing the most frequent base lesions, can all be caused by oxidative damage (Lindahl, 1993).

DNA glycosylases that remove oxidized base residues can be divided into two functional subgroups: those that repair oxidized purines (e.g. *E. coli* Fpg (formamidopyrimidine-DNA glycosylase) or MutM) and those that repair oxidized pyrimidines (e.g. *E. coli* EndoIII (Endonuclease III or Nth) and EndoVIII (Endonuclease VIII or Nei). In human cells, five DNA glycosylases for removal of oxidized bases have been cloned and characterized: hNth (human EndoIII); hOGG1 (human OGG1), hNEIL1, hNEIL2, (human Nei-like1/2) and SMUG1 (Bjoras et al., 1997; Hazra et al., 2002a; Hazra et al., 2002b; Ikeda et al., 1998; Masaoka et al., 2003; Radicella et al., 1997; Takao et al., 2002; Wibley et al., 2003). hNTH1, hNEIL1, hNEIL2, and hSMUG1 catalyze excision of oxidized pyrimidines, such as 5-OHC, whereas hOGG1 removes oxidized purines, such as 8-oxoG. However, hNEIL1 also appears to be an alternative glycosylase for the removal 8-oxoG. Because hSMUG1 is a member of the UDG family of glycosylases and its major task in cells is to initiate repair of deaminated bases, its structure and base recognition mechanism will be discussed with those enzymes. Each of the human DNA glycosylases involved in repair of oxidized damages, except from hSMUG1, also removes imidazole ring-fragmented Fapy (formamidopyrimidine) residues, which block replication and thereby are cytotoxic.

With the exception of SMUG1, glycosylases that repair oxidative damages fall into two structural families, determined by the presence of either an HhH or an H2TH motif and unrelated to the type of oxidized base recognized. Representative structures have been determined for both families. *E. coli* EndoIII was one of the first DNA repair protein elucidated (Kuo et al., 1992), however, the cocrystal structure with DNA was not determined until quite recently (Fromme and Verdine, 2003b). Other HhH structures for oxidative damage-sensing glycosylases have included hOGG1 and

MutY (Bjoras et al., 2002; Bruner et al., 2000; Fromme et al., 2004; Guan et al., 1998) and those carrying the H2TH motif include MutM, Fpg, EndoVIII, and Neil1 (Doublie et al., 2004; Fromme and Verdine, 2002, 2003a; Gilboa et al., 2002; Serre et al., 2002; Sugahara et al., 2000; Zharkov et al., 2002). Structures determined in the presence of damaged bases have provided invaluable details regarding specific lesion recognition, and these will be discussed briefly according to the human glycosylases in the following sections.

Human 8-oxoguanine-DNA glycosylase 1 (hOGG1)

Several distinct enzymes recognize 8-oxoG in different contexts because it is a major mutagenic base lesion, pairing in *syn* conformation with A rather than in *anti* with C and thereby causing G:C to T:A transversion mutations upon replication (Lindahl, 1993). Mutations in hOGG1 have been linked to lung cancer in humans (Le Marchand et al., 2002; Park et al., 2004). The structure of a catalytically inactive hOGG1 enzyme core bound to 8-oxoG-containing duplex DNA revealed that hOGG1 contains an HhH motif and flips the 8-oxoG base out of the double helix into a specific recognition pocket (Bruner et al., 2000). hOGG1 discriminates 8-oxoG from G using a single hydrogen bond between the Gly42 carbonyl and the purine N7, which is protonated only in 8-oxoG, and no direct contacts are made to the 8-oxo moiety.

hOGG1 is a bifunctional glycosylase/AP lyase, and Lys249 has been proposed to attack C1′ and promote β-elimination. Interestingly, hOGG1 AP-lyase activity is stimulated by free 8-bromoG or 8-aminoG (Fromme et al., 2003; Morland et al., 2005), but inhibited by free 8-oxoG base (Morland et al., 2005). The preference for bases opposite 8-oxoG is C>T>G>>A, and the preference for C was shown to result from specific hydrogen bonds donated by Arg154 and Arg204 to N3 and O2 of the cytosine base. A structure of apo hOGG1 showed that, in the absence of DNA, the overall enzyme conformation is conserved but that catalytic residues, such as Lys249, are positioned improperly for catalysis (Bjoras et al., 2002). Binding of the correct substrate is proposed to be coupled to reorientation of these side chains and subsequent catalysis.

The adenine-DNA glycosylase human MutY homolog (hMYH)

The adenine-DNA glycosylase, MYH, is the human homolog of bacterial MutY and aids the protection against 8-oxoG lesions. MutY and its functional homologues recognizes 8-oxoG:A pairs and excises the misincorporated adenine. Then cytosine can be inserted opposite the 8-oxoG lesion that in *E. coli* is repaired by the functional homolog of OGG1, Fpg (MutM), thus avoiding propagation of a G:C to T:A mutation upon further replication (Michaels and Miller, 1992). hMYH has been implicated in one form of hereditary colon cancer named MAP (MYH-associated colon cancer), that closely resembles familial adenomatous polypsis (FAP) (Lipton and Tomlinson, 2006). The contribution of MYH to the multiple adenoma phenotype was first documented in a study of a Welsh Caucasian sibship with multiple colorectal adenomas and carcinomas but no inherited APC or mismatch repair mutations (Al-Tassan et al., 2002). The third bacterial enzyme in protection of 8-oxoG lesions, MutT, recognizes free 8-oxoG deoxyribonucleotide triphosphates and catalyzes the removal of pyrophosphate, thus preventing misincorporation of this base into DNA (Michaels and Miller, 1992). Mice with MutT homolog (Mth1) deficiency display increased frequency of spontaneous tumors (Tsuzuki et al., 2001), underscoring the importance of averting misincorporation in protecting against 8-oxoG damage.

MutY and its homologues are monofunctional HhH family members with the N-terminal catalytic core holding an iron sulfur cluster that has been proposed to play a role in DNA damage sensing (Boon et al., 2003). The C-terminal domain of MutY is MutT-like (Fromme et al., 2004; Volk et al., 2000), and seems to play an active role in 8-oxoG:A recognition of MutY since truncation of the MutT-like domain resulted in loss of 8-oxoG:A versus G:A mispair discrimination (Gogos et al., 1996; Noll et al., 1999). Structures of MutY have been determined for: 1) the catalytic active core domain alone (Guan et al., 1998); 2) an inactive mutant alone and in complex with free adenine, which is an inhibitory product (Guan et al., 1998); 3) other regulatory and catalytic mutants of the core domain (Zharkov et al., 2000); and 4) full-length MutY crosslinked to DNA containing 8-oxoG base pair (Fromme et al., 2004). In the adenine-bound structure, the base is bound in a deep pocket surrounded by a large positively charged groove, ideal for DNA binding. MutY makes specific hydrogen bonds similar to Watson-Crick base pairing using Arg26 and two water molecules that discriminate against other bases in the adenine binding pocket.

The structure of full-length MutY complexed to DNA has contributed answers to many long-standing questions regarding how the protein specifically recognizes both 8-oxoG and adenine bases (Fromme et al., 2004). The catalytic core and MutT-like domains encircle the DNA strand together. Whereas the adenine is flipped out similarly to its position in the MutY core:adenine structre, the 8-oxoG moiety is not flipped out of the base stack. Although 8-oxoG makes extensive contacts with the MutT-like domain, the contacts are quite different from those with MutT (Massiah et al., 2003). The 8-oxoG *anti* conformation is stabilized in the MutY complex structure rather than the *syn* conformation, which is normally the energetically favored conformer when paired opposite A. The 8-oxoG base is specifically recognized through a hydrogen bond to N7 from the Ser308 hydroxyl group, which in turn, is oriented by a hydrogen bond to the hydroxyl of Tyr88. The O8 atom may also form a hydrogen bond to the backbone amino group of Ser308, although the geometry is not ideal. Notably, the residue in humans corresponding to Tyr88 is equivalent to one of the germline mutations found in hMYH in some MAP patients (Al-Tassan et al., 2002).

Human EndoIII homolog 1 (hNTH1)

The second major DNA glycosylase for correcting oxidized base lesions in human is hNTH1, which was first cloned and characterized a decade ago (Aspinwall et al., 1997; Hilbert et al., 1997; Ikeda et al., 1998; Liu and Roy, 2002). hNTH may initiate BER of a wide range of oxidized pyrimidine derivates, such as thymine glycol (Tg), 5-hydroxycytosine, dihydrouracil (DHU), urea, and at least six other oxidized pyrimidines in addition to AP-sites, and the ring-opened structure of 1, N^6-ethenoadenine reviewed in Hazra *et al*., (Hazra et al., 2007). It has been demonstrated that the structure specific endonuclease XPG both interacts with and stimulates the activity of hNTH1 (Bessho, 1999; Klungland et al., 1999), and, interestingly, cells from patients with Cockayne syndrome due to XPG deficiency possessed reduced global repair of Tg lesions (Klungland et al., 1999). EndoIII contacts the backbone of both the lesion- and the non-lesion-containing DNA strand (Fromme and Verdine, 2003b). In contrast, the structurally related HhH/GPD proteins AlkA and hOGG1 bind only the lesion containing DNA strand. The trapped complex of bacterial EndoIII and DNA also provides structural evidence that the redox-inert iron sulfur

cluster (Fu et al., 1992) is likely to be involved in DNA binding, as suggested by mutagenesis and the apo crystal structure of EndoIII (Thayer et al., 1995).

Human Nei-like proteins 1, 2, and 3 (hNEIL1, 2, and 3)

Three potential human functional orthologs of Endo VIII (Nei) have been identified (Bandaru et al., 2002; Hazra et al., 2002a; Hazra et al., 2002b). DNA glycosylase/AP lyase activity has, however, only been detected in hNEIL1 and hNEIL2. The two enzymes recognize different types of oxidized DNA lesions. Whereas NEIL1 preferably recognize and initiate repair of ring-opened purines-formamidopyrimidine (Fapy)-A and –G and thymine glycol, NEIL2 prefer cytosine-derived lesions, such as 5-hydroxyuracil and 5-hydroxycytosine. A more detailed list of substrates is reviewed in Hazra *et al*., 2007 (Hazra et al., 2007). The Endo VIII proteins are of H2TH fold, and the trapped Nei-DNA complex revealed a MutM like acid/base chemistry by conserved proline and glutamate in the extreme N-terminus (Zharkov et al., 2002). The crystal structure of the enzymatically active C-terminal of hNEIL1 revealed that it possess an internal "Zn-less finger" element (Doublie et al., 2004), in contrast to the C-terminal Zn-holding finger motif present in other Nei orthologs like hNEIL2 (Das et al., 2004). In NEIL1, this element mimics the anti-parallel β-hairpin Zn-finger motif but lacks the loops holding the residues involved in Zn-binding. NEIL2 has higher affinity for DNA bubble structures compared to single or double-stranded DNA (Dou et al., 2003). NEIL1 is furthermore upregulated in S-phase whereas the level of NEIL2 is independent of cell cycle status. Taken together, this implies a role in replication and transcription associated repair for NEIL1 and NEIL2, respectively.

Uracil in DNA

Uracils occur in DNA at a frequency of 100-500 per cell per day (Lindahl, 1993), either by misincorporation of dUMP or by spontaneous deamination of cytosine (Lindahl and Nyberg, 1974; Tye et al., 1977). Deamination of cytosine can induce C:G to T:A transition mutations, as uracil preferentially base pairs with adenine during replication. The human glycosylases that remove uracil are uracil-

DNA *N*-glycosylase (UNG), thymine-DNA glycosylase (TDG), single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1), and methyl-CpG binding endonuclease 1 (MED1/MBD4). Recent research has revealed that UNG plays an important role in the vertebrate specific immune system. It was reported that activated induced deaminase (AID) is required for both somatic hypermutation (SHM) and class switch recombination (CSR), and that it probably works directly on DNA by deamination of cytosine to uracil in the Ig (immunoglobulin) locus, followed by uracil removal by the nuclear UNG2 enzyme (Petersen-Mahrt et al., 2002). Both UNG2 deficiency and AID deficiency are associated with a hyper IgM2 (HIGM2)-like phenotype both in a mouse model (Revy et al., 2000) and in human patients (Imai et al., 2003). In addition, UNG deficient mice were found to have a higher than normal morbidity beyond 18 months of age, accompanied by increased incidence of B-cell lymphonas (Nilsen et al., 2003).

Human uracil-DNA N-glycosylase (hUNG1/2)

UNG initiates base excision repair of uracil from single-stranded DNA and from double-stranded DNA, regardless of whether the opposite base is a G or an A (Paper 1: (Kavli et al., 2002)). UNG enzymes are highly specific for uracil in DNA, with negligible activity against T or C (Kavli et al., 1996) or naturally occurring uracil in RNA (Caradonna and Cheng, 1980; Talpaert-Borle et al., 1982). Other substrates reported for UNG are largely restricted to uracil analogs with minor modifications at the 5 position (Paper 1: (Kavli et al., 2002)). In humans, UNG is targeted to either nuclei (UNG2) or mitochondria (UNG1) by distinct N-terminal pre-sequences generated by alternative splicing and alternative promoter usage in the *UNG* gene (Nilsen et al., 1997). Thus, unique N-terminal regions determine the subcellular localization, while the catalytic domain, whose structure is known, is the same for both nuclear and mitochondrial forms.

Five conserved motifs have been described in Parikh *et al*., (1998) as being important for DNA recognition and catalysis by UNG enzymes: 1) the water-activating loop (144-GQDPYH-148; human UNG1 nomenclature); 2) the 5′-side backbone compression loop (165-PPPPS-169); the uracil recognition region (199-GVLLLN-204); 4) the 3′-side backbone compression loop (246-GS-247); and 5) the minor groove-intercalation loop (268-HPSPLS-273) (Parikh et al., 1998). UNG binds

to DNA using three rigid loops made up of motifs 2, 4, and 5. These three loops largely consist of serine and proline residues, which permit close approach of the polypeptide chain to the DNA backbone. The loops compress the backbone (pinch) and slightly bend the DNA, which becomes fully bent (~45°) and kinked (~2°) when a push from the minor groove intercalation loop and a pull from the complementary uracil specific recognition pocket flip the uridine into an extrahelical position (Parikh et al., 1998; Wong et al., 2002).

The highly conserved substrate-binding pocket provides the shape and electrostatic complementarity to fit uracil in an extrahelical conformation, but is too narrow to accommodate purines (Figure 6). Selection against thymine and 5-methylated pyrimidines is provided by the side chain of a tyrosine (Tyr147 in human UNG1). Specific hydrogen bonds provide discrimination against cytosine. The O2 carbonyl of uracil hydrogen bonds to the UNG main chain NH that joins a conserved Gly-Gln sequence (Gly143-Gln144). The amide side chain of a conserved asparagine (Asn204) makes specific hydrogen bonds to N3 and O4 of uracil, whereas cytosine is excluded by unfavorable interactions with its exocyclic amine N4. A water cluster at the base of the uracil-binding pocket provides interactions that fix the proper orientation of the amide group (Pearl, 2000).

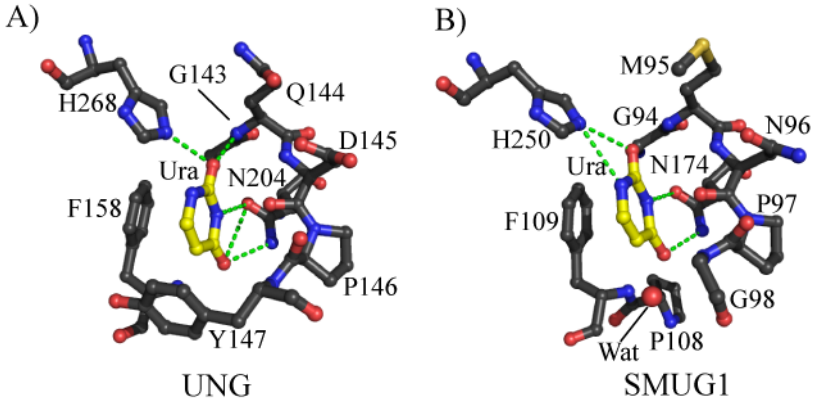

Figure 6: Specific recognition of uracil in A) UNG (PDB ID 1SSP) and B) SMUG1 (PDB ID 1OE5). The balls and sticks figures show the uracil lesion in yellow and active site residues in grey. The water molecule thought to be responsible for thymine rejection in the active site in SMUG1 is shown as a red sphere.

Human thymine-DNA glycosylase (TDG)

Human TDG (thymine-DNA glycosylase) was first discovered for its ability to hydrolyze the *N*-glycosidic bond of deoxythymidine in T:G mismatches (Brown and Jiricny, 1987). T:G mismatches in DNA arise mainly as a result of deamination of 5-methylcytosine (5-meC). TDG was later shown to remove thymine from C:T and T:T mismatches, but much less efficiently. More importantly, it removes uracil mispaired with guanine with ~10-fold higher activity than thymine (reviewed in (Cortazar et al., 2007; Hardeland et al., 2001)). Mismatch uracil-DNA glycosylase (MUG), the bacterial ortholog of human TDG, only removes uracil and not thymine mismatched with guanine (Gallinari and Jiricny, 1996). *E. coli* MUG is closely related to TDG (37% sequence identity), and the crystal structures reveal that they share the same core fold (Baba et al., 2005; Barrett et al., 1998). Whereas UNG is substrate specific, TDG is reported to excise a broad range of base lesions, such as uracils with modifications or substituents at the 5-carbon position, the cyclic alkylation product 3,N^4-ethenocytosine (εC), deaminated purines (e.g. hypoxanthine) and thymine glycol (a comprehensive list of TDG/MUG substrates is recently given in (Cortazar et al., 2007)). Remarkably, TDG can also remove 5-fluoruracil from single-stranded DNA, which was unexpected since TDG is double-strand specific for all other substrates tested.

The crystal structures of MUG (Barrett et al., 1998; Barrett et al., 1999; Pearl, 2000) reveal a similar overall fold as the family 1 UDGs. Two highly conserved motifs in UNG have topological and conformational equivalents in MUG: the water activating loop (GQDPY) and the minor groove-intercalating loop (HPSPLS). The corresponding motifs in MUG are 16-GINPGL-20 (identical in human TDG) and 140-NPSGLS-145 (MPSSSS in human TDG). The latter motif forms specific hydrogen bonds with the orphan guanine in a configuration that mimics Watson-Crick base pairing and constitutes the basis of the mismatch specificity (Barrett et al., 1999). The catalytic residues (underlined in the sequences above) in UNG are in both cases replaced by asparagines in *E. coli* MUG. The aspartate in the first motif in UNG activates a water molecule for nucleophilic attack on the C1´ of the deoxyribose. The asparagine in MUG and TDG in MUG cannot activate nucleophilic water, although a water molecule is found in almost the same position as seen in UNG.

The tyrosine residue that provides the barrier against thymine in UNG is replaced by a glycine (Gly20) in MUG. The preference for U:G over T:G in MUG is likely conferred by the position of a hydroxyl group in Ser23, which would clash with the 5′-methyl-group of thymine. In TDG, the residue corresponding to Ser23 is an alanine. The smaller alanine side chain allows better accommodation of thymine, explaining the increased specificity of TDG for T. The TDG/MUG specificity for G:U/T mispairs over canonical G:C base pairs results not from the scissile base itself as in the family 1 enzymes. Conversely, it results from a combination of the ease of flipping out a base from an unstable pair compared to flipping from a Watson-Crick C:G pair and from the deformability of DNA at non-canonical base pairs.

Human single-strand-selective monofunctional uracil-DNA glycosylase (SMUG1)

When first discovered in *Xenopus laevis*, the xSMUG1 enzyme was characterized as single-strand-selective monofunctional uracil-DNA glycosylase (Haushalter et al., 1999). However, in the initial characterization of xSMUG1 the strong product inhibition by the abasic site was not taken into account. In fact, human SMUG1 removes uracil efficiently from both U:G mismatches and U:A base pairs (Paper 1: (Kavli et al., 2002)). More recent studies on the substrate specificity of human and rat SMUG1 have revealed that SMUG1 initiates BER of 5-hydroxymethyluracil, 5-hydroxyuracil, and 5-formyluracil (Boorstein et al., 2001; Masaoka et al., 2003; Matsubara et al., 2003). Removal of these oxidized lesions might, in fact, be the major functions of SMUG1 *in vivo*.

In the crystal structure of xSMUG1 in complex with uracil-containing double stranded DNA, the enzyme had detached from the abasic end-product and rebound to the DNA end prior to crystallization (Wibley et al., 2003). End binding was also observed with the substrate analog βFU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-uracil]. At the 5′-end of the damage-containing strand, a cytosine adopts an extrahelical conformation and points towards the pyrimidine specificity pocket of xSMUG1. Upon replacing the 5′-end cytosine base with βFU, a mixed population of extrahelical cleaved abasic sites and βFU in a productive orientation in the active site was observed. Two motifs, the minor groove intercalating loop (251-PSPRN-255) and the following short α helix unique to the SMUG1 family (256-

PQANK-260), are inserted as a wedge into the DNA duplex, flipping the scissile nucleotide through the major groove. Penetration of both motifs into the base stack creates a more extensive disruption of the double stranded DNA than seen for the other glycosylases in the UDG family. A conserved arginine (in vertebrates and insects (Paper 2: (Pettersen et al., 2007)), Arg254, occupies the gap left from the flipped-out base, whereas a proline from the unique α helix pushes into the base stack on the distal strand.

In the crystal structures, a second SMUG1 enzyme is bound to the 3´-end of the damage-containing strand, where the base pairing remains intact and the SMUG1 active site is solvent accessible. Structures of the xSMUG1-dsDNA complex with free uracil or 5-hydroxymethyl-uracil (HmU) revealed the rather remarkable mechanism for achieving pyrimidine specificity in SMUG1. The uracil N3 imino and O4 carbonyl moieties hydrogen bond to the Asn174 side chain, and O2 accepts hydrogen bonds from the Met95 main chain NH group and the imidazole ring of His250 (Figure 6). This hydrogen bonding pattern implies that cytosine is rejected by SMUG1 in a manner analogous to that of UNG.

Rejection of thymine, however, is quite different in SMUG1 than in the family1 UDGs. The tyrosine that acts as the thymine barrier in UNG is substituted by a glycine (Gly98) in SMUG1. A well-ordered water molecule is found in place of the tyrosine side chain, which upon uracil binding retains a van der Waals contact with C5 and a hydrogen bond to the O4 carbonyl of the pyrimidine. Both lone-pairs of the water molecule accept hydrogen bonds from the NH groups of Gly98 and Met102, such that the water molecule specifically donates a hydrogen bond to the pyrimidine 4 position. This provides additional discrimination against cytosine, which has an amino group at this position. Furthermore, this tightly held water makes three hydrogen bonds in the absence of a base and would have to be displaced to accommodate a thymine in the binding pocket. HmU, however, is able to compensate for the energetic penalty of displacing the water molecule by binding in the same orientation and with the same hydrogen bonding patterns as uracil, with its hydroxyl group at the 5 position reinstating the hydrogen bonds of the water molecule.

Mismatch repair glycosylases-MIG and MBD4

Deamination of 5-methylcytosine to thymine is more rapid that deamination of cytosine to uracil (Lindahl, 1993). This lesion is counteracted by specific G:T mismatch repair glycosylases, such as the thermostable MIG and MBD4. Both MIG and MBD4 are HhH-containing glycosylases with structural homology to EndoIII rather than to MUG/TDG, despite sharing similar substrate specificities. MIG recognizes G:T mispairs and removes the thymine base (Mol et al., 2002), whereas MBD4 preferentially recognizes G:T, but will also excise uracil from G:U mispairs (Petronzelli et al., 2000). A mechanism for G:T mismatch recognition and glycosylic bond cleavage has been proposed and that is consistent with structural analysis, complementary biochemistry, and characterization of key site-directed mutants. These studies suggest that MIG bond cleavage is enhanced by a physical distortion of the nucleotide that imparts a ~90° twist to the thymine base, away from its normal *anti* position in DNA (Mol et al., 2002), similar to a model proposed for UNG (Parikh et al., 2000).

MBD4 is comprised of two DNA binding domains: a G:T mismatch specific DNA glycosylase and a methyl-CpG binding domain. This apparent fusion of functions results in an enzyme with preference for G:T mispair in a CpG context. As methyl-CpG steps often occur in clusters, this may lead to a local increase in repair enzyme concentration for damage sensing. NMR and crystal structures have been elucidated for domains homologous to the methyl-CpG domain (Ohki et al., 2001; Ohki et al., 1999; Wakefield et al., 1999) and for the MBD4 HhH-containing glycosylase domain (Wu et al., 2003), respectively. It remains to be shown how DNA might bind both domains simultaneously, particularly if the HhH-containing glycosylase domain bends DNA as significantly as the other HhH family members.

dUTPases

As a complement to BER, nucleotide pool "sanitizing" enzymes have been discovered that remove improper bases to prevent their misincorporation into DNA. Among these, dUTPases are of particular importance, as dUTP can be misincorporated opposite an A during replication but is often repaired to C,

introducing T:A to G:C transitions. Structures of several of these ubiquitous enzymes are now available, revealing two distinct groups, one trimeric and one dimeric. Structures of trimeric (Bjornberg et al., 2003; Cedergren-Zeppezauer et al., 1992; Chan et al., 2004; Dauter et al., 1999; Huffman et al., 2003; Mol et al., 1996; Prasad et al., 1996) and dimeric (Harkiolaki et al., 2004; Moroz et al., 2004) dUTPases reveal distinct yet highly conserved folds and dUTP binding pockets across kingdoms, as has been observed in the UDG family, reflecting the ancient and essential nature of protection against deamination damage.

Alkylation damage

The most common form of nonenzymatic methylation of DNA likely results from physiological exposure to endogenous S-adenosyl methionine (SAM), which is found in the nucleus and also participated in targeted enzymatic DNA methylation (Lindahl, 1993). The primary substrates for nonenzymatic methylation are ring nitrogens of purine residues, with 3-methyladenine (3-meA) and 7-methylguanine (7-meG) being the predominant lesions formed. 7-meG does not alter base pairing with C, but 3-meA blocks replication and is cytotoxic. Each of the alkylated bases bears a formal positive charge likely to be important for recognition. Four classes of enzymes initiate BER of alkylated bases, typified by the following enzymes: 1) *E. coli* TAG (3-meA-DNA glycosylase I); 2) *E. coli* AlkA (3-meA-DNA glycosylase II); 3) *Heliobacter pylori* MAGIII (3-meA-DNA glycosylase III); and 4) human AAG (alkyladenine-DNA glycosylase). TAG, AlkA, and MAGIII are HhH-containing enzymes, whereas AAG has an unusual fold not seen in other BER enzymes. Because 3-meA is so deleterious, each of the alkyl base glycosylases efficiently removes this lesion. AlkA and AAG recognize a broad spectrum of substrates, including deamination and cyclic etheno adduct products. TAG removes 3-meA preferentially and 3-methylguanine (3-meG) with lower affinity, but not 7-meG, and MagIII has highest affinity for 3-meA (Bjelland et al., 1993; O'Rourke et al., 2000).

At least two mechanisms for repair of simple alkylation damage that do not rely on BER have been described. Two such mechanisms involve direct removal of the damage without further modification of the nucleotide or DNA, by proteins using either “suicidal” (single turnover) (AGT or Ada) or “non-suicidal” (AlkB or FTO) reactions. These proteins have no homology to the BER enzymes or to each other.

AGT becomes covalently modified in the process of repairing O^6-alkylguanine lesions, and structures of the modified enzyme as well as a protein-DNA complex are discussed below (Daniels et al., 2000; Daniels et al., 2004; Hashimoto et al., 1999; Lin et al., 2001; Moore et al., 1994). Based on sequence homology, it was shown that the AlkB enzymes belong to a structural superfamily of 2-oxoglutarate (2OG) and iron-dependent oxygenases (Aravind and Koonin, 2001). The crystal structures of *E. coli* AlkB (Yu et al., 2006) and the functional human AlkB homolog 3 (hABH3) (Paper 5: (Sundheim et al., 2006)) have been elucidated. Interestingly, a very recent publication in Science by Schofield and collaborators demonstrates that the FTO (fat mass and obesity associated) protein demethylates 3-methylthymine (3-meT) in single stranded DNA using Fe(II) and 2OG as cosubstrates, suggesting a potential role for this enzyme in direct damage reversal of nucleic acids (Gerken et al., 2007).

Human alkylbase-DNA glycosylase (AAG)

AAG is the only known human alkylbase-DNA glycosylase, although other human enzymes exist that perform different types of alkylation damage repair, such as AGT and the human AlkB homologs 2 and 3 (hABH2 and hABH3). AAG is a structural outlier, with a topology unlike any of the other known BER glycosylases (Lau et al., 1998; Lau et al., 2000), consisting of a single α/β domain in which an antiparallel β sheet is surrounded by α helices. A β hairpin protrudes into the minor groove of DNA in cocrystal structures. A structure of AAG in complex with $1,N^6$-ethenoadenine (εA)-containing DNA substrate revealed that the base is flipped out and inserted into a deep pocket, as occurs in the other structural families of DNA glycosylases. Alkylbases are specifically recognized using planar stacking and cation-π interactions by Tyr127, His136, and Tyr159, and the chemical instability of the glycosidic bond in positively charged, alkylated nucleobases likely contributes to the catalytic specificity of AAG. Subsequent biochemical studies have revealed similarities between AAG and *E. coli* AlkA, particularly in their dependence on the weakened glycosidic bond of methylated bases for excision specificity (O'Brien and Ellenberger, 2004).

Human O(6)-alkylguanine-DNA alkyltransferase (AGT)

AGT and ADA are homologous proteins that directly remove alkyl groups from the O6 position of guanine in a stoiciometric suicide reaction. Structures have been determined for bacterial, archeal, and human AGT/ADA proteins (Daniels et al., 2000; Daniels et al., 2004; Hashimoto et al., 1999; Lin et al., 2001; Moore et al., 1994). Human AGT is of particular interest because it repairs damage induced by some anticancer chemotherapeutics. The crystal structure of native human AGT, as well as structures of the methylated and benzylated product complexes (Daniels et al., 2000), revealed a two domains α/β fold. The N-terminal domain consists of an antiparallel β sheet followed by two α helices. The C-terminal domain is comprised a β hairpin, four α helices, and a 3_{10} helix which harbors a conserved Pro-Cys-His-Arg motif. Human AGT also contains a novel zinc binding site not seen in the bacterial or archael homologs that is likely to play a structural role. The C-terminal domain also contains a helix-turn-helix motif, often used by DNA binding proteins for sequence-specific recognition (Wintjens and Rooman, 1996). The alkylated product structures in which Cys145 has a covalently attached benzyl or methyl group, established the active site as being near the recognition helix of the HTH motif. Surprisingly, the DNA-bound structures revealed that HTH motif is not used in the canonical way, with the recognition helix binding in the major groove where it can take a broad range of possible orientations that allow sequence-specific binding (Daniels et al., 2004). Instead, the recognition helix lies in the minor groove, which is likely to be advantageous for sequence-independent binding and nucleotide flipping. Another unexpected finding was that binding of AGT to DNA is cooperative and displays directionality, likely to be useful for targeting to areas of localized alkylation damage (Daniels et al., 2004). Furthermore, a 3´-phosphate twist mechanism by which a tyrosine is thought to facilitate nucleotide flipping was recognized and noted to be present in other base-flipping systems such as UNG, AlkA, AP endonuclease, and endonuclease IV.

The superfamily of Fe(II)/2OG dependent dioxygenases

The structural family of non-heme-Fe(II)/2OG dependent dioxygenases catalyses a variety of enzymatic reactions, including protein side chain modification, biosynthesis of antibiotics and plant products, metabolism related to lipids, biodegradation of a whole string of compounds, and repair of alkylated DNA by AlkB enzymes (reviewed in (Clifton et al., 2006; Hausinger, 2004)). However, only the hydroxylation reaction is identified in animals for the Fe(II)/2OG dependent dioxygenase enzymes. Common for most of the enzymes in this family is the coupling of substrate oxidation to the conversion of 2OG into succinate and CO_2. The members of this family share a conserved Fe(II) binding motif, HxD/Ex_nH, and a double stranded β-helix (DSBH) core fold. The DSBH fold is also found for proteins in the cupin and JmjC transcription factor families.

The E.coli AlkB enzyme

The expression *E. coli* AlkB is regulated by the adaptive response to alkylating agents. Transfer of the methyl lesion from methylphosphotriesters to Cys38 in the N-terminal domain of Ada turns this multifunctional protein into an active transcription factor, which turns on the *ada-alkB* operon as well as the *alkA and aidB* genes (reviewed in (Sedgwick and Lindahl, 2002)). Following the *in silico* classification of AlkB as a Fe(II)/2OG dependent dioxygenase (Aravind and Koonin, 2001), it was shown that *E. coli* AlkB directly reverses cytotoxic 1-methyladenine (1-meA) and 3-methylcytosine (3-meC) DNA lesions (Falnes et al., 2002; Trewick et al., 2002), that are mainly produced by S_N2 methylating agents. Additional minor substrates reported for the AlkB enzyme are the alkylated DNA bases 3-methylthymine (3-meT) and 1-methylguanine (1-meG) (Delaney and Essigmann, 2004; Falnes, 2004; Koivisto et al., 2004), larger alkyl groups such as DNA-ethyl, -hydroxyethyl, -propyl, and -hydroxypropyl (Duncan et al., 2002; Koivisto et al., 2003), and the lipid peroxidation products $1,N^6$-ethenoadenine (εA) and $3,N^4$-ethenocytosine (εC) (Delaney et al., 2005; Mishina et al., 2005).

The crystal structure of AlkB in complex with the trinucleotide d(T-1meA-T) was determined by Yu and collaborators in 2006 (Yu et al., 2006), providing final

evidence of a DSBH fold for the AlkB family and structural information on iron, 2OG and lesion binding in the active site pocket (Figure 7). The AlkB structure is discussed in detail in paper 5 (Sundheim et al., 2006).

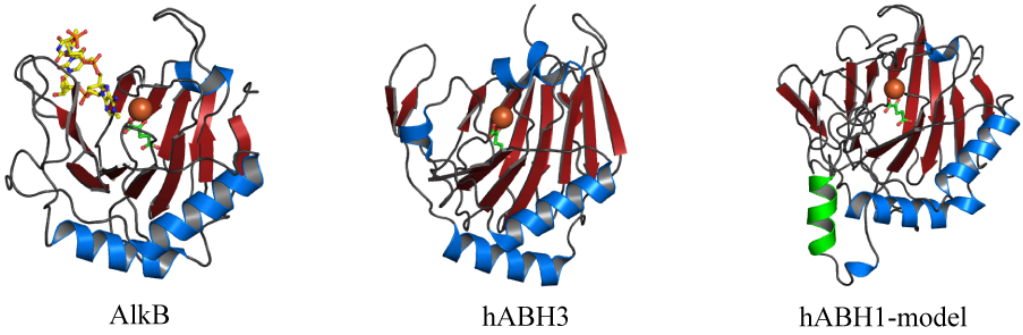

Figure 7: The AlkB proteins possess similar double stranded β-helix (DSBH) core fold, represented by *E. coli* AlkB (PDB ID 2FD8), hABH3 (PDB ID 2IUW), and a homology model of hABH1 (Marianne P. Westbye personal communication). The tri-nucleotide in AlkB and the 2OG cosubstrate are presented as balls and sticks colored yellow and green, respectively. The dark orange spheres illustrate the active site iron and α helices are blue and β strands dark red. The putative matrix-ancoring α helix in hABH1 model is colored green.

The human AlkB homologs 1-8 (hABH1-8)

Eight homologs of the AlkB enzyme are identified in the human genome (Drablos et al., 2004; Kurowski et al., 2003). hABH1 shares the highest degree of sequence similarity with the *E. coli* AlkB, and was reported to partially complement *E. coli* AlkB deficiency during treatment with methylating agents (Wei et al., 1996). Any AlkB like activity against 1-meA or 3-meC substrates was initially not found by us (Paper 3 (Aas et al., 2003)) or others (Duncan et al., 2002). However, our recent data reveal that hABH1 is predominantly a mitochondrial protein involved in direct reversal of 3-methylcytosine lesion in DNA and RNA (Feyzi *et al.*, personal communication). Furthermore, molecular modeling of hABH1 based upon the known structures of AlkB and hABH3 predicts an additional hydrophobic α helix that might be involved in anchoring the protein to the mitochondrial matrix (Figure 7). hABH2

and hABH3 are functional homologs of *E. coli* AlkB in reversal of both 1-meA and 3-meC lesions (Duncan et al., 2002; Aas et al., 2003), and these enzymes will be discussed in more detail below.

The catalytic functions of hABH4-8 have not yet been elucidated. Based on the knowledge of hABH1-3, the most obvious functions of these proteins are in direct reversal of alkylated nucleic acids. Nevertheless, one cannot dismiss the possibility that these proteins are catalytic active against different types of substrates, such as methylated proteins. Reversal of histone methylation has been reported for several enzymes. The nuclear flavin-dependent amine oxidase, LSD1, specifically demethylates mono- and di-methylated H3-lysine4 releasing the methyl group as formaldehyde (Shi et al., 2004). Interestingly, the Fe(II)/2OG dependent dioxygenases JHDM1 and JHDM2A demethylates mono- and di-methylated H3-lysine36 (Tsukada et al., 2006) and H3-lysine9 (Yamane et al., 2006), respectively, whereas JMJD2 demthylates trimethylated H3-lysines9 and 36 (Whetstine et al., 2006). Histone methylation is important in regulating chromatin structure and transcription, and to date 5 lysine residues in histone H3 (4 of them located in the tail) have been shown to be subject for mono- di- and tri-methylation ((Shi, 2007) with references therein). There is also a possibility that such a potential amino acid demethylase activity is linked to repair of damaged proteins, which could play an important role in cells that display a low protein turnover (Falnes et al., 2007).

The function of human AlkB homologues 2 and 3 (hABH2 and hABH3)

Both hABH2 and hABH3 catalyze hydrolytic demethylation of 1-meA and 3-meC in an Fe(II)/2OG dependent reaction (Paper 3: (Aas et al., 2003) and (Duncan et al., 2002)). In addition, both these and the AlkB are capable of repairing 1-meG and 3-meT (Falnes, 2004; Koivisto et al., 2004) while hABH3 repair εA lesion (Mishina et al., 2005). Our biochemical characterization revealed that hABH2 has a preference for lesions in dsDNA, whereas hABH3 displayed highest activity towards lesions in ssDNA (Paper 3 and 4: (Falnes et al., 2004; Aas et al., 2003)). Notably, both hABH3 and AlkB were also found to remove 1-meA and 3-meC lesions from RNA substrates (Falnes et al., 2004; Aas et al., 2003). This underscores a possible role for AlkB and hABH3 in repair of methylated RNA, as first suggested by Aravind and Koonin

(Aravind and Koonin, 2001). hABH2 is strictly localized to nuclei and colocalizes with PCNA in replication foci during S-phase. Conversely, hABH3 is found both in nuclei and the cytoplasm, but not in replication foci (Konishi et al., 2005; Aas et al., 2003). These results are compatible with a role for hABH2 in replication-associated repair of the replication-blocking 1-meA and 3-meC lesions and hABH3 in repair of single stranded DNA and RNA. A potential role for hABH3 and AlkB in repair of methylated mRNA and tRNA was investigated *in vitro* in the laboratory of Professor Falnes (Ougland et al., 2004). Both enzymes relieved methylation-induced mRNA translation blocks and reversed the aminoacylation and translation inhibition of methylated $tRNA^{Phe}$. Importantly, repair of 1-meA in tRNA by *E. coli* AlkB was also demonstrated experimentally *in vivo*. However, a definitive function for the AlkB enzymes in repair of damaged RNA *in vivo* has yet to be proven experimentally. The potential functions of AlkB-mediated RNA repair and RNA repair in general are reviewed in Falnes *et al*., (2007) (Falnes et al., 2007) and Feyzi *et al*., (To be published in Current Pharmaceutical Biotechnology).

Distinct functional roles of ABH2 and ABH3 were recently demonstrated in knock-out mice by comparing repair efficiencies of cell-free extracts using synthetic 1-meA- and 3-meC- containing DNA *in vitro* and by quantitating 1-meA from genomic DNA *in vivo* (Ringvoll et al., 2006). Whereas no detectable repair defects were observed in extracts lacking mABH3, mABH2 was found to be required to mediate efficient removal of these lesions from DNA. Although neither of the knock-out mice displayed any overt phenotype, accumulation of 1-meA lesions in liver DNA was observed in the mABH2-null mice.

Our integrated structural and biochemical study of hABH3 revealed a catalytic core domain of DSBH fold holding an iron and 2OG in accordance with its proposed iron and 2OG binding motifs (Figure 7) (Paper 5: (Sundheim et al., 2006)). Furthermore, structural investigations combined with site-specific mutations analysis suggested distinct recognition of the lesion in the active site and different composition of the nucleotide recognition lid compared to *E. coli* AlkB (Sundheim et al., 2006; Yu et al., 2006). Based on the location of the active iron center, we propose that also hABH3 use a β-hairpin in the putative nucleotide recognition lid to flip the damage base into active site pocket in consistence with the majority of nucleobase repair enzymes known.

A putative role for FTO in nucleic acid demethylation?

The human *FTO* (fat mass and obesity associated) gene encode a protein that is expressed in a wide range of human tissues (Frayling et al., 2007), and common variants in the first intron of FTO has been associated with obesity in both children and adults (Dina et al., 2007; Frayling et al., 2007; Scott et al., 2007; Scuteri et al., 2007). *Fto* mRNA in wild type mice is most abundant in the brain, particularly in hypothalamic nuclei governing energy balance. Moreover, the level of murine *Fto* mRNA in the arcuate nucleus is regulated by feeding and fasting (Gerken et al., 2007). In *silico* analysis of FTO revealed that it shares sequence motifs with the Fe(II)/2OG dependent dioxygenases within a predicted DSBH fold. It was also shown that recombinant murine FTO reverses 3-meT in single stranded DNA (Gerken et al., 2007). The structural model of human FTO based upon the crystal structure of hABH3 suggests that it contains a core domain DSBH fold and, interestingly, a nucleotide recognition lid similar to that of hABH3. Their sequence alignment of FTO enzymes and AlkB homologues reveal that the hairpin corresponding to β4-β5-hairpin in hABH3 (Sundheim et al., 2006) is of same length and 3 amino acids shorter to that predicted in hABH2. One might hypothesize that the composition of this hairpin-motif is important for single stranded versus double stranded specificity. It would also be interesting to know if the 3-meT demethylase activity is exclusively for DNA or whether FTO is able to reverse damaged RNA as well.

Aims of the study

In humans, there are four known glycosylases that initiate repair of uracils in DNA. These are UNG, TDG, SMUG1, and MBD4. It was proposed that the replication independent SMUG1 was the main enzyme initiating removal of deaminated cytosine, whereas UNG2 was responsible for replication associated repair of mis-incorporated dUTP (Nilsen et al., 2001). We aimed at elucidating the specific function of the two main human uracil-DNA glycosylases *in* vitro and *in vivo* to further clarify their distinct roles in repair of uracils in the genome (Paper I). In Paper II, we continued the in-depth analysis of the distinct roles of hUNG2 and hSMUG1. We wanted to investigate whether hUNG2 and hSMUG1 coordinated the subsequent step in BER differentially, to characterize active-site residues in hSMUG1, and to elucidate the role of the extended DNA minor-groove intercalating motif of hSMUG1 in binding of the complementary DNA strand.

Studies in our laboratory in the mid-1990's of the sequence upstream of the human *UNG* gene led to the discovery of a previously unrecognized gene in this region. Expression of the gene revealed that the product was translocated to the nucleus, and further bioinformatics analyses revealed weak but significant homology between the human protein (now called hABH2) and bacterial AlkB. An additional putative protein (hABH3) of weak homology was also identified in the human genome. Our biochemical characterization of the function of these gene products was guided by the finding that they belong to a family of enzymes functional dependent on Fe(II) and 2OG (Aravind and Koonin, 2001). The initial characterization of the two human proteins and comparison to *E. coli* AlkB resulted in Paper III. Discrepancies in substrate specificities reported by us and others led to a more extensive characterization of this enzyme with respect to preferred substrate (Paper IV). In addition to biochemical characterization of the human AlkB homologs we also initiated structural studies. The crystal structure of the catalytic core domain of hABH3 in complex with iron and 2OG and functional studies of residues within the active site are presented in Paper V.

Summary of results and discussion

Paper I: hUNG2 Is the Major Repair Enzyme for Removal of Uracil from U:A Matches, U:G Mismatches, and U in Single-stranded DNA, with hSMUG1 as a Broad Specificity Backup

The uracil-DNA glycosylases hUNG2 and hSMUG1 constitute the first step in the quantitatively dominating base excision repair (BER) pathway, and these enzymes are the only known human glycosylases that excise uracil from both single- and double stranded DNA. The C-terminal catalytic core domain of hUNG has been subject to extensive structural and biochemical characterization (Kavli et al., 1996; Mol et al., 1995; Parikh et al., 1998; Parikh et al., 2000; Slupphaug et al., 1996). However, the biochemical behavior of the full-length nuclear hUNG2 (Muller-Weeks et al., 1998) was yet to be elucidated, mainly because the regulatory N-terminal is easily proteolytically degraded both *in vivo* during heterologous expression as well as during purification. The degradation problem was overcome in our study by use of a mixture of protease inhibitors and careful treatment of the protein sample during bacterial lysis and purification. The full-length hUNG2 enzyme of a molecular weight of 34.6 kDa eluted as 52 kDa protein as judged from size exclusion chromatography, suggesting that the N-terminal is flexible and most likely unstructured. Purification of full-length hSMUG1 yielded in a fully functional truncated protein lacking 16 N-terminal amino acids. N-terminal sequencing of the hUNG2 enzyme revealed a mixture of full-length (60%) and hUNG2Δ1.

Surprisingly, both enzymes were stimulated by divalent magnesium at physiological concentrations. In the presence of Mg^{2+}, hUNG2 displayed catalytic activity 2-3 orders of magnitude higher than hSMUG1. Stimulation by Mg^{2+} stands in strong contrast to the behaviour of the catalytic core domain of UNG that is inhibited in presence of Mg^{2+}. Thus, the mechanistic property for Mg^{2+} stimulation of hUNG2 lies within or is aided by the regulatory N-terminal. Moreover, Michaelis-Menten kinetics revealed a weak preference (1.3-fold) for ssU substrate versus dsU substrate for hUNG2. Remarkably the ssU/dsU specificity ratio increased ~40 fold in the presence of Mg^{2+}. The corresponding ratios for hSMUG were 1.6 and 3.8 in the absence and presence of Mg^{2+}, respectively. These results show that hSMUG1 barely

justifies its name (single-strand-selective monofunctional uracil-DNA glycosylase) in the absence of Mg^{2+}. In the presence of physiological concentrations of divalent magnesium, hUNG2 is actually selecting ssU at a much higher rate than hSMUG1.

Furthermore, stimulation of uracil excision by APE1 was much more pronounced for hSMUG1 than for hUNG2. Actually, in the presence of APE1, the catalytic efficiency is higher for dsU than for ssU. DNA glycosylases were generally thought to protect the AP-site until the enzyme is alleviated and the damage is processed by BER enzymes downstream of the glycosylase. This behavior has previously been assigned to the catalytic core domain of UNG. Accordingly, AP-sites inhibit hSMUG1 activity. This was, however, not observed for the full-length hUNG2 enzyme. Thus, the strong stimulatory effect of APE1 on the catalytic efficiency of SMUG1 is likely caused by enhanced dissociation of hSMUG1 from AP sites in dsDNA.

Whereas the substrate specificity to hUNG2 is selective for uracil and uracil analogs with minor modifications at the 5-position, hSMUG1 is able to recognize and excise pyrimidines with bulky substitutions at the 3-, 4, and 5-positions. Our data shows that the substrate preference is U>>5-FU and U>HmU>>εC>5-FU for hUNG2 and hSMUG1, respectively.

In a previous work, a fraction of hUNG2 was shown to localize in replication foci in the S-phase (Otterlei et al., 1999). Here we applied fluorescently tagged hUNG2 (EYFP-hUNG2) and hSMUG1 (EYFP-hSMUG2) to study their localization during S-phase and outside the S-phase. hUNG2 was distributed to nucleoplasma outside the S-phase, a fraction accumulated in replication foci in S-phase. UNG2 also appeared to be excluded from nucleoli both in- and outside S-phase. Contrary, hSMUG1 was less strictly localized to nucleoli and is also found in cytoplasma. Moreover, hSMUG1 appeared to accumulate in nucleoli.

By knocking down the hUNG2 activity with neutralizing antibodies in human cell extracts, we provided evidenve that hUNG2 is the major cellular uracil-DNA glycosylase in repair of U:A and U:G DNA substrates. Preincubation of the cell extracts with neutralizing antibodies against hSMUG1 had no effect on the uracil excision activity but knocked out most of the repair activity of HmU. Knocking down both hUNG2 and hSMUG1 by neutralizing antibodies diminished all uracil glycosylase activity on U:A substrates, whereas a delayed repair of U:G substrates

was observed. A possible explanation for the latter is that a fraction of hUNG2 could be inaccessible for hUNG2 antibodies.

Summarized, the results presented in this study show that hUNG2 is the major uracil-DNA glycosylase initiating BER of U:A base pairs and U:G mismatches both in connection with replication and in resting cells. hSMUG1 seems to provide a broad specificity backup in the cellular defense of pyrimidine lesions in DNA, and may have a specific role in removal of uracil in nucleoli.

Paper II: Uracil-DNA glycosylases SMUG1 and UNG2 coordinate the initial steps of base excision repair by distinct mechanisms

The catalytic core domain of UNG and SMUG2 possess similar overall fold and belong to the same structural and functional family of DNA glycosylase. Lines of evidence strongly suggest that hUNG2 has a role in replication associated repair, e.g. it binds PCNA and RPA, it localizes to replication foci, and it is cell-cycle regulated with highest levels in S-phase (Haug et al., 1998; Nagelhus et al., 1997; Nilsen et al., 2000; Otterlei et al., 1999; Slupphaug et al., 1991). SMUG1 is neither involved in replication associated repair nor is it cell-cycle regulated (Paper I: (Kavli et al., 2002)). Moreover, it excises uracil with a much slower efficiency than UNG2, but has broader substrate specificity (Paper I: (Kavli et al., 2002) and (Boorstein et al., 2001)). SMUG1 was previously reported to be present in vertebrates and insects only (Aravind and Koonin, 2000; Nilsen et al., 2001). In this study, however, we found that some bacteria contain SMUG1 as their sole uracil-DNA glycosylase. Vertebrates contain both types of enzymes, suggesting a need for back-up systems in repair of U:G mismatches and/or distinct roles in initiating BER of U:G lesions. To further explore the distinct roles of hUNG2 and hSMUG1 we have studied how these BER enzymes recognize and process U:G mismatches.

We used an *in vivo* system in which U:G lesions were introduced by expressing AID in an Ung deficient *E. coli* strain and observed the effects when hUNG2 or hSMUG1 were expressed in this background. Surprisingly, only hUNG2 reversed the mutator phenotype, whereas co-expression of hSMUG1 inhibited cell growth. These results reveal that SMUG1 is unable to functionally replace Ung in U:G repair in proliferating *E. coli* cells. To further investigate the observed *in vivo* effects, we investigated the end-product AP-site binding subsequent to uracil excision

by using EMSA. Unlike hUNG2, hSMUG1 readily binds AP-sites in dsDNA context with a slightly higher affinity for AP:G than for AP:A. Next, we investigated the effect of hAPE1 on uracil excision from U:G substrate by hSMUG1 and hUNG2. In accordance with previous results obtained using U:A substrate (Paper I: (Kavli et al., 2002)), hSMUG1 was stimulated 2-3 fold by APE1, whereas hUNG2 was only weakly stimulated. Addition of hSMUG1 in molar excess inhibited both hAPE1 and bacterial ExoIII, suggesting that SMUG1 and AP endonucleases compete for binding to AP sites. Interestingly, hUNG2 had no effect on the activity of ExoIII, but stimulated the activity of hAPE1. Taken together, these results indicate that hSMUG1 binds and protects AP-sites in dsDNA until displaced by an AP endonuclease. hUNG2 does not bind AP sites, but aids and coordinates hAPE1 activity possible through specific interactions.

Site-specific mutations of putative important residues in the active site pocket only moderately affected on the catalytic activity of hSMUG1 compared to the effects of corresponding mutations in UNG. A tyrosine residue in UNG (UNG1 Tyr147) blocks the entrance of thymine to the active site pocket is replaced by a glycine residue in SMUG1. Nevertheless, mutating the glycine residue to a tyrosine in hSMUG1 totally abolished uracil excision by the protein. A plausible explanation for this behavior could be that the tyrosine side chain blocks the entrance of the active side due to slightly different conformation of the loops holding these residues in UNG and SMUG1.

The crystal structure of *X. laevis* SMUG1 revealed an α-helix following the DNA minor groove intercalating loop motif. The authors suggested that this α helix was involved in wedging the duplex DNA (Wibley et al., 2003). We showed by mutational analysis that this extended intercalating motif binds the distal strand preferentially with an orphan guanine, thus providing AP-site end- product binding properties to SMUG1.

Although hUNG2 and hSMUG1 are both uracil-DNA glycosylases with similar structural catalytic core folds, they possess distinct catalytic and BER coordination properties. The catalytically superior hUNG2 lacks product binding capacity and stimulates the subsequent AP site cleavage by specific “hand over” interaction with hAPE1. These properties are compatible with rapid and highly coordinated replication associated repair of U:G (pre-replicative) and U:A (post-

replicative). The role of the slow acting hSMUG1 is more likely in repair of deaminated cytosine (U:G) and other pyrimidine lesions in resting cells. The strong end-product binding suggest that hSMUG1 protects the potential harmful AP site until it is displaced by an AP endonuclease.

Paper III: Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA

Minor alkylation damage in the genome is repaired by BER- and DDR-mechanisms, including damage reversal by oxidative demethylation of 1-methyladenine and 3-methylcytosine by *E. coli* AlkB (Falnes et al., 2002; Trewick et al., 2002). In the present paper, we demonstrate that the two human AlkB homologues, hABH2 and hABH3, also are functional oxidative DNA demethylases. hABH2 prefers double stranded DNA, whereas hABH3 and *E. coli* AlkB acts more efficiently on single stranded nucleic acids. The identification of distant homologs of AlkB in plant RNA viruses (Aravind and Koonin, 2001) led the authors to hypothesize that AlkB might be involved in RNA repair. Encouraged by this, we tested *E. coli* AlkB, and the human AlkB homologs 2 and 3 in the ability to reverse 1-meA and 3-meC in RNA. Remarkably, both AlkB and hABH3 repaired alkylated poly(A) and poly(C), but no such repair activity was detected for hABH2. Furthermore, recombinant AlkB and hABH3 expressed in AlkB-deficient *E. coli* strain reactivated methylated RNA bacteriophage MS2 *in vivo*; suggesting that repair of RNA may have a biological relevance in living cells.

Transient transfection experiments of hABH2 and hABH3 in HeLa cells revealed that both enzymes localized to nuclei. hABH2 was homogenously distributed in the nucleoplasm with some accumulation in nucleoli outside S phase. In S phase, hABH2 accumulated in replication foci and colocalized with PCNA. In most cells, hABH3 was mainly distributed in nucleoplasm and some in cytoplasm as well, but was not observed in nucleoli. However, occasionally hABH3 accumulated in nucleoli as well as in the nucleoplasm, but no colocalization with PCNA was detected.

In this paper, we show that both hABH2 and hABH3 are functional homologs of *E. coli* AlkB. Different substrate specificities and subnuclear localization suggest distinct roles for hABH2 and hABH3 in defence against alkylation damage in cells. hABH2 may be involved in removing replication blocking 1-meA and 3-meC lesions

in the vicinity of replication forks, whereas hABH3 may be involved in repair of nuclear single stranded DNA as well as RNA.

Paper IV: Substrate specificities of bacterial and human AlkB proteins

E. coli AlkB and the human AlkB homologs 2 and 3 were previously identified as oxidative demethylases of 1-meA and 3-meC lesions in DNA (Duncan et al., 2002; Koivisto et al., 2003; Trewick et al., 2002; Aas et al., 2003). In addition, AlkB and hABH3 were also found to remove these lesions from RNA (Paper III: (Aas et al., 2003)). To further investigate the substrate specificities of AlkB, hABH2 and hABH3 we tested a panel of 1-meA and 3-meC lesions in different nucleic acids context.

Three different oligonucletide substrates were used to resolve ssDNA versus dsDNA specificity of the human and bacterial AlkB proteins. AlkB and hABH2 displayed moderate preference towards ssDNA and dsDNA, respectively, while hABH3 preferred ssDNA substrate. This pattern was consistent for all substrates tested, suggesting that the observed preference is sequence independent and a general property of the enzyme. The hABH2 dsDNA preference was only observed under physiological concentrations of divalent magnesium that had an inhibitory effect upon the activity against ssDNA substrates.

To gain more insight into the possibility that the AlkB proteins are involved in specific types of RNA repair, we investigated the preference for lesions in RNA/DNA hybrids and dsRNA substrates. In accordance with previous results, hABH2 preferred methylated DNA annealed to complementary RNA, over methylated ssDNA. Moreover, no significant repair of methylated dsRNA or methylated RNA annealed to complementary DNA was observed for hABH2. hABH3 preferred ssDNA over methylated-DNA:RNA hybrid substrate, and ssRNA over DNA:methylated-RNA and dsRNA duplexes. Although bacteria AlkB displayed a preference for ssDNA over dsDNA, similar activity was observed against ssDNA and methylated-DNA:RNA. The AlkB activity against RNA substrates was, however, similar to that of hABH3.

In the experiments designed by Trewick *et al*., AlkB displayed a preference for dsDNA over ssDNA (Trewick et al., 2002). However, they used unmethylated poly(dT) annealed to an ~310 bp methylated poly(dA) substrate. This likely produces a heterogeneous mixture of substrates containing both single- and double- stranded

regions, and possibly also substrates containing more than two single stranded molecules, such as various concatemers and branched molecules. Thus, this substrate may not be ideal for comparing ssDNA versus dsDNA preference for AlkB activity, and thereby likely explain the discrepancy between their conclusion and ours.

Duncan *et al*., (Duncan et al., 2002) did not report any ssDNA versus dsDNA preferences for neither hABH2 nor hABH3, and concluded that they had similar functions in DNA repair. We show in this study that the dsDNA preference of hABH2 is only displayed in the presence of Mg^{2+}. This observation may in part explain the discrepancy in the reports by Duncan and collaborators and us ((Duncan et al., 2002), paper III: (Aas et al., 2003), and paper IV (Falnes et al., 2004)).

Paper V: Human ABH3 structure and key residues for oxidative demethylation to reverse DNA/RNA damage

hABH3 and AlkB have been shown to directly reverse cytotoxic 1-meA and 3-meC lesions in DNA and RNA. In this study, we present the crystal structure of the catalytic core domain of hABH3 (N-terminal truncated hABH3Δ69) in complex with iron and 2OG. The high resolution structure (1.5 Å) was solved using phases from a multiwavelength anomalous dispersion (MAD) experiments with a single Pt derivative. hABH3Δ69 contains a central double stranded β-helix (DSBH) core fold made up of β7-β11. In hABH3Δ69 the DSBH is extended with β1 in the smaller sheet and β2, β3, and β6 in the larger sheet. Two helices buttress the larger sheet. The active site iron is likely in the inactive Fe(III) state in the aerobically grown crystal, and is coordinated by His191, Asp193, His257, and the 2OG molecule. β4 and β5 form a hairpin that creates a lid over the active site. We hypothesize that the positively charged groove formed between the hairpin and the DSBH core domain constitute the DNA/RNA binding cleft.

The crystal structure of *E. coli* AlkB in complex with a 1-meA-containing trinucleotide was published during the revision of our paper. Comparison of the structures reveals that the overall folds are similar dispite the relatively low sequence similarity between them. However, significant differences are observed in the substrate binding pockets and in other key regions in the vicinity of the active sites of the two enzymes. The substrate binding pocket of AlkB is predominantly

hydrophobic and 1-meA is sandwiched between His131 and Trp69 (Yu et al., 2006), whereas the substrate binding pocket in hABH3 is noticeably more polar. The more hydrophobic substrate binding pocket may explain the broader substrate specificity of AlkB compared to hABH3. AlkB binds the tri-nucleotide in a rather unusual conformation that not easily conforms to the hABH3 structure. Thus, we propose that the conformation of the trinucleotide is an artifact caused by the short oligonucleotide in combination with alternative binding of this substrate compared to longer biologically relevant oligonucleotides or that hABH3 and AlkB must bind oligonucleotides differently.

We combined structural information and biochemical characterization of site specific mutants of hABH3 to assign important residues involved in iron and 2OG binding, and residues located in the DNA/RNA binding groove likely to be involved in substrate recognition and nucleotide flipping. According to our data, the flexible β4-β5 hairpin is likely involved in both nucleotide flipping and ss/dsDNA discrimination.

hABH3 Leu177 is located in the active site and is essential for demethylation activity against 1-meA, and a leucine in this position is conserved in AlkB, hABH2, and hABH6. This leucine was found to be modified in the refined crystal structure, and MALDI-TOF MS analyses of tryptic peptides revealed a mass shift of +14 and +16, corresponding to a mixture of hydroxyl and carbonyl group at Leu177 Cδ atom. A corresponding modification was also observed in recombinantly expressed and purified AlkB, hABH2, and hABH6 proteins. We found that the oxidation of Leu177 likely results from hydroxyl radicals formed by uncoupled decarboxylation of 2OG in the absence of primary substrate. Unable to separate un-oxidized protein from oxidized protein, a series of L177 mutants were made and analyzed for their 1-meA and 3-meC activities. The mutational data reveal that Leu177 likely serves as a "buffer stop" to prevent pyrimidines to be positioned too deep into the catalytic pocket. The mutants mimicking the oxidized leucine, L177Q and L177E, were found to possess no activity neither against 1-meA nor 3-meC. This strongly suggests that self-hydroxylation of this leucine residue inactivates the enzyme, maybe to avoid generation of reactive oxygen species and potentially harmful side reactions in the cell.

In this study, we provide structural and biochemical information yielding new insights into the catalytic mechanism and divergent substrate preferences of nucleotide repair enzymes in the AlkB family. Moreover, we discovered a novel self-hydroxylation of an active site leucine that may used to knock out the ability to produce harmful oxidative side reaction in the absence of primary substrate.

Future perspectives

In general, direct visualization of repair proteins in complex with nucleotides both in solution and in crystal diffraction experiments provide a molecular-level understanding based upon the discovery of testable general themes and principles for DNA/RNA base damage recognition, processing, and coordination. The structural biology of nucleobase damage and repair protein:DNA/RNA complexes furthermore gives an initial understanding of how dynamically assembled macromolecular machines and reversible complexes may control pathway progression and selection. Currently high-resolution structures coupled to characterization of protein active sites and protein interfaces are providing a mechanistic understanding of how single-site mutations can lead to degenerative diseases such as cancer. As one example, structures of the DNA base repair enzyme UNG provided the basis to understand damage specificity an to see how single site mutations in the UNG base specificity pocket can result in a mutator phenotype (Kavli et al., 1996).

In paper I, we showed that the full-length human UNG2 enzyme displays distinct properties compared to the catalytic core domain alone in respect to Mg^{2+} influenced catalytic activities and ss/ds DNA preference and to end product binding properties. Although extensive attempts to crystallize full-length hUNG2 have been undertaken this has not yet succeded. We have faced several obstacles in the process, especially regarding N-terminal proteolytic degradation. Crystals have been obtained using apparently homogenous purified N-terminal his-tagged hUNG2. However, the molecular replacement solution using the catalytic core domain of UNG as search model revealed that ~100 N-terminal residues including the his-tag were missing in the structure. The packing arrangement in the crystal furthermore suggested that these residues were cleaved off prior to crystallization, and this was confirmed with coomassie blue stained polyacrylamid gel electrophorese and mass spectrometry analyses of tryptic peptides. It remains to be determined if this is caused by protealytic cleavage due to contaminants in the purified solution or by a self-cleavage process. Either way, to crystallize the apo-enzyme is probably not the best approach to achieve structural information of the regulatory N-terminal domain, since it is most likely flexible and unstructured in non-physiological *in vitro* buffer solutions.

Therefore, structural analysis of the regulatory domain of hUNG2 will also be attempted by other techniques such as SAXS (small angular x-ray scattering).

Schär and Steinacher proposed that the N-terminal of TDG forms a flexible DNA holding "clamp" and that in this state TDG may slide along the DNA in search for a G mismatched lesion (Cortazar et al., 2007). It is not inconceivable that the N-terminal of hUNG2 also forms a "sliding clamp", which upon binding of Mg^{2+} optimizes its conformation to slide along ssDNA. hUNG2 is a multifunctional glycosylase that removes uracils near replication forks and in resting cells, and is important for affinity maturation of antibodies in B-cells. Much of this dynamicity resides in the regulatory N-terminal, which is reported to contain at least one binding motif for replication protein A (RPA) as well as a PiP-box binding motif for PCNA (Otterlei et al., 1999). In addition, we have very recently published a study in wich hUNG2 is shown to be stepwise phosphorylated through the cell cycle, most likely by cyclin dependent kinases. Each of these phosphorylations confer distinct properties to UNG2 (Hagen et al., 2007). Utilizing this biochemical knowledge will be important to guide successful experiments that will provide structural pieces to elucidate the regulated and dynamic functionality of hUNG2.

In paper II, we used the crystal structures of xenopus SMUG1 and hUNG to design site-specific mutations to characterize the distinct properties of hSMUG1 compared to hUNG2. Insolubility even at low concentration of hSMUG1 has been great obstacle to overcome in retrieving crystals of hSMUG1. However, it will be of great importance to elucidate the structure of SMUG1 with a productive binding of DNA in the active site to further explore the properties of DNA binding motifs and find clues how SMUG1 regulates the subsequent processing of the AP-site in the BER pathway.

In paper III and IV we report some of the first biochemical characterizations of bacterial AlkB and the human AlkB homologues 2 and 3. Interestingly, we found that AlkB and hABH3 may have a possible role in RNA repair. The crystal structure of AlkB in complex with a 1-meA-containing tri-nucleotide was solved by Yu and colleagues in 2006 (Yu et al., 2006) and we reported the structure of hABH3 later that year (Paper V: (Sundheim et al., 2006)). Several features of these enzymes were elucidated by these crystal structures, but still there are properties such as ss/dsDNA/RNA discrimination to be elucidated. We thus aim to crystallize protein:DNA complexes of the human Fe(II)/2OG nucleobase demethylases.

Moreover, we will pursuit the functions of the uncharacterized N-terminal regions of these enzymes as well as the biological functions of the remaining human AlkB homologs.

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106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
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108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.

109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

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110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
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112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
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118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
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121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
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126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
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131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

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132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
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137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.

139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

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141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
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143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
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157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
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160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
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165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
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168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
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174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
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183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
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188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
197. Tore Amundsen: PERFUSION MR IMAGING IN THE DIAGNOSIS OF PULMONARY EMBOLISM

198. Nanna Kurtze: THE SIGNIFICANCE OF ANXIETY AND DEPRESSION IN FATIQUE AND PATTERNS OF PAIN AMONG INDIVIDUALS DIAGNOSED WITH FIBROMYALGIA: RELATIONS WITH QUALITY OF LIFE, FUNCTIONAL DISABILITY, LIFESTYLE, EMPLOYMENT STATUS, CO-MORBIDITY AND GENDER
199. Tom Ivar Lund Nilsen: PROSPECTIVE STUDIES OF CANCER RISK IN NORD-TRØNDELAG: THE HUNT STUDY. Associations with anthropometric, socioeconomic, and lifestyle risk factors
200. Asta Kristine Håberg: A NEW APPROACH TO THE STUDY OF MIDDLE CEREBRAL ARTERY OCCLUSION IN THE RAT USING MAGNETIC RESONANCE TECHNIQUES

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
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224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
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229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
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231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
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238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
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246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS

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254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
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267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS

280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
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283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
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286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OG PATHOLOGICAL GAMBLING IN NORWAY
288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
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