Torkild Visnes

DNA excision repair of uracil and 5-fluorouracil in human cancer cell lines

Torkild Visnes

Doktoravhandling

NTNU
Norges teknisk-naturvitenskapelige universitet
Avhandling for graden philosophiae doctor
Faculty of Medicine
Department of cancer research and molecular medicine

NTNU
Det skapende universitet

NTNU
Det skapende universitet

NTNU

Torkild Visnes

DNA excision repair of uracil and 5-fluorouracil in human cancer cell lines

NTNU
Det skapende universitet

Torkild Visnes

DNA excision repair of uracil and

5-fluorouracil in human cancer cell lines

Contents

2

Sammendrag på norsk

DNA er et tilsynelatende stabilt molekyl, som overføres så å si uten endringer fra unnfangelse til alderdom og fra generasjon til generasjon. Men arvestoffet vårt er ikke så uforanderlig som det kan se ut som. DNA kan endres kjemisk ved å reagere med en rekke stoffer som er påført utenfra eller som normalt finnes inne i enhver celle. DNA består av repeterende enheter av nukleotider, som igjen består av fosfat-, sukker- og basegrupper. Fosfat- og sukkergruppene danner en ryggrad, mens basene parer med andre baser på en motstående DNA-tråd. Fokus for denne avhandlingen er baseskadene uracil og 5-fluorouracil. Uracil dannes ved at den normale basen cytosin reagerer med vann. Dette resulterer i et uracil:guanin base-par. Eventuelt kan både uracil og 5-fluorouracil inkorporeres i stedet for den normale basen thymin under DNA-replikasjon. Dette resulterer i uracil paret med adenin, mens 5-fluorouracil kan pare med adenin eller guanin.

For å unngå at skader på DNA resulterer i mutasjoner finnes det flere mekanismer i cellen som erstatter og reparerer skadd DNA. En av de viktigste reparasjonsmekanismene er base eksisjonsreparasjon (BER). BER initieres ved at en DNA-glykosylase kutter en skadd eller unormal base fra DNA. Hos mennesker er det identifisert fire forskjellige glykosylaser som alle kan initiere reparasjon av uracil og 5-fluorouracil: UNG, SMUG1, TDG og MBD4. En AP-endonuklease kutter så sukker-fosfat ryggraden ved å kutte ved siden av den nå base-løse sukkergruppen, og et nytt nukleotid settes inn av en DNA polymerase. Restene av sukkergruppen kan så fjernes direkte av DNA-polymerasen, før ryggraden på DNA-tråden bindes sammen av en DNA ligase. I sum blir dermed en skadd base erstattet med en normal (en-nukleotid BER). I enkelte tilfeller klarer imidlertid ikke polymerasen å fjerne det som er igjen av sukkergruppen, dette skjer gjerne når sukkergruppen har blitt redusert eller oksidert. Da vil polymerasen sette inn flere nukleotider, slik at den skadde sukkergruppen fortrenges. Dette skaper en spesiell struktur som gjenkjennes av en flap endonuklease, som kutter ut den fortrengte biten, før en DNA ligase knytter DNA-tråden sammen igjen. Dermed fører reparasjon av en skadd base til at flere nukleotider erstattes (fler-nukleotid BER).

Inntil nylig har ikke fler-nukleotid BER vært observert i cellenes mitokondrier, som har sitt eget DNA å ta vare på. Hvordan ville i så fall mitokondriene håndtere skader som i cellekjernen repareres av fler-nukleotid BER? Dette har vi undersøkt i det første arbeidet, hvor vi fant at også mitokondriene kunne utføre fler-nukleotid BER.

Uttrykket av glykosylasen UNG varierer mellom forskjellige mennesker, organer og cellelinjer. I det andre arbeidet viser vi at hastigheten til BER sporet som helhet kontrolleres på det første trinnet, det vil si av mengde og aktivitet av DNA-glykosylasen som initierer reparasjonen. UNG initerte all observerbar reparasjon av uracil paret med adenin, mens reapasjon av uracil paret med guanin ble initiert hovedsakelig av UNG, med et relativt stort bidrag fra TDG i en av cellelinjene.

I det tredje arbeidet har vi studert hvordan 5-fluorouracil repareres i DNA og hvilken betydning DNA-reparasjon har å si for virkningsmekanismen for 5-fluorouracil. Vi fant at BER, initiert av UNG2, står for det aller meste av reparasjonen når 5-fluorouracil er paret med adenin. Når 5-fluorouracil er paret med guanin utfører BER, initert av UNG2, SMUG1 eller TDG det meste av reparasjonen, mens mismatch-reparasjon ser ut til å være av mindre betydning. Nedregulering av de nevnte glykosylasene og hemming av BER-sporet påvirket imidlertid ikke kreftcellelinjers følsomhet for 5-fluorouracil. Dermed later det til at i dette tilfellet spiller ikke inkorporering i DNA og påfølgende DNA-reparasjon noen stor rolle for celledød. I stedet ser det ut som om 5-fluorouracil heller dreper celler via inkorporering i RNA, samt ved at dannelsen av thymidin-nukleotider hemmes.

Acknowledgements

List of Papers

Paper I:

Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis.

(Akbari M, Visnes T, Krokan HE and Otterlei M).

Paper II:

The rate of base excision repair of uracil is controlled by the initiating glycosylase.

(Visnes T, Akbari M, Hagen L, Slupphaug G and Krokan HE)

Paper III:

Cytotoxicity of 5-fluoropyrimidines is mainly through RNA incorporation and thymidylate synthase inhibition rather than DNA fragmentation from DNA excision repair

(Pettersen HS, Visnes T, Vågbø CB, Doseth B, Kavli B and Krokan HE)

List of Abbreviations

4-AN	4-amino-1,8-naphthalimide
5-FU	5-fluorouracil
5-FdUMP	5-fluoro-2'-deoxyuridinemonophosphate
5-F(dU)	5-fluoro-2'-deoxyuridine
5-FdUTP	5-fluoro-2'-deoxyuridine triphosphate
5-F(rU)	5-fluorouridine
8-oxoG	7, 8-dihydro-8-oxoguanine
9-1-1	Rad9-Rad1-Hus1 heterotrimer
AID	Activation-Induced Deaminase
ALKBH1-8	alkB, alkylation repair homolog (E. coli) 1-8
AP-1	Activator protein-1
APC	Adenomatous polyposis coli
APE1	APEX nuclease (multifunctional DNA repair enzyme) 1
ApnI	AP endonuclease 1(S. cerevisiae)
APOBEC	Apolipoprotein B mRNA editing enzyme
AP-site	Apurinic or apyrimidinic site
ATPase	Adenosinetriphosphatase
Bcl-2	B-cell CLL/lymphoma 2
BER	Base excision repair
cccDNA	Covalently closed circular DNA
CHO	Chinese hamster ovary
CIN	Chromosomal instability
COX IV	Cytochrome c oxidase subunit IV
CpG	Cytosine-phosphate-Guanine
CS	Cockayne syndrome
CSR	Class-switch recombination
DHFR	Dihydrofolate reductase
DNA2	DNA replication helicase 2 homolog (yeast)
DNA-PK	Protein kinase, DNA-activated
DNMT3b	DNA (cytosine-5-)-methyltransferase 3 beta
dRP	Deoxyribosephosphate
dRPase	Deoxyribosephosphate phosphodiesterase
DSB	Double-strand break
dsDNA	Double-stranded DNA
dUTPase	Deoxyuridine triphosphatase
EXO1	Exonuclease 1
FADD	Fas (TNFRSF6)-associated via death domain
FaPyA	4,6-diamino-5-formamidopyrimidine
FaPyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FEN-1	Flap structure-specific endonuclease 1
FTO	Fat mass and obesity associated
GEN1	Gen1 homolog 1 endonuclease (drosophilia)
GG-NER	Global genomic nucleotide excision repair
H2AXγ	H2A histone family, member X (phosphorylated)
HIGM	Hyper-IgM Syndrome

5-hm(dU)	5-hydroxymethyl-2'-deoxyuridine
HMGB1	High-mobility group box 1
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Homology-directed repair
HR23B	RAD23 homolog B (S. cerevisiae)
Hsp70	Heat-shock protein 70kDa
Hus1	HUS1 checkpoint homolog (S. pombe)
IR	Ionising radiation
Ku70,80	Ku antigen 70 and 80 kDa
LIG1	DNA ligase I
LIG3	DNA ligase III
LIG4	DNA ligase IV
LP	Long patch BER
M1G	Pyrimido[1,2-a]purin-10 (3H) –one
MAP	MUTYH-associated polyposis
MBD4	Methyl-CpG binding domain protein 4
MCM7	Minichromosome maintenance complex component 7
MDA	Malondialdehyde
MED1	Methyl-CpG binding endonuclease 1 (aka MBD4)
MEF	Mouse embryonic fibroblast
MGMT	O-6-methylguanine-DNA methyltransferase
MLH1,2,3	MutL homolog 1, 2 and 3
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MNNG	N-Methyl-N'-Nitro-N-Nitrosoguanidine
MNU	N-methyl-N'-nitro-N-nitrosoguanidine
MPG	N-methylpurine-DNA glycosylase
Mre11	Meiotic recombination 11 homolog A (S. cerevisiae)
MRN	Mre11-Rad50–Nbs1 heterotrimer
MSH	MutS homolog
MSI	Microsatellite instability
mtDNA	Mitochondrial DNA
MTH1	MutT-homolog 1
MutLα	MLH1-PMS2 heterodimer
MutLβ	MLH1-MLH2 heterodimer
MutLγ	MLH1-MLH3 heterodimer
MutSα	MSH2-MSH6 heterodimer
MutSβ	MSH2-MSH3 heterodimer
MUTYH	MutY homolog (E. coli)
MX	Methoxyamine
NAD+	Nicotinamide adenine dinucleotide
Nbs1	Nijmegen breakage syndrome 1 (nibrin)
NEIL1,2,3	Nei endonuclease VIII-like 1,2 and 3 (E. coli)
NEM	N-Ethylmaleimide
NER	Nucleotide excision repair
NF-kB	Nuclear factor-kB
NHEJ	Non-homologous end-joining

NIR	Nucleotide incision repair
NTHL1	Nth endonuclease III-like 1 (E. coli)
OGG1	8-oxoguanine DNA glycosylase
p53	Tumour protein p53
PAR	Poly (ADP-ribose)
PARG	Poly (ADP-ribose) glycohydrolase
PARP-1	Poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PMS2	PMS2 postmeiotic segregation increased 2
PNKP	Polynucleotide kinase 3'-phosphatase
POLαβτδελ	DNA polymerase α, β, γ,δ,ε,λ
PUA	3'phospho-α,β-polyunsaturated aldehyde
Rad1	RAD1 homolog (S. pombe)
Rad50	RAD50 homolog (S. cerevisiae)
Rad52	RAD51 homolog (RecA homolog, E. coli)
Rad9	RAD9 homolog (S. pombe)
RAR	Retinoic acid receptor
RFC	Replication factor C
ROS	Reactive oxygen species
RPA	Replication protein A
RXR	Retinoid X receptor
SHM	Somatic Hyper-mutation
siRNA	Small interfering RNA
SMUG1	Single-strand-selective monofunctional uracil-DNA glycosylase 1
SN	Single-nucleotide BER
SSB	Single-stranded DNA break
ssDNA	Single-stranded DNA
SUMO	Small ubiquitin-like modifier
TC-NER	Transcription-coupled nucleotide excision repair
TDG	Thymine-DNA glycosylase
THF	N5,N10-methylenetetrahydrofolate
TMZ	Temozolomide
TS	Thymidylate synthase
TTD	Trichothiodystrophy
UDG	Uracil-DNA glycosylase
Ugi	Uracil-DNA glycosylase inhibitor
UNG 1, 2	Uracil-DNA glycosylase 1 and 2
VDAC1	Voltage-dependent anion channel 1
WRN	Werner syndrome protein, RecQ helicase-like
XLF	XRCC4-like factor
XP	Xeroderma Pigmentosum
XRCC	X-ray repair complementing defective repair 1
YB-1	Y box binding protein 1

1. INTRODUCTION

A human embryo develops from a single cell at the time of conception into a multitude of different cells that comprise the adult body. Decades later, these cells will collectively have the experiences of a lifetime, while the genetic material will remain essentially unchanged. Genetic information is also stable at far longer timescales, as the genetic information that constitutes a human, chimpanzee, mouse or whale show far more similarity than morphology would suggest. Yet, while DNA is apparently exceedingly stable, it is far from chemically inert. The chemical structure of DNA is altered through chemical reactions with a multitude of exogenous chemicals such as those found in cigarette smoke, as well as the exposure to ionising and ultraviolet radiation. Furthermore, compounds found in the cellular environment may also damage DNA. The most abundant of these is water, which is present at a concentration of ~55 M.

1.1 Base loss

Water can react with DNA in several ways. The N-glycosidic bond between base and sugar is particularly susceptible to hydrolysis, resulting in base loss and the generation of an exposed deoxyribose site called an apurinic/apyrimidinic (AP) site in DNA [1]. Purines are lost from DNA at a higher rate than pyrimidines. Guanine hydrolyses at a slightly higher rate than adenine, while the loss of pyrimidines is ~20 times slower than that of guanine. The rate of depurination is 4 times higher in ssDNA than in dsDNA [2]. It has been estimated that this takes place approximately 10000 times per human diploid cell per day [2,3].

1.2 Deamination

Water can also react with exocyclic amino groups in DNA bases, most frequently at 5-methylcytosine and cytosine to produce thymine and uracil, respectively. The former deaminates at a four times higher rate than the latter, and the deamination rate is several hundred-fold increased in single-stranded DNA (ssDNA). Estimates on the formation of uracil from cytosine range from 70 to a few hundreds per cell per day, depending on

how much of the DNA is assumed to be single-stranded ([4] and references therein). While 5-methylcytosine deaminates at a higher rate than cytosine, it is much rarer in the human genome. Thus, approximately 10% of cytosine deaminations occur at 5-methylcytosines [1]. In addition, the exocyclic amino groups of adenine and guanine are vulnerable to hydrolytic deamination, producing xanthine and hypoxanthine, respectively. However, these products are formed at only about 2-3% the rate of cytosine deamination. These exocyclic amino groups are involved in Watson-Crick base pairing, so the products of deamination will be mutagenic. Deaminated (5-methyl) cytosine pairs with adenine, while hypoxanthine pairs with cytosine. Xanthine is non-coding (Figure 1) [1].
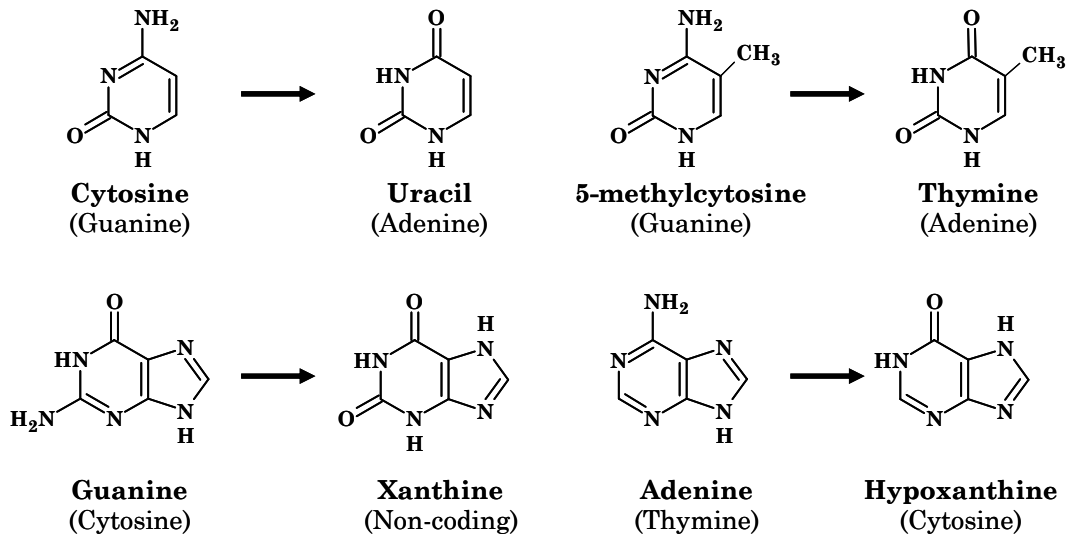
Figure 1: Hydrolytic deamination of DNA bases generates base analogues that are mis- or non-coding. The preferential base-paring partner is given in parentheses. Note that the deamination of 5-methylcytosine results in a base normally present in DNA.

1.3 Reactive oxygen species

Many processes produce reactive oxygen species (ROS) in the cell. Up to 0.2% of the oxygen consumed in normal oxidative respiration in mitochondria are converted to superoxide ions (O_2^-) [5], which is further converted to hydrogen peroxide (H_2O_2) and the very reactive hydroxyl radicals ($^\bullet OH$) inside the cell. Furthermore, similar ROS are created as a consequence of ultraviolet light, inflammation, cell injury, phagocytosis, and the hydroxylation of steroids and drugs [6]. ROS oxidation of DNA results in

single- and double-strand breaks, AP-sites, as well as a multitude of modified bases [6,7]. For pyrimidines, the double bonds between 5 and 6 positions are especially vulnerable, as are methyl groups in thymine and 5-methylcytosine. Purines are frequently oxidised in the 8-position, which may create ring-opened formamidopyrimidines (FaPyA and FaPyG), and a multitude of other lesions [7]. Many of these appear to be generated in large amounts endogenously in mammalian cells, and may be mis- or non-coding, mutagenic and/or cytotoxic. Quantitation of these lesions is problematic, however, and estimates vary by several orders of magnitude. Furthermore, reactions of ROS with polyunsaturated membrane lipids produce potent DNA-reactive agents as by-products. These yield several mutagenic etheno- and propanobase adducts [8], the best studied is malondialdehyde (MDA), which predominantly produces pyrimido[1,2-*a*]purin-10 (*3H*) -one, abbreviated M1G, in DNA [9]. Additionally, ionising radiation (IR) produces ROS in large amounts. IR is naturally present in the environment, as a result of the disintegration of naturally occurring radionuclides, or may be extra-terrestrial in origin. IR damages DNA directly through the excitation and ionisation of bases and sugars in DNA or indirectly through the generation of ROS. IR induces localised base damage, single- and double-strand breaks, and is used in the treatment of cancer [10].
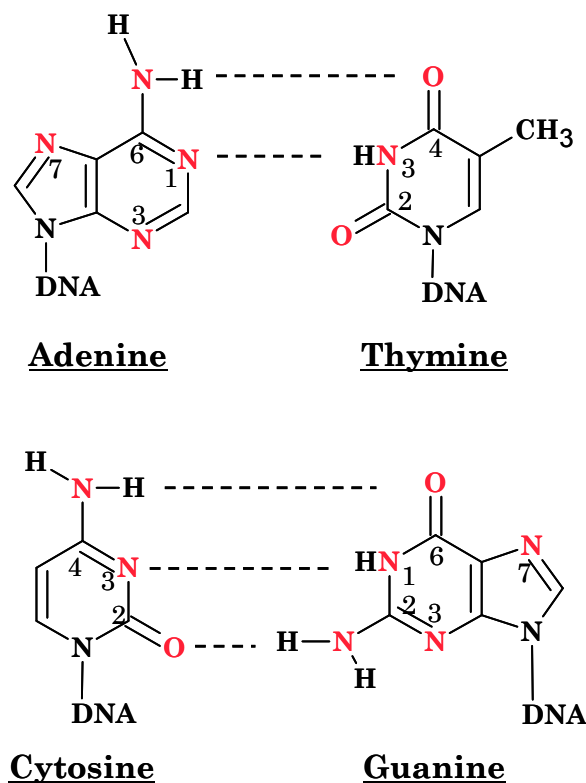
Figure 2: Susceptibility of alkylation at extracyclic oxygens and ring nitrogens in DNA bases. Many alkylations interfere with Watson-Crick hydrogen bonding, and generates mis- or non-coding adducts.

1.4 Alkylating agents

Endogenous alkylating agents participate as methyl-donors in many biochemical reactions, and are ubiquitous in mammalian cells. Exogenous alkylating agents exist in cigarette smoke, environmental toxins and products of incomplete burning of biomass. Many are carcinogens, such as benzo(a)pyrene. Both endo- and exogenous alkylating agents may interact and adduct nucleophilic centres in DNA bases, i.e. at positions occupied by oxygen and nitrogen atoms (Figure 2) [10,11]. One of the most abundant endogenous methyl donors is S-adenosyl-L-methionine, which has the potential to alkylate bases in DNA, predominantly resulting in 4000 7-methylguanines, 600 3-methyladenines, as well as 10-30 O^6-methylguanines per human cell per day [12]. These, and other examples of methylated bases, are found in samples from human cells at steady state levels at around one per 10^7 nucleotides, give or take an order of magnitude [13]. The biological consequences of these lesions are diverse. While 7-methylguanine is thought to be rather harmless, 3-methyladenine blocks replication and is highly cytotoxic, and O^6-methylguanine is highly mutagenic and cytotoxic [1]. While

many of the alkylating agents are carcinogenic, such as the tobacco-specific nitrosoamines [14], they may also be used to treat cancer. Monofunctional agents (e.g. carrying one reactive group) such as temozolomide (TMZ) methylate DNA bases, while bifunctional agents, i.e. those carrying two reactive groups such as melphalan, also have the capacity to crosslink two different bases that can be on the same or different strands of DNA [10]. Thus, exogenous alkylating agents are of vital importance for both the generation and treatment of human cancer.

1.5 Misincorporation by polymerases

In addition to the threats posed by reactive compounds constantly present in the cellular environment, enzymes that exert their normal function may also alter or damage the sequence of DNA. One example of this includes the introduction of mismatches by DNA polymerases, which has a small probability of introducing mismatched nucleotides during DNA synthesis. A suboptimally balanced nucleotide pool may further decrease the replication fidelity. Under these conditions, a high or low concentration of one or more nucleotides may lead to the formation of non-Watson-Crick base pairing (reviewed in [15]). Furthermore, replicative DNA polymerases tend to incorporate dNTPs carrying a base with similar structure as the four canonical bases. Hence, dGTP which is easily oxidised in the 8-position (8-oxo-dGTP) is readily incorporated into DNA. During replication, its incorporation is precluded by MutT-homolog 1 (MTH1), which hydrolyses 8-oxo-dGTP to 8-oxo-dGMP [16].

A similar preclusive mechanism acts on dUTP, which is a normal intermediate during *de novo* synthesis of dTTP. dUTPase hydrolyses dUTP to dUMP, which is in turn is converted to dTMP by reaction with N5,N10-methylenetetrahydrofolate (THF) catalysed by thymidylate synthase (TS). The inhibition or lack of either enzyme or THF due to dietary factors, results in an increased dUTP/dTTP ratio. As the replicative polymerases have a similar K_M towards these nucleotides, dUTP is readily incorporated into DNA resulting in U:A base pairs according to standard Watson-Crick base pairing. These are not mutagenic by themselves, but because the repair of U:A to T:A may employ a polymerase with a higher error frequency than replicative polymerases, the resulting repair of U:A may well result in mutagenesis [17]. Furthermore, the

replacement of uracil with thymine in DNA alters the binding of transcription-factors [18]. While substantial amounts of thymine can be replaced with uracil in the genomes of genetically engineered *E. coli* and *S. cerevisiae*, they will eventually stop dividing due to a "general failure of macromolecular biosynthesis" [19,20]. Furthermore, replacing about ~1% of thymines with uracil in *S. cerevisiae* results in a mutator phenotype characterised by AT to CG transversions [21].

Mammalian cells accumulate uracil in their genomes too. This is aggravated by treatments which result in a higher dUTP/dTTP ratio, i.e. methotrexate inhibits dihydrofolate reductase (DHFR), thus reducing the availability of THF to be used as a methyl-donor in dTMP synthesis [22]. Folic acid and vitamins B6 and B12 are all required for the biosynthesis of THF. THF will not be replenished if the supply of these nutrients is low enough, ultimately leading to an increased uracil misincorporation into DNA of mammalian cells. Importantly, folate deficiency in humans are linked to several disorders including colon cancer, neurodegeneration and birth defects ([23]; reviewed by [24]).

The manipulation of TS activity is exploited in the treatment of cancer, as treatment with fluoropyrimidines leads to inhibition of TS. The fluoropyrimidines are inter-converted to a variety of fluorinated ribonucleotides and deoxyribonucleotides inside the cell. 5-fluoro-2-deoxyuridine monophosphate (5-FdUMP) binds with high affinity to TS and inhibits the enzyme. This depletes the level of dTTP, necessary for DNA synthesis, Moreover, the dUTP/dTTP ratio increases, which results in insertion of dUMP into DNA. Finally, imbalanced nucleotide pools may lead to the generation of mispairs by replicative polymerases [25,26,27]. However, this is not the only proposed cytotoxic mechanism of fluoropyrimidines, as 5-FU is incorporated into both RNA and DNA [28]. 5-FU pairs most efficiently with adenine, but may also pair with guanine through a pH-dependent ionisation of the base [29]. The removal of 5-FU by DNA repair processes could contribute to the cytotoxicity of the drug [25] either as a consequence of repair, or indirectly as a consequence of utilising a skewed nucleotide pool for repair [15]. Finally, the incorporation into RNA disrupts rRNA, tRNA and mRNA, as well as the processing of uridine into pseudouridine [30,31,32,33,34,35]. All

14

of these mechanisms can probably contribute to cytotoxicity to some extent, but even after half a century of clinical use the relative contribution of each is still a matter of dispute.

While the examples mentioned so far have described damage to DNA due to interaction with endogenously occurring reactive substances in a more or less random manner, endogenously encoded enzymes may also specifically damage DNA. Human cells contain a number of enzymes in the apolipoprotein B-editing catalytic polypeptide (APOBEC) family, that deaminates cytosine to uracil in nucleic acids, thus potentially yielding a CG->TA mutation [36]. The best studied of these is the activation-induced deaminase (AID), which specifically deaminates cytosine residues in immunoglobulin loci in maturating B-cells. This is required for class-switch recombination (CSR) as well as somatic hypermutation (SHM) [37,38]. Others, e.g. APOBEC3G, deaminates retroviral genomes in the cytosol, thereby restricting their replication [39].

Thus, even if we disregard exogenous threats such as IR and environmental chemicals, the DNA of human cells are under constant assault from reactive components of the cellular environment, in sum totalling at the very least a few ten thousands DNA lesions per cell per day, most of which are potentially mutagenic. Yet the DNA of human cells are replicated with an impressive accuracy - less than one of the $3.2 \cdot 10^9$ base pairs in the human genome are mutated per replication [40]. However, several DNA repair mechanisms maintain the chemical and sequential integrity of the genome by removing DNA damage prior, during and after replication.

1.6 DNA damage and cancer

In contrast to somatic cells, which replicate their DNA with high fidelity, are cancer cells characterised by the accumulation of mutations of all types. The most striking examples are provided by the fact that most cancer cells are not diploid, i.e. they carry an abnormal number of chromosomes, which in turn alters the expression of thousands of genes [41]. Alternatively, chromosomes may also contain insertions, deletions, amplifications, rearrangements or translocations of large chromosomal segments. These may generate oncogenic fusion proteins, or put normally coding genes under the control

of different promotors [42]. Such chromosomal changes are referred to as chromosomal instability (CIN). Yet another example of genetic instability is hereditary non-polyposis colon cancer (HNPCC). HNPCC is characterised by a rather stable number and structure of chrommomsomes, but is associated with changes in the number of simple repetitive sequences 1-6 nucleotides in length. This may potential result in inactivating frameshift mutations. Such repetitive sequences are called microsatellites and hence, variation in the number of repeats is referred to as microsatellite instability (MSI) [43]. Additionally, tumour cells tend to accumulate point mutations more frequently than normal tissue [44,45,46]. And even when the nucleotide sequence is preserved, epigenetic changes in methylation status may very well alter the expression of genes that promote tumourigenesis, as demonstrated for the human *MLH1*-gene [47].

Is the documented genomic instability a cause of or consequence of cancer? Is it an early or late event in carcinogenesis? It has been argued that the sheer volume of genetic changes observed in cancer cells is so large that it could not have arise as a result of a normal mutation rate. Thus, an enhanced mutation rate brought about by random mutations in genes responsible for the stability of DNA (e.g. DNA repair genes) could well be an early event in tumourigenesis [46,48]. This hypothesis is, however, debated [49]; some maintain that an instability at the chromosome-level is sufficient to explain cancer [41], others argue that a mutator phenotype – at any level – is not necessary at all. In this scenario, rare mutations in genes that confer some kind of growth advantage to the cell will be selected for. Thus, given enough cell divisions and natural selection, they argue that normal mutation rates may well account for the genetic variability of human cancers [50].

2.0 DNA REPAIR MECHANISMS

The DNA in a human cell is continuously challenged by various modifications and alterations even in the absence of exogenous DNA damaging agents. Yet, human cells are able to cope with these challenges and replicate with high fidelity by employing enzymatic systems that detect and repair damaged DNA. In many cases the repair is error-free, returning DNA to the state it was in before the lesion. Repair may, however, also be error-prone, thus 'repairing' DNA to something else than the original state. About 150 human genes are currently identified as or (suspected to be) implicated with DNA repair. A frequently updated table summarising these genes, and containing links to relevant databases are found at http://www.cgal.icnet.uk/DNA_Repair_Genes.htm [51,52].

2.1 Direct reversal of DNA damage

The simplest imaginable mechanism of direct damage reversal is demonstrated by ligases, which re-join strand breaks generated by e.g. oxidative damage [10].

The AlkB-homologue family, of which there are at least nine members in the human genome, provides an example of direct repair of methylated bases in nucleic acids [53,54]. The bacterial AlkB-enzyme removes alkyl-groups from N-1 position of adenine, and the N-3 position of cytosine, in a process requiring Fe^{2+}, 2-oxoglutarate and molecular oxygen. The offending alkyl group is oxidised to an unstable hydroxyalkyl-moiety, which spontaneously de-associates from the base as formaldehyde. This restores DNA to its original state, at the energetic expense of the concurrent conversion of 2-oxoglutarate to succinate and CO_2 [55], reviewed in [56]. All the nine known human AlkB-homologues are expressed [54,57], but biochemical activities have hitherto only been identified for the fat and obesity associated protein (FTO), ALKBH1, 2 and 3 [54,58,59,60].

The protein O^6-methylguanine-DNA methyltransferase (MGMT) recognises and repairs guanine alkylated at the O^6-position or thymine alkylated the O^4-position. These lesions are formed by reaction with endogenous and exogenous alkylating agents. These are

pre-mutagenic and pre-toxic lesions, as they preferentially form base pairs with thymine and guanine, respectively [61,62]. MGMT removes the offending alkyl-group by permanently transferring it to a cysteine residue in the active reaction site. As a consequence, the protein is inactivated, ubiquitinylated [63] and swiftly degraded by the proteasome [64]. Thus, the repair of a single alkylation requires the synthesis and degradation of a whole 22 kDa protein. It follows from this that the number of MGMT molecules per cell dictates the capacity to repair such alkylated lesions directly. MGMT upregulation increases the cells' tolerance to alkylating agents significantly [65]. On the other hand, if MGMT is knocked down [66,67] or out [68] the cells become hypersensitive (reviewed in [69]).

2.2 Repair of double strand breaks

Double-strand breaks (DSB) are cytotoxic lesions where the backbones of both DNA strands are cleaved. DSBs come in two forms; two-ended DSBs describe a simple fracture of DNA, where one DNA double helix is broken in two. This may happen at any stage of the cell cycle, as a consequence of IR, physical stress or the repair of closely positioned lesions at opposite strands. On the other hand, one-ended DSBs are generated during S-phase or G2, and happens when a replication fork encounters a single-strand break (SSB) [70]. The severity of DSB can be seen when there is a failure to repair them, which may lead to cell death or large scale chromosomal rearrangements in the form of insertions, deletions and translocations [71].

Human cells have at least two distinct mechanisms for the repair of DSBs, non-homologous end-joining (NHEJ) and homology-directed repair (HR). As the names may suggest, the former process is less accurate than the latter, and is simpler mechanistically. In NHEJ, a heterodimer of Ku70 and Ku80 binds to each DSB. These in turn recruit DNA-dependent protein kinase (DNA-PK), which becomes activated and
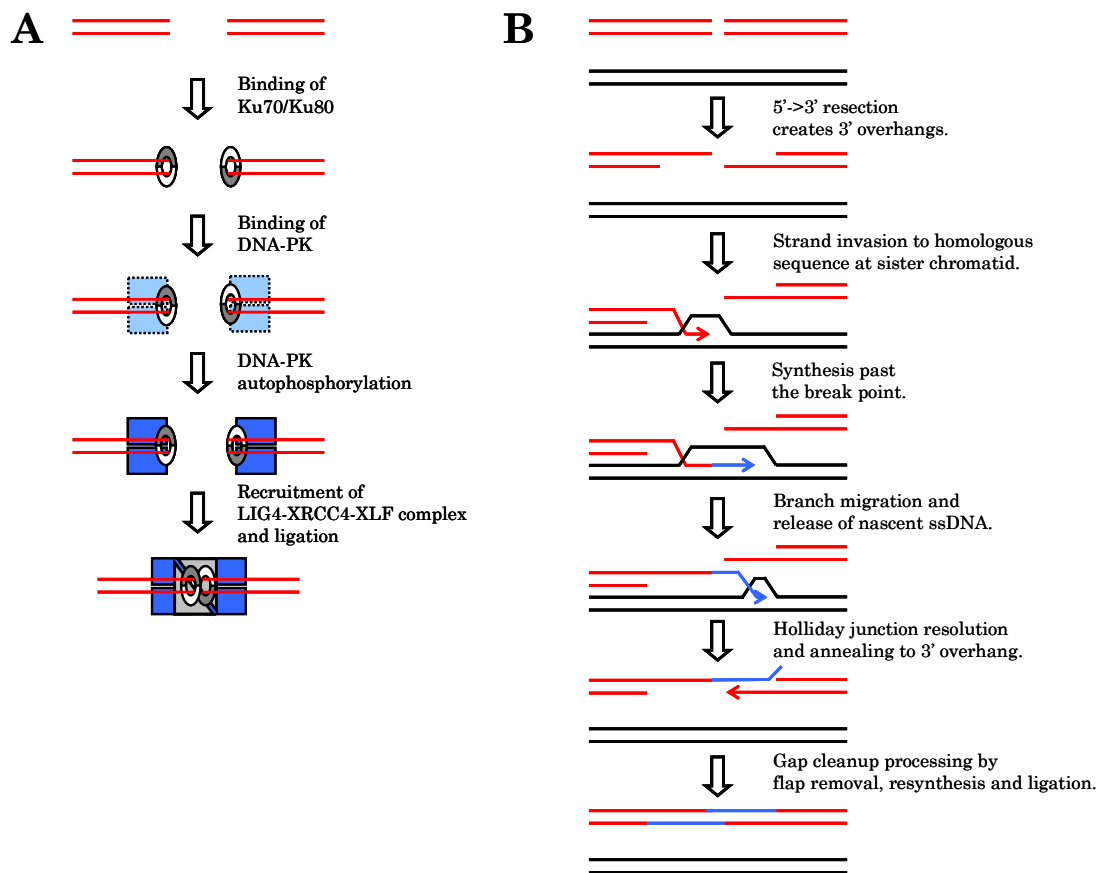
18

Figure 3: Repair of double-strand breaks. (A) Non-homologous end-joining of a double strand break. Ku70/Ku80 heterodimers and DNA-PK are sequentially recruited to the double strand breaks, followed by (auto)phosphorylation of DNA-PK and nearby proteins. The two broken strands are brought together and ligated by a complex containing LIG4, XRCC4 and XLF. **(B)** Homology-directed repair of a double-strand break. 5' ends are degraded, and the resulting 3' overhang invades a DNA strand containing a homologous sequence, e.g. in the sister chromatid. DNA is synthesised past the break point (blue lines), followed by branch migration. The nascent DNA is released and allowed to anneal to the other side of the strand break, thus connecting the two ends of DNA. After the Holliday junction has been resolved, flaps, gaps and nicks are processed.

phosphorylates itself and other proteins when two ends of DNA are positioned opposite each other. Finally, the two DNA ends are joined by a complex containing DNA ligase IV (LIG4), XRCC4 and XLF [70]. If the DSBs arose from IR it is likely that both strands of DNA contain multiple lesions (so-called dirty ends), in which case additional processing is required to rejoin ends. Several proteins seem to be involved in processing of dirty ends, including – among others - Aprataxin, the Werner syndrome protein (WRN), Artemis, Mre11-Rad50–Nbs1 (MRN) -complex and DNA polymerases μ and λ [70,72]. NHEJ has limited specificity in that it joins two ends of DNA, thus potentially joining 'wrong' ends, which may lead to gross chromosomal rearrangements. Additionally, a few base pairs may be lost during the joining process. HR, on the other

hand, is able to rejoin ends in an error-free manner and even restores missing sequence information. It achieves this by using a homologous template located elsewhere in the cell, preferentially on the sister chromatid. The MRN-complex, which degrades one of the strands in the 5'->3' direction initiates this process [73]. RAD51 and associated proteins then bind the remaining ssDNA and guide it to a homologous sequence elsewhere in the genome [74,75]. The free 3' end on the invading strand primes DNA synthesis, which continues past the break point of the original homologous sequence, thus generating a Holliday-like structure. This allows the recessed side of the other strand break to anneal to the newly replicated strand. The original sequence is restored after the Holliday junction has been resolved by symmetrical nicking of both strands by Gen homolog 1 (GEN1) [76], then if necessary followed by removal of flaps, gap resynthesis and nick ligation [10,70]. The examples given above are, however, only one of several possible DSB-repair mechanisms.

2.3 Mismatch repair (MMR)

DNA is usually replicated at a very high fidelity, with the four canonical bases in DNA binding to each other in a manner described by Watson-Crick base pairing [77]. That is, however, not always the case, as mispairing can be introduced by incorporating the wrong nucleotide during DNA synthesis, strand slippage during replication of repeat sequences, recombination involving non-identical sequences or chemical alteration of bases [78]. These lesions are all potentially mutagenic and substrates for mismatch repair, which removes the mismatch along with a relatively large fragment of DNA followed by re-synthesis. Furthermore, MMR is involved in many diverse processes, including antibody diversification, regulation of recombination and crossovers, as well as the DNA damage response [79]. Mechanistically, the obvious challenge for MMR is to distinguish the newly replicated strand containing an erroneous base from the template strand. The human MMR system is initiated by a MutS heterodimer, of which there are at least two in human cells. MutSα comprises the MutS homologues (MSH) 2 and 6 and recognises simple base-base mismatches and small insertion-deletion loops (≤ 2 bases), while MutSβ
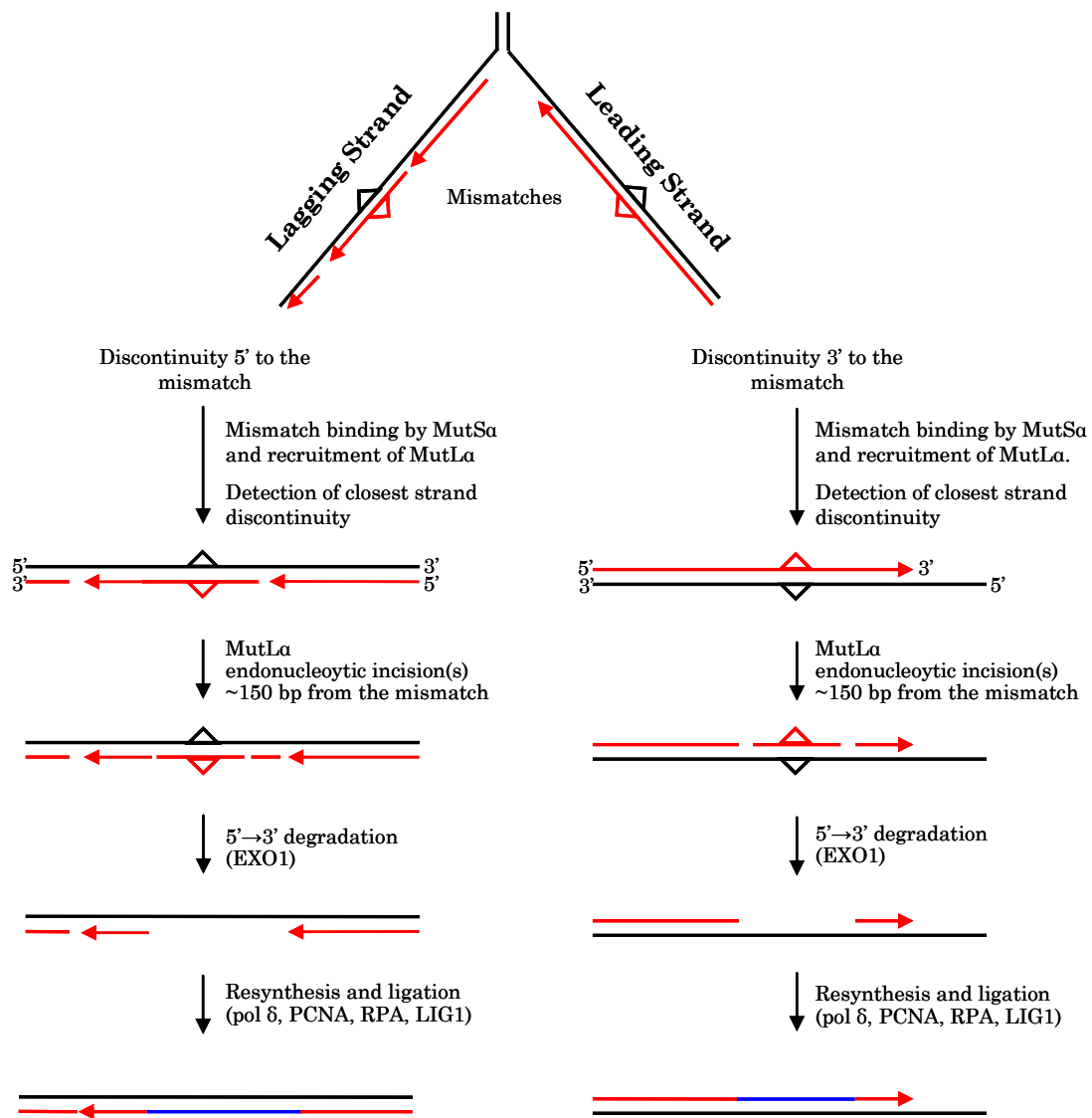
Figure 4: Correction of replicative mismatches by MMR. Mismatches are generated during semi-conservative DNA synthesis (red). They are recognised and bound by MutSα followed by recruitment of MutLα. These genereate sliding clamps which translocates along DNA until a strand discontinuity is encountered. In the leading strand this strand discontinuity is located 3' to the mismatch, while in Okazaki fragments, the strand discontinuity may be 3' or 5' to the mismatch. If the closest strand discontinuity is located 5' to the mismatch (left), EXO1 will be loaded onto the SSB and degrade the all DNA between the discontinuity and ~150 nucleotides past the mismatch. However, EXO1, which is exclusively 5'→3', can not act directly if the closest discontinuity is located 3' to the mismatch (right). Here, MutLα will generate one or more incisions around the mismatch in the strand that harbours the discontinuity and load EXO1, which then degrades the strand containing the mismatch in the 5'→3' direction. In both cases resynthesis (blue) and ligation are performed by POLδ, PCNA, RPA, RFC and LIG1.

(containing MSH2 and 3) recognises larger insertion-deletion loops [78]. The binding of a MutS-heteroduplex to a mismatch leads to the recruitment of one of three MutL-heteroduplexes, and the formation of a ternary complex containing the mismatch and the MutS-MutL heteroduplexes. This forms a sliding clamp that translocates along the DNA in either direction at the expense of ATP hydrolysis until it encounters a strand

break that acts as a signal to discriminate the nascent and template strands [79]. If the strand break is positioned 5' to the mismatch, the strand between the nick and the mismatch, as well as some 100-150 nucleotides past the mismatch, is degraded by exonuclease 1 (EXO1) [80,81,82]. If the strand break is 3' to the mismatch, an endonuclease in the PMS2 subunit of MutLα is activated, which incises the nascent strand ~150 nucleotides 5' to the mismatch [80,83] followed by EXO1 degradation. Repair is then completed by the synthesis of a new strand by DNA polymerase δ or ε, aided by proliferating cell nuclear antigen (PCNA), replication factor C (RFC) and replication protein A (RPA), followed by ligation by DNA ligase I (LIG1). The proteins mentioned above are sufficient to reconstitute both 5' and 3' nick-directed MMR *in vitro* (Figure 4). However, many additional factors are shown to interact with the central MMR machinery [84], and the mechanisms briefly outlined above are therefore probably more intricate *in vivo*.

Deficiencies in the core MMR components are mutagenic, and may lead to point mutations as well as MSI, characterised by variations in the number of repeats at repetitive sequences. The consequence of this at the level of the mammalian organism is HNPCC [43]. In addition, MMR deficient cells tolerate many DNA damaging agents. Examples include S_N1-alkylating agents (MNNG, MNU), intrastrand cross-linking agents such as cisplatin [85,86], antimetabolites such as 6-thioguanine [87,88] and fluoropyrimidines [89,90,91,92]. When challenged with these agents, MMR proficient cells arrest in G2/M [93,94] and may eventually undergo apoptosis [95], while MMR deficient cells continue to divide at the expense of genomic stability. Two not mutually exclusive hypotheses exist to explain this. The first notes that many of these agents damage both strands of DNA. Since MMR is directed towards the newly replicated strand, it is unable to repair damage in the template strand. Thus, MMR may excise and try to repair the non-damaged strand, leading to the generation of another mismatch, thus initiating a cascade of repeated misincorporations opposite the offending base [96]. This concept has been termed futile repair (reviewed in [27]). Alternatively, the recognition (and repair) of lesions by MMR may initiate ATM- and/or ATR-mediated signalling cascades, which in turn arrest the cell in G2/M, and may guide the cell towards apoptosis [93,94].

2.4 Nucleotide excision repair (NER)

NER excises DNA lesions as part of an oligonucleotide, which is about 30 nucleotides in length in humans. NER does not appear to recognise DNA damage in itself, rather it detects distortions of the DNA double helix. These tend to be pyrimidine dimers introduced by UV-light, or bulky lesions introduced by chemotherapeutics or environmentally encountered chemicals (e.g. benzo(a)pyrene) [10]. Inactivating mutations in human NER genes are associated with xeroderma pigmentosum (XP), a cancer-prone syndrome resulting in epithelial skin cancer induced by exposure to sunlight, as well as Cockayne syndrome (CS) and trichothiodystrophy (TTD) [97]. NER is a multi-step process, where 20 to 30 known proteins participate in a well defined and orderly fashion (Figure 5) (reviewed in [10,98,99]. Global genomic NER (GG-NER) is initiated by recognition of the helix distortion by a heterotrimer consisting of XPC, RAD23 homolog B (HR23B) and centrin 2 [100,101], followed by binding of XPA and RPA to the damaged area (reviewed in [98]). Alternatively, if RNA polymerase II is blocked by a DNA lesion in actively transcribed genes, the CS genes A and B recruit the rest of the NER machinery and remove the stalled RNA polymerase. This mode of NER is called transcription-coupled NER (TC-NER) (reviewed in [102]). Irrespective of how repair was initiated, the next steps are thought to be identical for GG- and TC-NER. Following damage recognition, the multi-protein complex that is transcription factor IIH (TFIIH) unwinds the DNA sequence surrounding the lesion using XPB and XPD helicases, which are part of the TFIIH complex. The resulting single-stranded bubble-structure is stabilised by RPA. Then endonucleases XPG and XPF cuts the DNA backbone 5 or 6 nucleotides 3' to the lesion and 20 to 22 nucleotides in the 5' direction, respectively, thus releasing an oligonucleotide. Replicative DNA polymerases then fill in the resulting gap, using the un-damaged strand as template [103,104].
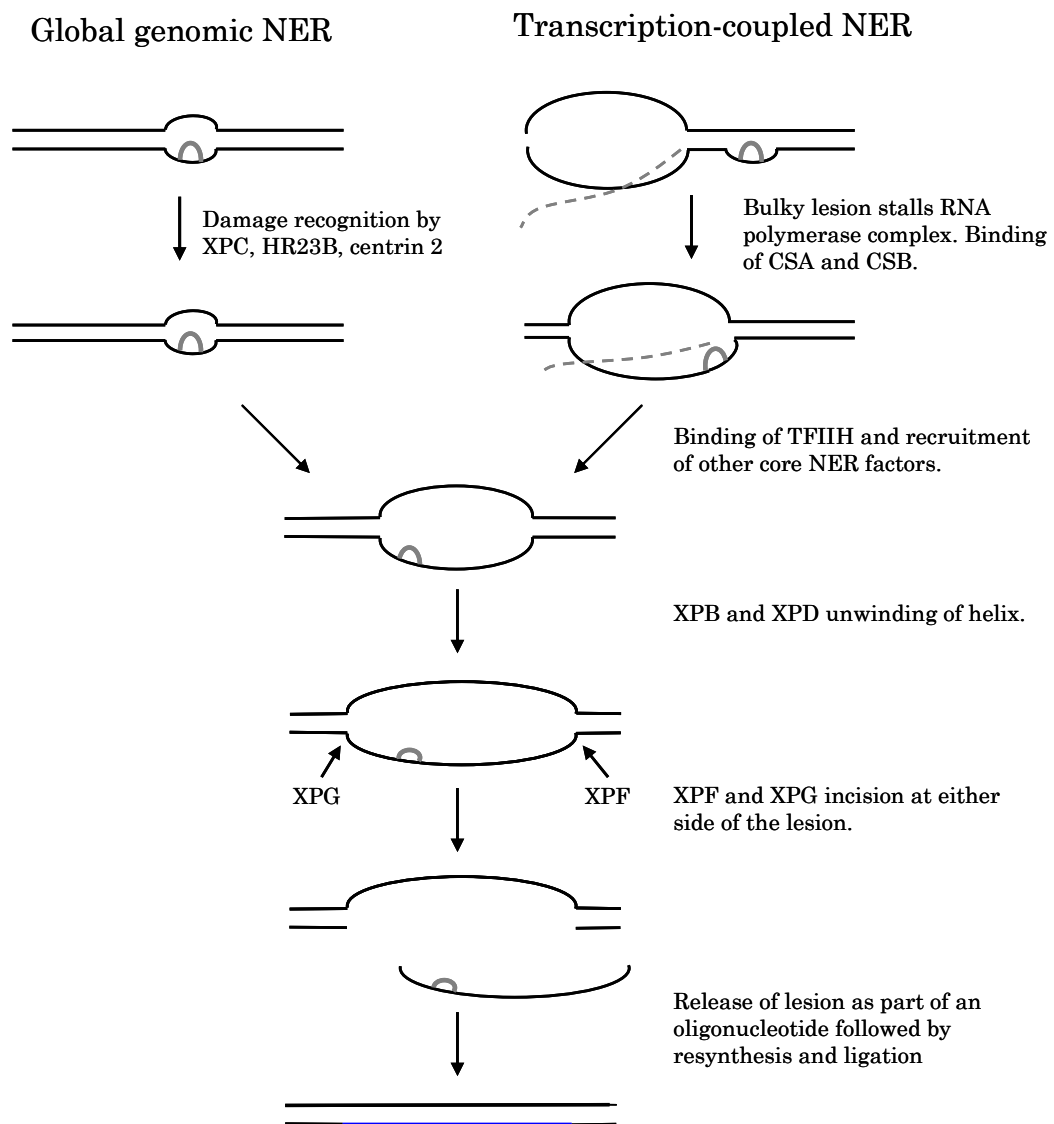
Figure 5: Nucleotide Excision Repair of bulky lesions in DNA. Bulky, helix distorting lesions anywhere in nuclear DNA are recognised by a complex of XPC, HR23B and centrin 2 (upper left branch), which then recruits core NER protein complexes (middle branch). Additionally, these bulky lesions stalls RNA polymerase on actively transcribed DNA strands (upper right branch), in which case CSA and CSB replaces the stalled RNA polymerase with core NER components (middle branch). XPB and XPD helicases unwinds the double helix surrounding the lesion, followed by incisions on either side of the lesion by endonucleases XPF and XPG. The damaged DNA is then removed as part of an oligonucleotide, followed by resynthesis and ligation.

2.5 Base excision repair (BER)

BER is initiated by a damage specific glycosylase, which recognises and excises an offending base, resulting in a free base and an AP-site. It is thought to be the quantitatively most important mode of DNA repair in mammalian cells [105]. A few glycosylases are bifunctional, in that they display an additional lyase activity that

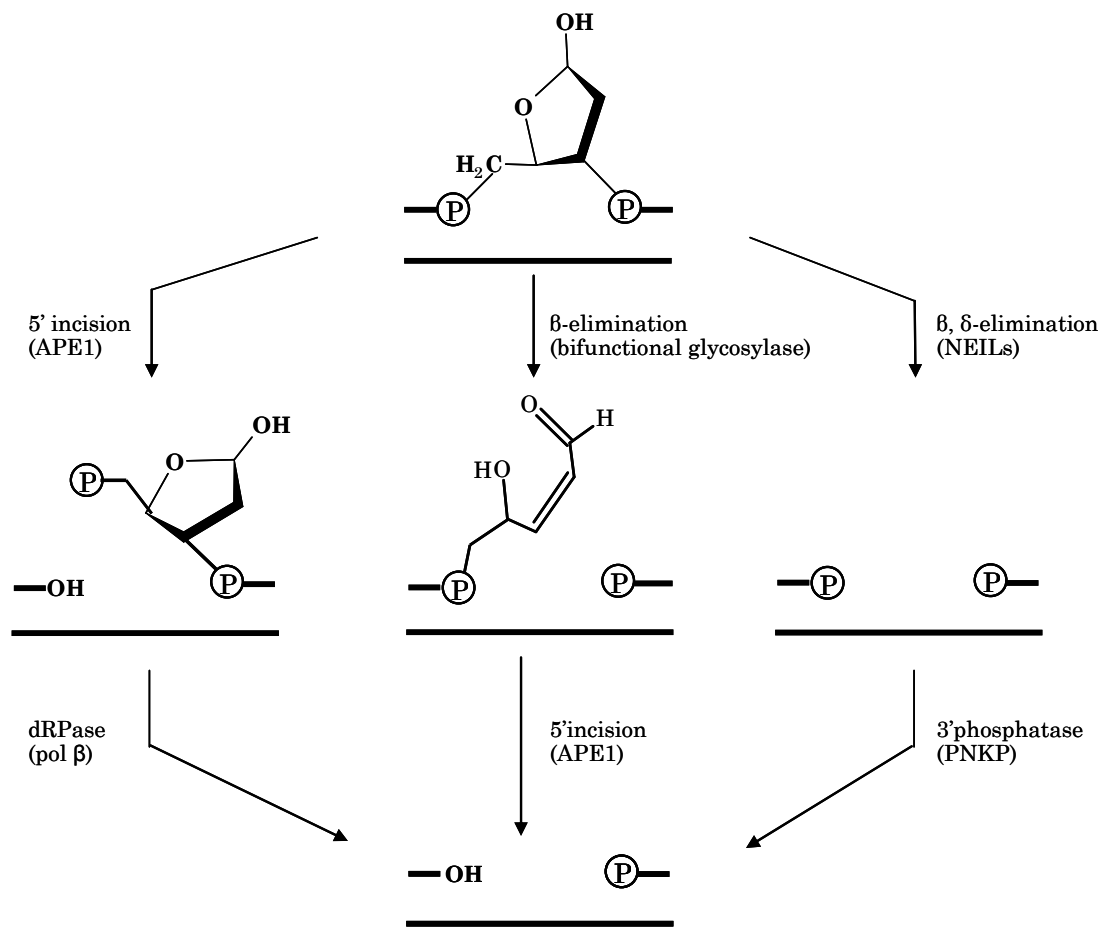incises the DNA backbone 5' and/or 3' to the deoxyribose (β and δ-elimination, respectively) (Figure 6).

Figure 6: Gap tailoring during BER. Monofunctional glycosylases generate a natural AP-site (top), at which point the DNA backbone still is intact. The resulting AP-site is then incised by APE1 at the 5' side of the phosphate, generating a 3'OH group and a 5' deoxyribosephosphate (dRP) fragment (middle, left). Bi-functional glycosylases carry associated lyase activity able to incise the DNA backbone by β-elimination, resulting in the generation of a 3'phospho-α,β-polyunsaturated aldehyde (PUA) and a 5' phosphate group (middle, middle). The PUA is released by the 3'-phospho-diesterase activity of APE1, again generating a 3'OH group. Glycosylases of the NEIL-type, are able to carry out β,δ-elimination, leaving a 1-nucleotide gap flanked by phosphates on either side (middle, right). While APE1 may act on this lesion as well, through its associated 3' phosphatase activity, it is more likely that this may be mediated by the more potent PNKP [106], or aprataxin [107].

Next, AP endonuclease 1 (APE1) incises the DNA backbone 5' to the deoxyribose, followed by the incorporation of one or several nucleotides, removal of the remaining deoxyribose fragment and ligation. This may take place by at least three slightly different sub-pathways, defined by the number of nucleotides that are incorporated (Figure 7).
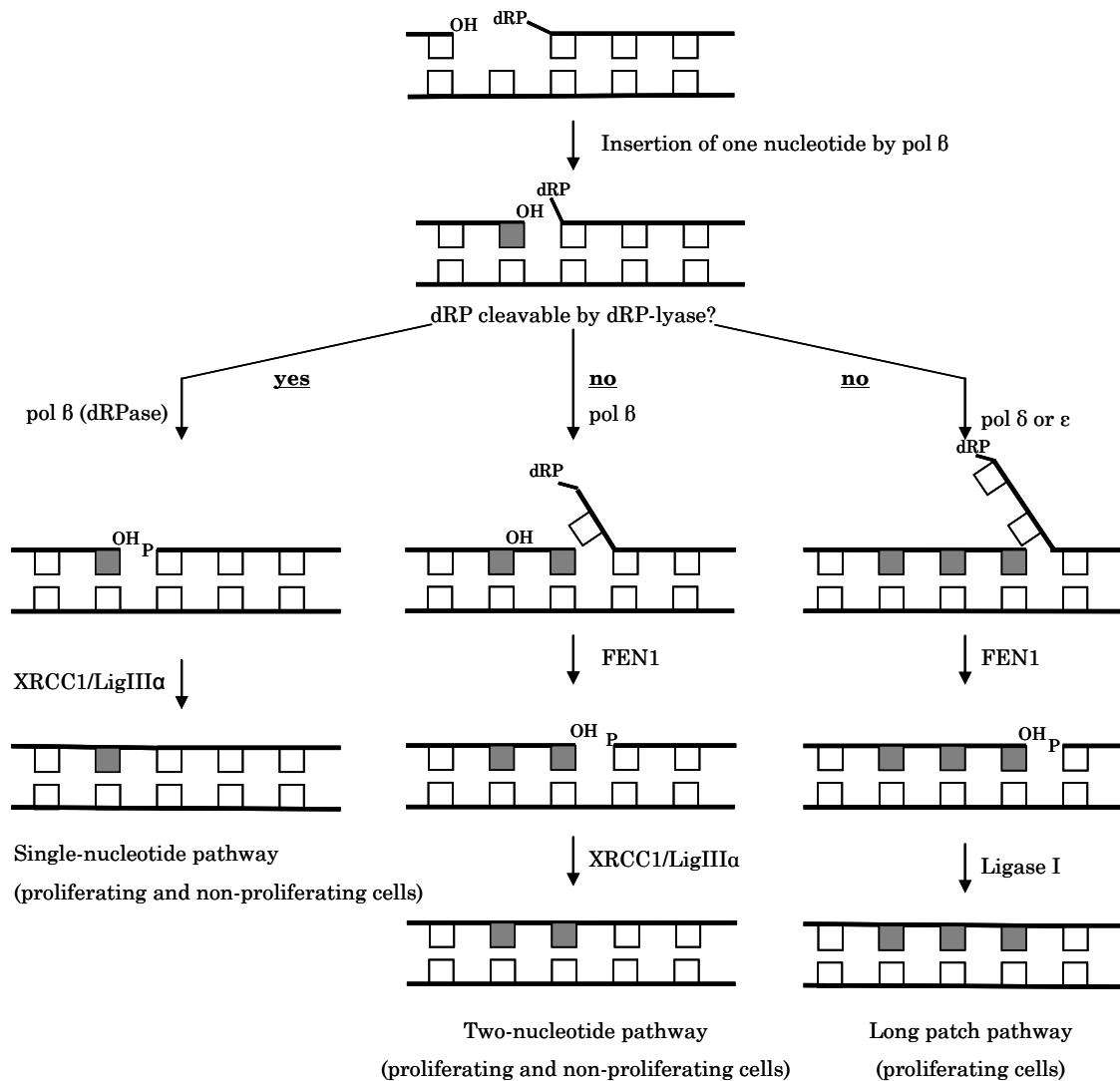
Figure 7: Replacing the excised nucleotide: SN and LP. Following gap tailoring, BER may be completed by the insertion of one or several nucleotides followed by ligation. POLβ inserts the first nucleotide in all cases. Provided that the dRP-fragment can be removed by the inherent dRP-lyase activity of the polymerase, the resulting nick can then be closed by XRCC1/Ligase IIIα complex. This is the single nucleotide pathway (left branch). If, however, the dRP-fragment is resistant to dRP-lyase removal, POLβ may insert a second nucleotide. This allows the dRP-fragment to be removed as part of a small flap (middle branch). Alternatively, a switch to replicative POLδ or ε may occur, and these may insert a longer patch of nucleotides (in this case, three) downstream of the original lesion. The displaced strand is then cleaved off by FEN-1, and the resulting nick ligated by DNA ligase I (right branch). The latter (right) pathway is exclusive to proliferating cells, while the single- and two-nucleotide pathways are employed in both proliferating and non-proliferating cells.

Single-nucleotide (SN) and long patch (LP) pathways have been successfully reconstituted *in vitro* using purified proteins. In the SN-pathway, one nucleotide is incorporated by POLβ, followed by the generation of a ligatable end by 3' deoxyribose lyase (dRPase) activity residing in the 8 kDa fragment of the same polymerase. Finally, DNA ligase IIIα in conjunction with XRCC1 ligates the nick [108]. Alternatively, the dRP-fragment may be removed as part of a single-stranded 'flap' generated by strand-

displacement synthesis. This happens if the dRP-fragment is modified in such a way that it becomes resistant to the dRPase activity of POLβ. In non-proliferating cells this is performed by POLβ, which inserts another nucleotide, followed by flap removal by flap endonuclease 1 (FEN-1) and ligation (two-nucleotide pathway) [17,108]. Alternatively, POLδ or ε (together with RFC and PCNA) may incorporate an even longer patch. The dRP-fragment is removed by FEN-1, along with the displaced nucleotides, followed by ligation by LIG1 (LP-pathway) [109,110]. The latter pathway is apparently exclusive to proliferating cells, while single- and two nucleotide pathways can be carried out in non-proliferating cells as well [17].

2.5.1 Human uracil-DNA glycosylases

The human genome contains four known genes encoding glycosylases capable of removing uracil from DNA. These are uracil-DNA glycosylase (UNG), single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1), thymine-DNA glycosylase (TDG) and methyl-CpG binding domain protein 4 (MBD4). UNG, SMUG1 and TDG adopt the same α/β core fold and belong to the same super family [111].

2.5.2 Uracil-DNA glycosylase (UNG)

The human UNG gene encodes two open reading frames driven by separate promotors and encodes the 304 amino acids in UNG1 and 313 amino acids in UNG2. They share the C-terminal 269 amino acids that are necessary and sufficient for catalytic activity, but differ in their N-terminal sequences that contain mitochondrial (UNG1) and nuclear (UNG2) localisation signals, respectively [112,113].

Uracil in both single and double-stranded DNA are the main substrates for the UNG-proteins, and they are exceptionally active relative to other glycosylases [114]. UNG-enzymes may also excise uracil-analogues with modifications in the 5' and/or 6' position that are small enough to fit into the catalytic active site of the enzyme, although at lower efficiency. Examples include 5-fluorouracil (5-FU), isodialuric acid, 5-hydroxyuracil and alloxan [115,116]. Among the biologically relevant substrates, the catalytic domain of the human UNG are most active on uracil in ssDNA, followed by uracil in dsDNA

opposite guanine, then adenine [117]. However, the surrounding base sequence has a significant effect on catalytic efficiency [117,118].

UNG1 mRNA is expressed in all tissues examined, whereas *UNG2* mRNA is mainly associated with proliferating tissues [119]. Following serum starvation, both mRNA's, as well as total activity are upregulated at the entry of S-phase, seemingly independent of ongoing DNA synthesis [119,120,121]. The protein level of UNG2 is upregulated in S-phase and degraded in G2/M or early G1. hUNG1, on the other hand, is apparently expressed rather stably through the cell cycle [119,122,123,124,125,126]. UNG2 co-localises with PCNA and RPA in replication foci, where UNG2 probably acts on misincorporated uracil [122]. Co-immunoprecipitation experiments support these observations, as not only proteins necessary for SN- and LP-pathways co-immunoprecipitate with UNG2, but also the replication-associated proteins cyclin A, MCM7 and DNA polymerase α [127,128]. Furthermore, specific inhibition of UNG-proteins remove nearly all activity on U:A base pairs in extracts from human cells [116,127,129]. In addition, the apparent inverse expression pattern of TDG and UNG2 [124], suggests that UNG2 may be the major activity acting on deaminated cytosines during S-phase. UNG1 appears to be the only uracil-DNA glycosylase in mitochondria [129], and would therefore be responsible for uracil repair in all contexts.

UNG2 is modified by post-translational phosphorylation at Ser23, Thr60 and Ser64 [123]. These phosphorylations regulate cellular turnover, as the two latter residues appears to form part of a phosphodegron, which is a signal for ubiquitinylation and proteosomal degradation subsequent to phosphorylation. Furthermore, phosphorylation at any of these sites increases activity by up to 30%. Finally, the binding to RPA is increased by phosphorylation at Ser23, and diminished by phosphorylation at Thr60 and Ser64, while binding to PCNA is relatively unaffected by phosphorylation at any of these sites [123].

Gene-targeted *Ung$^{-/-}$* knockout mice appear to develop normally, but display lymphoid hyperplasia early in life and a 22-fold increase in B-cell lymphomas at a later age compared to wild type [130,131]. A variety of cells derived from *Ung$^{-/-}$* mice

28

accumulate genomic uracil and display a slight increase in mutation rate and frequency [130,132,133,134,135,136,137]. Omitting folate from the growth medium results in a mild increase in genomic uracil and mutation rate in cultured $Ung^{-/-}$ MEFs, compared to wild type [135]. While brain cells (but not colon epithelial cells) from $Ung^{-/-}$ mice fed with a folate-deficient diet also accumulated genomic uracil, they did not display the (mild) mutator phenotype. Nevertheless, they appear more vulnerable to neurodegeneration, which may be consistent with some cognitive and behavioural changes displayed by $Ung^{-/-}$ animals that were fed a folate-deficient diet [135]. Moreover, following brain damage provoked by cerebral ischemia, murine Ung-mRNA and protein activity were upregulated, especially in the cytoplasmic/mitochondrial fraction. Moreover, in $Ung^{-/-}$ mice, brain ischemia and reperfusion resulted in an increase in infarct size compared with wild type [133]. This was further aggravated when the animals were fed a folate-deficient diet [138]. These results suggest that murine UNG may protect neurons from tissue damage brought about by folate deficiency or ischemia.

Another deviation from wild type is apparent in the acquired immune system of $Ung^{-/-}$ mice. Here, B-cells from $Ung^{-/-}$ mice display an altered mutation spectrum at dC and dG sites during somatic hypermutation (SHM). Moreover, $Ung^{-/-}$ mice exhibit defects in class-switch recombination (CSR), i.e. an altered balance of serum immunoglobulin isotypes, with higher levels of IgM and lower levels of IgG3 compared to wild type [139]. These defects in the murine acquired immunesystem may be explained mechanistically by UNG2-mediated excision of uracil, generated through cytosine deamination by AID [139]. However, since the overexpression of mutants of UNG2 with <1% wild type activity in $Ung^{-/-}$ B-cells efficiently restores CSR, the canonical catalytic activity of UNG2 may not necessarily be required for CSR [140,141]. Thus, it has been suggested that UNG2 may play a structural rather than catalytic role in CSR, possibly acting as a scaffold for other repair factors [142,143]. Humans that carry recessive inactivating mutations in the UNG-gene show a similar phenotype as $Ung^{-/-}$ mice, especially with respect to CSR and SHM. Together these defects manifest themselves as a form of hyper-IgM syndrome (HIGM), interestingly similar to that caused by deficiencies in the AID-gene. In humans, however, the CSR defect is more

severe than in *Ung*-knockout mice, while the SHM mutation spectrum is affected in a similar manner in both species, i.e. only at cytosine and guanine [144]. These inactivating mutations either result in UNG2 proteins that are catalytically inactive or that are excluded from the nucleus [145].

The activity of the UNG proteins shows considerable variation between tissues as well as individuals [146,147,148]. Furthermore, cancer cells appear to display a large variation in the level and activity of both total UNG and UNG2 proteins, in contrast to un-transformed fibroblast cell lines that show a quite low and uniform expression of UNG proteins. The variation between cancer cell lines is not caused by polymorphisms in the coding regions of the *UNG*-gene, but rather reflects variations in the level of protein expression of UNG2 [149]. The effect of this variation on BER is unclear, although a single study suggests that forced overexpression of UNG2 results in a significant growth inhibition as response to inhibitors of TS at short – but not long – incubation times. Concurrently, a significantly elevated level of single-strand breaks as estimated by the comet assay was observed. This indicates that overexpressed UNG2 removed misincorporated uracil in excess of what the rest of the BER pathway could process, thus resulting in an accumulation of repair intermediates [150]. This was not sufficient to affect cell death or clonogenicity, but nevertheless suggests that high expression levels of UNG-proteins could contribute to genetic instability.

2.5.3 Single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1)

SMUG1 has a broad substrate specificity, recognising uracil as well as a number of different uracil-analogues with substitutions in the 5' and 6' positions. Major substrates for SMUG1 are ethenocytosine, 5-fluorouracil and in particular 5-hydroxymethyluracil, for which it is the major activity in human cells [116]. When APE1 and Mg^{2+} are present in the reaction mixture the preferred substrate for SMUG1 is actually uracil in double-stranded DNA, in spite of the nomenclature [116]. SMUG1 is localised in nuclei with high levels in nucleoli and is constitutively expressed throughout the cell cycle [116] and similarly expressed in both proliferating and non-proliferating tissues [132]. Knocking down *Smug1* in MEFs with siRNA results in a mutator phenotype, especially

G:C to A:T transitions, in a manner which is non-redundant with UNG2 [136]. Additionally, *Smug1*-knockdown MEFs are sensitive towards ionizing radiation and 5-FU [136,151]. SMUG1 forms foci with DNA polymerase λ (POLλ) in irradiated mouse cells, and these enzymes co-immunoprecipitate, indicating that the glycosylase is involved in repair of oxidised pyrimidines, in addition to repair of deaminated cytosines [152].

2.5.4 Thymine-DNA glycosylase (TDG)

The search for an enzyme able to repair deaminated 5-methylcytosines, which results in the canonical base thymine mispaired with guanine, led to the discovery of TDG. This glycosylase binds and excises thymine in T:G mispairs, thus initiating BER [153,154]. While the activity on the T:G mispair is rather small, TDG is up to ~100 times more active on uracil in U:G mispairs. The highest known activity is that of 5-FU opposite guanine, for which the turnover number is up to ~1000 times higher than on T:G mispairs [155]. This is to a large degree sequence specific, with the highest activity when a guanine is present 3' to the mispaired thymine (or other lesion), thus mimicking a deaminated 5-methylcytosine in a CpG context [155]. In addition to deamination products, TDG can also excise oxidised bases (hydroxyuracil, 5-methylhydroxyuracil) and products of lipid peroxidation (ethenocytosine) [156]. Once TDG has excised its target base it binds extremely tight to the product AP-site [157,158], to such an extent that the purified enzyme does not turn over (reviewed in [159]). The full product inhibition is alleviated by the next enzyme in the BER pathway, APE1, which displaces TDG from the AP-site [158], and by the covalent addition of a Small Ubiquitin-like Modifier (SUMO, 1 or 3) [160] to the C-terminus of TDG [161]. This induces a conformational change in TDG, which radically alters the way it interacts with DNA. The SUMO-conjugated protein can no longer bind AP-sites, thus facilitating catalytic turnover and resulting in an enhanced processing of U:G. Concurrently, however, the ability to process T:G is reduced [162]. Release from the AP-site is aided by physical interaction with the XPC-HR23B complex [163]. The T:G glycosylase activity is further stimulated by the physical association with the RAD9-RAD1-HUS1 heterotrimer (abbreviated 9-1-1 complex) [164]. 9-1-1 is similar to PCNA in that it acts as a sliding clamp and interacts with many downstream BER proteins [165,166,167,168,169].

While *TDG*-mRNA is expressed at similar levels in all tissues studied [170], and at similar levels throughout the cell cycle [121], the protein level is tightly linked with the progression of the cell cycle. TDG is highly expressed in G1 and G2/M, but is degraded at the entry of S-phase by the proteasome. This is the opposite expression profile to that of UNG2 [124]. TDG has so far not been implicated in carcinogenesis. Unlike most other mice with targeted deletions of other glycosylases, *Tdg*^{-/-} mice lose viability halfway through the gestational period [159]. However, cells derived from knockout embryos are tolerant to 5-FU, indicating that the TDG could mediate fluoropyrimidine cytotoxicity. Overexpression and knockdown of TDG in HeLa-cells exposed to 5-FU results in sensitation and tolerance, respectively, thus mirroring the murine phenotype [171]. Furthermore, forced expression of TDG during S-phase is apparently harmful to the cell and results in S-phase arrest [124]. While one might invoke several explanations why TDG-initiated DNA repair in S-phase would be detrimental, one should keep in mind that TDG is also established as a regulator of transcription. TDG interacts with transcription factors such as c-Jun, retinoic acid receptor (RAR) and retinoid X receptor (RXR), estrogen receptor α, p53 and others [172,173,174,175] (reviewed in [159]). Furthermore, recent evidence hints at a role for TDG in the maintenance of methylated CpG sequences, as TDG interacts with DNMT3b (DNA (cytosine-5-) - methyltransferase 3β) [176].

2.5.5 Methyl-CpG binding domain 4 (MBD4)

Two different groups discovered MBD4 independently. One group found the protein in a database search for candidate proteins that could bind to methylated CpG-sequences [177], and another in a yeast two-hybrid screening that used human MLH1 as bait [109]. It later turned out that these two studies had identified one and the same protein, which contains an N-terminal methyl-CpG binding domain that targets the protein to methylated CpG-sequences *in vivo* and *in vitro* [177] and a C-terminal domain similar to bacterial glycosylase/lyases. The C-terminal domain harbours monofunctional glycosylase activity on thymine, uracil and 5-FU when paired with guanine, which is especially active in methylated CpG contexts [178,179]. Like most other glycosylases,

the activity of MBD4 is strongly inhibited by its slow dissociation from the AP-site [180].

The human *MBD4*-gene contains a polynucleotide stretch of 10 adenines that is frequently mutated in human MSI-positive cancers (reviewed in [109,179,181,182]. The loss or gain of an extra adenine results in a premature stop codon in the mRNA, which is translated to a truncated protein that lacks the glycosylase domain. However, the methyl-CpG binding domain is still intact and able to bind methylated CpG-sequences. *In vitro,* the truncated MBD4 acts as a dominant negative inhibitor of the wild type MBD4 activity as well as other UDGs, most likely through steric hindrance. When the truncated MBD4 is transfected into human cell lines, it confers an increase in mutation frequency, a predisposition to structural chromosomal rearrangements and altered clonogenic response to cisplatin and etoposide [183]. Knockout mice have an increased mutation frequency, especially at CpG-sequences [184,185]. They also display reduced apoptosis in the murine intestine in response to several DNA damaging agents, including 5-FU, to which *Mbd4*$^{-/-}$ MEFs were considerably more tolerant [186,187]. The absence of apoptosis and tolerance to cytotoxic agents are thought to be mediated by interactions with Fas-associated death domain protein (FADD) and/or MLH1 [109,188].

2.5.6 8-oxoguanine DNA glycosylase (OGG1)

A major mutagenic DNA-lesion induced by oxidative damage is 7, 8-dihydro-8-oxoguanine (8-oxoG), which may form base pairs with adenine and result in G:C → T:A transversion mutations [189,190,191]. OGG1 excises 8-oxoG from DNA when paired with cytosine, thus initiating BER. It carries an additional lyase activity cause incision at the DNA backbone 3' to the AP-site, and is therefore classified as a bifunctional glycosylase [192]. Alternative splicing of *OGG1*-mRNA generates two major isoforms in human cells. The α-isoform is targeted to the nucleus, and the β-isoform is targeted to mitochondria [193]. OGG1 expression is apparently not cell cycle regulated, at mRNA or protein levels [121,194], and OGG1 is not induced by oxidative stress [195].

OGG1 binds tightly to the AP-site after base excision, and there is consequently little turnover of the purified enzyme. However, APE1 stimulates dissociation of OGG1 from the AP-site, thus allowing the enzyme to turn over. The presence of APE1 results in an increase in glycosylase activity [196] and uncoupling of glycosylase and lyase activities [197]. SN-BER of 8-oxoG has been reconstituted using only four enzymes (OGG1, APE1, POLβ and LIG1) [198], and studies on extracts suggest that this is the predominant mode of repair for this lesion [172,199].

Gene-targeted *Ogg1*-knockout mice have an increased level of 8-oxoG in DNA, increased G:C to T:A transversion mutations, and a predisposition to lung adenoma/carcinoma in old animals [189,200,201]. Polymorphisms in the human *OGG1*-gene are present in the human population and may contribute to lung carcinogenesis, although the literature is rather ambiguous (see review in [183,202]). Regulating the expression of OGG1 in human cells exposed to oxidative stress modulates survival. Overexpression of OGG1 in irradiated human cells is apparently harmful, while suppression of expression leads to increased survival. This is thought to be caused by double strand breaks created by simultaneously occurring repair processes close to each other on opposite strands, as overexpression of OGG1 protects against H_2O_2, and suppression of OGG1 sensitises cells [203,204].

2.5.6 MutY homolog (MUTYH)

8-oxoG that escapes excision and repair by OGG1 is premutagenic because replicative polymerases may incorporate an adenine opposite this lesion [205]. This generates the substrate for the MUTYH glycosylase, which excises the normal adenine base when it is paired with 8-oxoG [206,207]. This initiates BER, which cause insertion of a cytosine opposite the 8-oxoG, generating the substrate for OGG1, thus eventually resulting in a repaired G:C base pair [208]. In addition, the human MUTYH excises adenine when mispaired with guanine [209,210], although the functional significance of this activity is not clear. MUTYH is a bifunctional glycosylase, but the lyase activity of the enzyme is dramatically lower than the glycosylase activity. Human cells contains at least two different isoforms localised in the nucleus and mitochondria and no less than 10 different mRNAs, indicating that even more isoforms could be present [211].

APE1 stimulates the glycosylase activity of MUTYH by alleviating AP-site product inhibition, and these two proteins interact directly [210,212]. Expression of MUTYH is upregulated in G1/S and MUTYH co-localises and interacts directly with PCNA and RPA in replication foci [212,213]. However, the steps following APE1 incision are not known at present, as no direct interactions between MUTYH and POLβ or δ have so far been observed [212]. These polymerases would, however, tend to re-incorporate adenine rather than cytosine opposite 8-oxoG, and thus probably initiate a futile repair cycle [214]. Rather, DNA POLλ may be a good candidate to ensure the incorporation of cytosine opposite 8-oxoG, as the presence of PCNA and RPA increases both the selectivity for cytosine opposite 8-oxoG , as well as the efficiency of POLλ. Concurrently, PCNA and RPA repress the efficiency of POLβ at an 8-oxoG template [215]. Consistent with this, murine POLλ is localised to sites of oxidative damage, and *Poll*-knockout MEFs are hypersensitive to oxidative DNA damage [152]. MUTYH has also been shown to interact and co-localise with MSHα [216] as well as the 9-1-1 complex, especially after oxidative stress [10].

Mutyh$^{-/-}$-mice show no obvious phenotype, with a lifespan and tumour frequency close to the wild type [217]. However, when crossed with *Ogg*$^{-/-}$ animals to generate the double knockout, the offspring have reduced lifespan, an accumulation of 8-oxoG in lung tissue, small intestines and liver and a dramatic increase in tumour incidence in lungs, small intestines and ovaries [217,218]. This demonstrates in principle that the repair of 8-oxoG is important to prevent carcinogenesis in mammals. Consistent with this, variants of the human *MUTYH*-gene coding for catalytically weakened proteins confer a recessive predisposition to colorectal cancer, as well as an accumulation of G:C to T:A transversions in the gene encoding Adenomatous polyposis coli (APC) [219]. This disorder has been termed *MUTYH*-associated polyposis (MAP) (reviewed in [220]). Biallelic inheritance of *MUTYH*-variants results in a 93-fold excess risk of colorectal cancer, and may account for ~2% of colorectal cancers diagnosed before 40 years of age, and less than ~1% of patients diagnosed before 55 years of age [221].

2.5.7 Nth endonuclease III-like 1 (E. coli) (NTHL1)

NTHL1 is a bifunctional glycosylase that excises oxidised pyrimidines from DNA, most notably thymine glycol, 5-hydroxycytosine, FaPyG and dihydrouracil [222,223,224,225,226]. The β-lyase activity is several-fold lower than the preceding glycosylase activity [227]. NTHL1 interacts directly with PCNA, p53 and XPG, and its glycosylase activity is stimulated by the two latter enzymes [228], as well as by APE1 and Y-box binding protein 1 (YB-1) [227,229]. Interestingly, NTHL1 also stimulates itself through a DNA-independent dimerisation [230].

The expression of *NTHL1* is cell cycle regulated, with the highest level of mRNA observed during S-phase [226]. It is localised in the nucleus as well as mitochondria [225,231]. Mice with targeted disruptions in the *Nthl1*-gene have been generated, but show no overt phenotype compared to wild type even when exposed to IR or H_2O_2 [232,233]. However, the expression level of NTHL1 is an important determinant for cellular survival in irradiated human cells. Here, induced overexpression of NTHL1 leads to an increased mutation frequency, double strand breaks and lower survival [203]. Downregulation of *NTHL1*-mRNA and protein by siRNA results in fewer double strand breaks in response to IR, but a survival similar to that of overexpressing cells, indicating that the protein level of NTHL1 has to be tightly regulated. Interestingly, the response to H_2O_2 is different, as overexpression increases survival while downregulation sensitises cells [204].

2.5.8 Nei endonuclease VIII-like 1 & 2 (E. coli) (NEIL1 and 2)

Like the well-characterised bacterial endonuclease VIII (Nei; endonuclease eight), the NEILs are bi-functional glycosylases that excise oxidised bases from DNA. They have the ability to carry out β- and δ-elimination on the resulting AP-site, resulting in a 1-nucleotide gap flanked by 3' and 5' phosphates [234,235,236]. The resulting 3' phosphate must me removed prior to nucleotide insertion, but this is a very poor substrate for APE1. Instead, polynucleotide kinase 3' phosphatase (PNKP) removes the 3' phosphate. Thus, BER can be performed independently of APE1 [106,236,237]. It has also been suggested that NEIL1 and NEIL2 could participate in the removal of

deoxyribose-fragments in short patch BER, a task which is normally performed by POLβ [238].

NEIL1 recognises and excises a large variety of oxidised pyrimidines. It is the only known enzyme able to act on FaPyA [223], and it can also excise thymine glycol, 5-hydroxycytosine, 5-hydroxyuracil, 5,6-dihydrothymine and 5,6-dihydrouracil [233,234,236]. It is present in the nucleus as well as mitochondria [239]. The expression of NEIL1 is strongly induced during S-phase [235]. Furthermore, it is active in fork- and bubble-structures, unlike OGG1 and NTHL1. NEIL1 interacts with PCNA, which also stimulates its activity [240,241]. Hence, it is likely to play a role in genomic surveillance at or near the replication fork. NEIL1 is also shown to interact with and – in most cases - be stimulated by all subunits of the 9-1-1 complex as well as WRN, CSB, FEN-1, POLβ and LIG3α [106,164,241,242,243].

Downregulation of *NEIL1*-mRNA and protein in human and hamster cells by siRNA results in an increased level of oxidative DNA damage and mutation rate, both spontaneously and after oxidative stress [244]. SiRNA-mediated knockdown of *Neil1* in MEFs results in sensation to IR. *Neil1*-knockout mice develop normally until 7 months of age, when males, and to a lesser extent females, have been reported to develop severe obesity along with obesity-associated ailments, but not cancer [245]. Furthermore, $Nthl1^{-/-}Neil1^{-/-}$-double knockout mice show a very high incidence of lung and liver tumours compared to either single knockout. This was accompanied by an accumulation of FaPyA and to a lesser extent FaPyG in DNA from mice lacking *Neil1*, while the level of 8-oxoG in DNA was rather similar [246]. However, the latter group did not observe the obese phenotype in $Neil1^{-/-}$ mice. Although these groups report conflicting phenotypes for the *Neil1*-knockout animals, they both report that the observed phenotype (be it cancer or obesity) is less severe for females than for males [245,246].

NEIL2 excises oxidised pyrimidines, with the most pronounced activity towards 5-hydroxyuracil, and somewhat less on 5,6-dihydrouracil and 5-hydroxycytosine. It is localised exclusively in the nucleus [234,236]. Like NEIL1, it is active in bubble structures of varying sizes, indicating that it could be active in transcription and/or

replication [240] and its expression is rather unaffected by the cell cycle [236]. NEIL2 appears to interact directly with POLβ and LIG3α, and co-immunoprecipitates with these two as well as with PNKP and XRCC1 – but not APE1. These proteins are in concert able to repair 5-hydroxyuracil in plasmid DNA in an APE1-independent manner, either when added together as recombinant proteins or as a multi-protein complex immunoprecipitated from cells [237]. Furthermore, NEIL2 interacts with YB-1, which also stimulates NEIL1 activity [247].

While NEIL3 has extensive homology to *E. coli* Nei, NEIL1 and NEIL2 [234], no glycosylase activity has so far been associated with the protein. The only known activity of NEIL3 is an AP lyase-activity on ssDNA. Thus, it might be relevant in the response to oxidative damage, as the protein can partially rescue Nei- and Fpg-deficient *E.Coli* from oxidative stress [248].

2.5.9 N-methylpurine-DNA glycosylase (MPG)

MPG is a monofunctional glycosylase that removes purine bases methylated in N3- and N7-positions, as well as hypoxanthine, N6-ethenoadenine and 3-ethenoguanine (reviewed in [105,249]. *MPG*-mRNA and activity are up-regulated during the S-phase [121]. MPG interacts with PCNA, which also stimulates its activity [121]. Furthermore, its glycosylase activity is stimulated by APE1 and HR23B [250,251] and it interacts with Methyl-binding domain 1 protein (MBD1) and XRCC1 [252,253].

Monofunctional methylating agents such as methyl methanesulfonate (MMS) and TMZ induce methylations at purine bases, with the relatively harmless 7-methylguanine as the quantitatively most important lesion [254,255]. The direct cytotoxicity is mediated by generation of another cytotoxic and mutagenic lesion, 3-methyladenine. Although rather counter-intuitive, induced overexpression of MPG does not protect against these agents, but confers a sensitation to the cells [256,257,258]. This effect is, however, not seen when exposed to methylating agents that yield almost exclusively 3-methyladenine [258]. Thus, it would appear that the accumulation of toxic BER intermediates stemming from the repair of relatively harmless adducts by overexpressed MPG mediates cell death. Agents that target BER intermediates, such as methoxyamine (MX)

and PARP-1 inhibitors, aggravates the cytotoxicity of methylating agents (reviewed in [259]), also when MPG is overexpressed [257]. Furthermore, while *Polb*^{-/-} MEFs are hypersensitive to alkylating agents, they are as sensitive as wild type when the *Mpg*-gene is knocked out as well [260,261].

2.5.10 APEX nuclease (multifunctional DNA repair enzyme) 1 (APEX1, APE1)

APE1 is a multi-functional enzyme that modifies DNA with several known activities on damaged DNA. It was first identified as an AP-endonuclease that incises the DNA backbone at AP-sites, generating a 3'OH and a 5' deoxyribosephosphate (dRP) – fragment [262,263]. APE1 also contains a 3' phosphodiesterase activity generating a 3'OH from 3'phospho-α,β-polyunsaturated aldehyde (PUA), the product of bifunctional glycosylases and a 3' phosphatase activity which releases phosphate and generates a 3' OH (reviewed in [264]). Furthermore, APE1 contains a 3'->5' exonuclease activity that excises mismatches at 3' nicked or gapped DNA structures [216]. It also contains an endonuclease activity acting on oxidised bases, which incises the DNA backbone in a glycosylase-independent manner, generating a 3'OH group and a "dangling" 5' damaged base. The latter process has been termed nucleotide incision repair (NIR) [265,266]. Additionally, APE1 is a regulator of transcription factors, including AP-1, NF-κB, p53 and numerous others. In many cases the transcription factors are actively kept in an active reduced state through interaction with the N-terminal redox domain of APE1 (reviewed in [264,267]).

Given the many roles of APE1, it comes as no surprise that attempted generation of knock-out *Apex*^{-/-} mice results in embryonic lethality [268]. Haploinsufficient *Apex*^{+/-}-mice are viable, but they have increased spontaneous mutation rates [269] and an increased cancer incidence when exposed to oxidative stress [270]. Furthermore, reducing the APE1 expression level by anti-sense RNA results in hypersensitivity to a range of different DNA damaging agents, but not UV [271]. SiRNA-mediated silencing of *APE1*-mRNA and protein in human cells results in an accumulation of AP-sites, followed by blocked cell proliferation and apoptosis. This may be reversed by the

expression of *S. cerevisiae* Apn1, that has a similar AP endonuclease activity, but are structurally unrelated to human APE1 and unable to regulate transcription factors or interact with other human BER proteins [272]. On the other hand, an increase in the already high endogenous level of APE-1 leads to resistance against many DNA damaging agents, again with UV as an exception ([273], reviewed in [264]). APE-1 is furthermore upregulated during S-phase [274], and as a consequence of genotoxic stress (reviewed in [264]).

Most of the known human glycosylases bind tightly to the product AP-site and are inhibited by them, including SMUG1, TDG, MBD4, MUTYH, OGG1, NTHL1 and MPG [158,180,196,210,227,251,275]. APE1 alleviates the product inhibition and stimulates the turnover of most of these glycosylases by displacing them from the AP-site. If bifunctional glycosylases are displaced prior to the relatively slow β-elimination reaction, then they act as being monofunctional and hand over an intact AP-site to APE1 [197,210,227]. This type of glycosylase stimulation by displacement may be a "passive" process, in which APE1 decreases the amount of AP-sites available for glycosylases through its enzymatic mechanism [197], or alternatively an active process that displaces glycosylases from the AP-site through formation of a temporary glycosylase-APE1-DNA complex [276,277]. These models are not mutually exclusive. While APE1 stimulation of glycosylases is well established, the effect of a glycosylases on APE1 activity has not been thoroughly examined. A few studies show that the presence of a glycosylase tightly bound to the AP-site weakly inhibits endonuclease activity of APE1 [158,275], in effect slowing down repair of AP-sites. UNG2 does not bind to AP-sites but its activity is nevertheless reported to stimulate APE1 [116,275].

Similar to the glycosylases, APE1 remains bound to the product of its reaction, the 5' dRP-fragment [278,279]. APE1 promotes binding of POLβ, the next enzyme in the SN BER pathway, to nicked AP-sites, and stimulates its dRP-ase activity. Reciprocally, POLβ stimulates the AP-endonuclease activity of APE1 [278,280]. These bilateral interactions between glycosylases and APE1, and APE1 and POLβ prompted the suggestion that BER intermediates are bound by the preceding enzyme in the pathway until the next protein arrives, at which point the intermediate is shuttled from one repair

enzyme to the other, much like the baton in a relay [281]. In addition to DNA glycosylases and POLβ, there is evidence for a rather extensive network of protein-protein interactions regarding APE1 and BER proteins. APE1 is shown to stimulate and/or physically interact with PCNA, LIG1 and FEN-1 (all involved in LP-BER) [282,283], as well as the 9-1-1 complex, XRCC1, p53, high mobility group box 1 protein (HMGB1), Heat-shock protein 70 (Hsp70) and WRN [167,284,285,286,287,288]. Many of these factors are associated with APE1 as part of BER-competent multi-protein complexes, which may be formed even in the absence of DNA damage [127,128]. Negative regulators of APE1 include BCL-2, which inhibits endonuclease activity and disrupts the APE1-XRCC1 complex [289], and granzyme A, which degrades APE1 by proteolytic cleavage, directing the cell towards apoptosis [290].

Several polymorphisms in the APE1 gene have been identified in the human population, some in the coding regions of the gene [291]. As the phenotype of $Apex^{+/-}$ mice and downregulation by siRNA may suggest, inactivating mutations could play a role in mutagenesis and carcinogenesis. However, results from epidemiological studies are so far too ambiguous to suggest a causal relationship between APE-1 polymorphisms and cancer (reviewed in [259,292]).

2.5.11 Polynucleotide kinase 3'phosphatase (PNKP)

As mentioned, the phosphate-flanked 1-nt gaps generated by NEIL glycosylases are poor substrates for APE1. Here, the 3' phosphatase-activity of PNKP comes into play, thereby unveiling the 3'OH group required for nucleotide-insertion by polymerases [106]. A multi-protein complex proficient in SN BER has been isolated from human cells, containing (among others) NEIL2 and PNKP but not APE1 [237]. The 'end-cleaning' properties of PNKP are also involved in the repair of double- and single-strand breaks [293,294]. *PNKP*-mRNA and protein downregulation by siRNA confers sensitivity to several genotoxic agents as well as a ~7-fold increase in spontaneous mutation frequency [295].

2.5.12 DNA polymerase β (POLβ)

POLβ has two main activities in BER, localised in two different domains. The polymerase activity is contained in the 31 kDa C-terminal domain. This activity is the main polymerase in SN BER [296,297,298], and may also be responsible for incorporation of the first nucleotide in LP-BER [299]. In addition, POLβ contains lyase activity in an 8 kDa N-terminal domain required for removing the dRP fragment generated by APE1 during SN BER [298,300]. As this latter activity is the catalytically slowest of the BER enzymes it was proposed that excision of dRP is the rate-limiting step in SN-BER [301]. Furthermore, it appears to be the clearly dominant dRPase activity in mammalian cells [302,303]. POLβ takes part in an extensive network of interactions with BER proteins, among them APE1, PNKP, PCNA, XRCC1, PARP1 and 2, LIG1, and FEN-1 (reviewed in [304]) also as part of multi-protein complexes. XRCC1 and LIG1 seem to inhibit strand-displacement synthesis by POLβ [108,198], while WRN and FEN-1 stimulates it [305,306]. This may suggest that the presence of BER proteins close to the repair site affects the extent of LP strand-displacement. The functional significance of PCNA and PARP-1 and 2 on POLβ is unclear, or there are conflicting results in the literature (discussed in [307]).

While attempted generation of $Polb^{-/-}$ mice results in embryonic lethality [308], $Polb^{-/-}$ embryonic fibroblasts are able to grow in culture. They show an extreme sensitivity to MMS, which can be reversed by expressing the 8 kDa-lyase domain of the murine POLβ alone [302]. Haploinsufficient (i.e. $Polb^{+/-}$) mice are viable, with an increased mutation rate when exposed to alkylating agents [309]. The spontaneous mutation frequency is not affected, but increased tumour formation and CIN are observed in the heterozygous animals [310]. POLβ is frequently overexpressed in human cancers (compared to normal tissue) [311]. POLβ overexpression confers a mutator phenotype and an enhanced tumour formation to chinese hamster ovary cells [312,313]. Furthermore, POLβ is frequently mutated in a range of different human tumour specimens, and some of these mutations are in coding regions of the gene [314]. This may result in proteins with altered properties, such as lower replicative fidelity and/or dRPase-activity [315,316]. Expression of some of these are mutagenic and sufficient for

cellular transformation of mouse cells [317]. Additionally, deletion mutants found in human cancers lead to a dominant negative phenotype, where the truncated protein hinders the wild type from binding to DNA [318].

2.5.13 DNA Polymerase δ and ε (POLδ and ε)

In addition to their roles in semi-conservative DNA synthesis, these polymerases are also involved in gap filling in long-patch BER, MMR and NER. In extracts from *Polb*-deficient murine cells, both POLδ and ε can perform both LP and slow SN BER, in a process requiring PCNA and RFC [319,320]. The current understanding of the process is that POLβ inserts the first nucleotide in both sub-pathways, followed by the insertion of several more by POLδ or ε resulting in LP repair [299]. LP-BER has been reconstituted with APE1, POLδ or ε, PCNA, RFC, FEN-1 and LIG1 as necessary and sufficient factors [110,321]. Furthermore, both polymerases co-immunoprecipitates with core BER factors in a BER competent multi-protein complex [128].

2.5.14 Flap Structure-specific endonuclease 1 (FEN-1)

When replicative polymerases encounter another strand of nucleic acid on the template, they can continue replicative DNA synthesis by displacing the obstructing strand. This generates a structure with a protruding single-stranded 5'end. This structure arises during lagging strand DNA synthesis, LP BER and HR. The single-stranded protruding 5' fragment can be removed by FEN-1 by cleaving at the junction between double- and single-stranded structures, thus generating a ligatable end [322]. FEN-1 also contains a 5'→3' exonuclease activity that degrades nicks, gaps and recessed ends [323], and a gap endonuclease activity that cleaves the single-stranded regions of gaps (e.g. stalled replication forks), thus generating a double-strand break [324].

Oxidised or reduced AP-sites in the BER-pathway cannot be processed by the lyase activity of POLβ [325]. Instead, FEN-1 removes these modified AP-sites as part of an oligonucleotide, which is generated by strand-displacement synthesis by POLβ, δ or ε [306,326]. FEN-1, alone or in conjunction with PARP-1, stimulates POLβ-mediated strand-displacement synthesis at modified AP-sites [327]. PCNA stimulates FEN-1

binding to substrate and subsequent catalysis [328], an effect which is antagonised, at least *in vitro,* by the expression of the PCNA binding protein p21 [329]. FEN-1 activity is also stimulated by APE1, POLβ, PARP-1, WRN and 9-1-1 [165,282,327,330].

Fen1^{-/-} knockout mice are not viable, but heterozygotes display an apparently normal phenotype. However, when an additional *Apc*-allele is mutated in a *Fen*^{+/-} background, the animals develop MSI-positive tumours [331]. Many human cancers frequently overexpress FEN-1 [332], and furthermore, cancer-associated mutations in the human *FEN1*-gene have been identified. Many of these mutations lead to a protein that is inactive for 5' exonuclease and gap endonuclease activities. Mice homozygous for these partially inactive proteins have increased autoimmunity, chronic inflammation and cancer incidence [333].

2.5.15 DNA ligases in BER

Two of the three known DNA ligases in humans have been implicated in BER. These are DNA ligase I (LIG1) and DNA ligase III (LIG3), in PCNA-associated excision repair and XRCC1-associated short patch repair, respectively. The third known ligase (DNA ligase IV, LIG4) appears to function only in NHEJ and V(D)J recombination [334]. All human ligases derive their energy from ATP, in contrast to bacterial ligases, which are dependent on NAD^+ [10].

LIG1 interacts with the DNA clamps 9-1-1 complex and PCNA. The latter protein recruits LIG1 to replication foci, where it functions to join Okazaki-fragments in lagging strand synthesis [335]. LIG1 is present in both proliferating and non-proliferating cells, but the mRNA level increases markedly when cells are induced to proliferate [336]. The discovery of the PCNA interaction led to the notion that LIG1 was involved in PCNA-dependent LP BER, a position which was strengthened by reconstitution experiments with purified proteins [110,321,326] and its presence in multi-protein BER-competent complexes [128,337]. Furthermore, a mutant cell line defective for LIG1 has significantly longer *in vitro* repair tracts in LP BER, while SN BER is unaffected [338]. Finally, LIG1 has been proposed to be a patch size mediator for POLδ and ε [110]. The 9-1-1 complex interacts *in vitro* and *in vivo* with LIG1, an

interaction that is stimulated by UV light. The 9-1-1 complex coordinates and stimulates the sequential activities of FEN-1 and LIG1 [339], suggesting that it functions as a DNA damage-induced PCNA-like factor in DNA repair. LIG1 also interacts with APE1 and POLβ [283,337].

The *LIG3*-gene encodes two isoforms, referred to as α and β. The LIG3β isoform is apparently specific for testis, while the LIG3α isoform is expressed in all other cells and tissues as well [340]. Furthermore, the *α*-mRNA contains two translation start codons. These result in proteins with different N-termini that specifically directs them to the nucleus or mitochondria [341]. Most of the nuclear LIG3α is in a complex with XRCC1, and this interaction is apparently required for its stability, as unbound LIG3α is swiftly degraded in the absence of XRCC1 [342,343]. Thus, cells deficient for XRCC1 are also functionally deficient for LIG3α. Both XRCC1 and LIG3α binds to PARP-1 and poly(ADP-ribose), and are thus recruited to DNA with strand-breaks [344]. LIG3α is thought to perform DNA ligation in SN BER and single-strand break repair. Consistent with this, LIG3α is found in BER-competent multi-protein complexes [237,293,345], and it is one of four core proteins sufficient for complete SN BER in reconstitution experiments [108].

2.5.16 X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1)

The XRCC1 protein harbours no known enzymatic activity, but cells deficient in this protein are nevertheless sensitive to a range of DNA damaging agents, e.g. IR as well as oxidative and methylating agents (reviewed in [346]). XRCC1 binds rapidly to sites of damaged DNA *in vivo* [347] and purified XRCC1 binds to BER intermediates *in vitro* including small gaps and nicks [348], natural AP-sites as well as 5' and 3' incised AP-sites (i.e. the product of monofunctional glycosylases, APE1 and bifunctional glycosylases, respectively) [349].. Extracts from *XRCC1*-deficient Chinese hamster ovary (CHO) cells have a 2 to 4 fold reduced ligation efficiency [350]. Furthermore, XRCC1 has the ability to interact with and/or stimulate BER proteins at all steps of the pathway, including MPG, OGG1, NEIL2, APE1, POLβ, LIG3α, PCNA, PARP-1 and -2

and PNKP [108,237,253,286,293,342,351,352,353,354]. XRCC1 has also been found as a member of several multi-protein complexes, at least some of which are probably formed independent of DNA damage [127,128,355]. Together these observations suggest a role in single-strand break repair and BER as a scaffolding protein, which stimulates, orchestrates and recruits other repair proteins to the site of DNA damage.

Gene-targeted mice deficient for *Xrcc1*, or for that matter mice that overexpress murine XRCC1, have not been obtained due to embryonic lethality [356,357], thus underlining the importance of a tightly regulated expression of XRCC1 in murine cells. In the human population, single-nucleotide polymorphisms in the coding regions of the *XRCC1*-gene are relatively widespread, but the epidemiological significance regarding cancer is rather ambiguous [292,358].

2.5.17 Proliferating cell nuclear antigen (PCNA)

PCNA is a donut-formed homotrimeric protein that encapsulates DNA and acts in replication forks as a sliding clamp for replicative polymerases. It is loaded onto DNA by RFC. Like XRCC1, PCNA do not contain any (known) enzymatic activities, but rather seem to function as a scaffolding protein that keeps various replication and repair factors in close proximity to DNA. PCNA interacts with numerous BER proteins, including UNG2, MUTYH, NTHL1, NEIL1, APE1, XRCC1, POLβ, FEN-1, DNA LIG1, PARP-1 and WRN [122,212,228,241,251,282,328,329,354,359]. PCNA is required for LP BER by POLδ and ε [110,321]. When the cyclin-dependent kinase inhibitor p21 is expressed, it binds to PCNA and sequesters it from repair factors, thus inhibiting LP repair [329].

2.5.18 Poly(ADP-Ribose) Polymerase 1 and -2 (PARP-1 and -2)

PARP-enzymes catalyse the polymerisation of NAD^+ to chains of poly(ADP-ribose) (PAR), which may be elongated up to 200 units in length. PARP-1 is responsible for most of the PARP-activity in mammalian cells, and binds as a dimer with high affinity to DNA containing strand-breaks. This activates the enzyme [360], leading to auto-

46

ribosylation of PARP-1 itself, as well as nearby acceptor proteins (e.g. a number of histones, p21, p53, XRCC1, POLβ and LIG3α). Auto-ribosylation of PARP-1 leads to its dissociation from DNA due to charge repulsion between the negatively charged PAR and the phosphate groups in DNA [361]. This dissociation is necessary for DNA repair enzymes to access the lesion. Thus, in the absence of NAD^+, or in the presence of PARP-1 inhibitors, the binding of PARP-1 to damaged DNA inhibits repair. This leads to accumulation of DNA repair intermediates in the form of strand-breaks [346,362]. Furthermore, it has been shown that PARP-1 binding to DNA recruits and stabilises XRCC1 and associated proteins into nuclear foci at strand-breaks [363]. PARP-1 inhibitors sensitises cells to any agent that directly or indirectly produces strand-breaks [346], an effect which can be exploited in cancer chemotherapy. Following the dissociation of auto-ribosylated PARP-1 from DNA, PAR is rapidly degraded by PAR glycohydrolase (PARG), thus allowing re-binding of PARP-1 to damaged DNA. The binding of PARP-1 to damaged DNA is therefore a rather transient and dynamic phenomenon. If this process is allowed to repeat itself, for instance if the strand-break is not easily repaired, or the number of strand-breaks is so large that they overwhelm the cellular repair capacity, PARP-1 will soon consume all intracellular NAD^+. This is a signal for cell death, which may be dependent or independent of caspases, depending on the metabolic status of the cell in question (reviewed in [364]).

While PARP-1 modifies several BER proteins covalently, it can also interact with them directly, as demonstrated for POLβ, PCNA, XRCC1, WRN and LIG3α, thereby recruiting them to sites of damage (reviewed in [304]). Several of these are stimulated by this interaction. Furthermore, under conditions where ATP is scarce it has been suggested that ATP for ligation are derived from the PAR-polymer, and that this constitutes the rate-limiting step of BER [365].

Eighteen proteins in the PARP-family have so far been identified in humans, of which PARP-1 is the most abundant and accounts for at least 85% of the PARP-activity in living cells, while PARP-2 accounts for some ~10% [366,367]. These are the only family members that are responsive to damaged DNA. Neither *Parp-1* nor *Parp-2*

knockout mice display grave aberrant phenotypes in the absence of DNA damaging agents, but attempted double knockouts are embryonic lethal [368].

2.5 Mitochondrial DNA repair

Each human cell contains several mitochondria, and each mitochondrion contains several copies of their own 16,569 bp genome (mtDNA). Each copy of the mtDNA genome encodes 22 tRNAs, 2 rRNAs and 13 polypeptides that participate in the electron transport chain. The lack of histones and the proximity to oxygen radicals produced by the "leaky" electron transport chain makes the mtDNA especially exposed to oxidative damage. Consequently, quite a few of the estimated ~2000 proteins that populate mitochondria must be involved in DNA repair. The best established DNA repair pathway in mitochondria is BER. Five glycosylases have so far been reported in mammalian mitochondria: UNG1, OGG1, MUTYH, NTHL1 and NEIL1 [112,193,211,231,239]. Furthermore, mitochondria contain APE1, APE2 and LIG3α. Together with the only known polymerase activity in mitochondria, DNA polymerase γ (POLγ), which also contains a dRP-ase activity, these enzymes are sufficient to complete BER by the insertion of a single nucleotide, while LP BER requires FEN-1 or DNA2 to excise the flap generated by strand displacement [129,369].

Other excision repair pathways are not as well characterised. NER is not thought to be active in this organelle, as pyrimidine dimers are apparently not repaired in mtDNA [370,371,372]. HR and NHEJ may be active in mitochondria [373,374,375], although mitochondria are apparently unable to repair some of the lesions that are removed by these pathways in nuclei [376]. Direct repair proteins, specifically MGMT and ALKBH1, are also present and active in mitochondria [60,376]. Finally, mammalian mitochondrial extracts appears to harbour a functional MMR pathway [377]. However, none of the core MMR-proteins seem to be directed to mammalian mitochondria. Rather, mitochondrial MMR seems to be initiated by YB-1 [378].

3.0 AIMS OF THE STUDY

The base excision repair pathway has traditionally been subdivided into single-nucleotide and long patch subpathways. Mechanistically, these pathways differ in two ways; by the number of nucleotides that are inserted, and the way in which the AP-site is removed. In SN-BER the AP-site is removed by beta-elimination, which is performed by POLβ and γ in the nucleus and mitochondria, respectively. These activities are, however, not able to remove modified AP-sites [325]. Instead, reduced or oxidised AP-sites are removed as part of an oligonucleotide 'flap' that is displaced from dsDNA by long patch synthesis. The dominant activity on 'flap' substrates in nuclear DNA is FEN1. However, prior to paper I, neither FEN1 nor FEN-1 like activities had been demonstrated in mitochondria. Indeed, mitochondrial BER was thought to proceed exclusively by the SN-pathway [379,380]. Thus, the aim of this study was to examine the capacity of mitochondria for repair of lesions that in the nucleus require LP BER. This work is presented in paper I.

Four uracil-DNA glycosylases have been identified in human cells. These are UNG, SMUG1, TDG and MBD4. Of these four, UNG is apparently the quantitatively most important glycosylase. There is a substantial variation in uracil-excision activity among different tissues, individuals and cell lines [146,147,148,149]. The significance of this variation on the rest of the BER pathway is not clear, especially since the rate-limiting step of BER have been proposed to reside at every step of the pathway, except for nucleotide insertion. In murine male germ cells, uracil-repair seems to be limited by the expression of UNG in young animals and APE1 in older [381]. Furthermore, the catalytically slowest process in SN-BER is the removal of the dRP-fragment by POLβ, so in reconstitution experiments the rate is determined by the level of POLβ [301]. Finally, the ligation step has also been suggested to be rate-limiting [365]. Furthermore, DNA glycosylase overexpression is cytotoxic and/or inhibits growth in cells that are challenged with treatments that specifically generates their substrates in DNA [150,203,204,257,258]. Using nuclear extracts from human cell lines known to vary in the expression of UNG2, we aimed to examine which of the known nuclear BER

proteins, if any, acted as bottle necks in the repair of uracil in DNA in human cell lines. This work is presented in paper II.

5-FU has been in clinical use for over half a century, but the exact mechanism by which it kills cells is still a matter of debate. The current understanding of the cytotoxic mechanism involves inhibition of TS and incorporation of fluorinated uracils into RNA and DNA [26]. The latter aspect has received considerable attention in recent years, as a variety of cells in which DNA repair proteins are downregulated or absent display an altered sensitivity to 5-FU or one of its metabolites [25]. Several DNA repair pathways can initiate repair of 5-FU from nuclear DNA. The ability to excise 5-FU from DNA is demonstrated for UNG2, SMUG1, TDG and MBD4 [116,151,155,157,382,383] , which initiate the BER pathway. Alternatively, the MMR pathway has also been shown to act on 5-FU in DNA [384]. We aimed to determine the relative significance of each DNA glycosylase to initiate BER *in vitro*, and compare the efficiency of BER and MMR to the repair of 5-FU from DNA. Furthermore, we examined what effect downregulation of the DNA repair enzyme that was quantitatively dominant *in vitro* had on fluoropyrimidine cytotoxicity. This work is presented in paper III.

4.0 SUMMARY OF PAPERS AND GENERAL DISCUSSION

4.1 Paper I: Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis.

Akbari M, Visnes T, Krokan HE and Otterlei M.

The mitochondrial genome is under continuous attack from ROS due to its proximity to the electron transport chain. ROS oxidises and modifies only bases in DNA, but also AP-sites that are generated by spontaneous hydrolysis or as BER intermediates. These modified AP-sites cannot be excised by beta-elimination employed by the dRP lyase activity of POLβ in nuclei and POLγ in mitochondria [325]. Hence, oxidised AP-sites are refractory to repair by the SN-pathway. The only known pathway to excise these modified AP-sites in human cells is to remove them as part of a 'flap' oligonucleotide during LP-BER [299]. Yet, mitochondrial BER was thought to take place exclusively by SN-BER [379,380]. So how would oxidised AP-sites be repaired in mitochondria?

An essential and critical factor in the study of mitochondrial BER is to prepare mitochondrial extracts free of nuclear contaminants, in this case especially those involved in DNA repair. We did this by treating partially purified intact mitochondria from HeLa and HaCaT cells with proteinase K. The mitochondrial double membrane served as a barrier that protected mitochondrial proteins from degradation. Proteinase K was then partially removed by centrifugation. Residual proteinase K was inhibited with proteinase inhibitor cocktail, thereby allowing extraction of mitochondrial proteins free of nuclear BER proteins. The purity of the extract and absence of nuclear contaminants was then confirmed by the following observations: 1) Absence of the nuclear proteins POLδ, PCNA, lamin A+C and UNG2, as judged by Western blot analysis. 2) Presence of VDAC1, COX IV and UNG1, specific for mitochondria, as judged by Western blot analysis 3) All UDG-activity and complete U:A BER in the extract were inhibited by neutralising antibody against the catalytic domain of UNG, indicating that SMUG1, TDG and MBD4 were not present in the extract. 4) We detected DNA polymerase activity in the presence of aphidicolin but not *N*-ethylmalemide (NEM), indicating that the polymerase activity stemmed from the mitochondrial POLγ. (Aphidicolin inhibits

POLα, δ and ε, while NEM – at the concentration used here – inhibits POLα, γ, δ, and ε but not β).

By exposing cccDNAs containing an AP-site in a specific position to mitochondrial extracts from HeLa and HaCaT, we observed incorporation of labelled deoxynucleotides not only at the lesion site, but also at a few nucleotides 3' of the lesion, suggesting mitochondrial LP-BER. Omitting ATP from the reaction mixture resulted in an increased incorporation in the 3' fragment, indicating that DNA ligase activity was a patch size mediator. Furthermore, by purifying the substrate DNA after the reaction and treating it with T4 DNA ligase, we observed a nearly complete conversion of repair intermediates to ligated fragment, indicating that the LP-repair intermediates were ligatable and hence could not have contained any dRP- or flap-fragments. In addition, we demonstrated the efficient repair of tetrahydrofuran, a modified AP-site that is resistant to dRP-lyase activity and consequently repaired exclusively by LP-BER. These observations were consistent with mitochondrial LP-BER, and furthermore – suggested the presence of a flap endonuclease activity in the mitochondrial extract, analogous to nuclear BER. However, at the time, there was no such enzyme(s) associated with mitochondria.

We therefore constructed a circular DNA substrate that imitated the flap-containing BER intermediate by annealing two overlapping oligonucleotides, to a circular ssDNA molecule followed by strand elongation and ligation. The oligonucleotide upstream of the overlap junction was radiolabelled at the 5' end, while the other oligonucleotide served as a flap. The ligation of these two oligonucleotides would be indicative of complete repair, which requires the removal of the overlapping flap. After treating the substrate with mitochondrial extract and cutting the DNA 18 and 33 nucleotides on either side of the flap, we detected a shift in gel migration of the radiolabelled oligo, which was inhibited by the addition of EDTA. This shift indicated that the two oligonucleotides had been ligated, and that the obstructing flap must have been removed. Furthermore, this strongly suggested that mitochondria harboured an enzyme that could resolve flap-structures from DNA. We could, however, not detect the quantitatively dominant nuclear flap endonuclease, FEN-1, in the mitochondrial extracts

by Western blot analysis, although we were able to detect FEN-1 by Western blot analysis in a nuclear extract. In order to increase sensitivity, we also attempted to immunoprecipitate FEN-1 from mitochondrial extract. However, even after immunoprecipitation we were still not able to detect mitochondrial FEN-1, whereas this was easily done in nuclear immunoprecipitates. In addition, the FEN-1-immunoprecipitate from nuclear extracts was active on flap-DNA substrates, while the FEN-1-immunoprecipitate from mitochondrial extracts was not. Finally, we showed that the flap-removing activity in mitochondrial extracts was of an endonucleolytic nature, as it released the expected 5-mer from a substrate containing a 5 nucleotide flap, similar to nuclear extracts and FEN-1 immunoprecipitate. In addition to the 5-mer flap, we also detected smaller fragments, which probably represent exonucleic digestion after the flap release. However, we cannot exclude the possibility that at least some of the flap-removing activity processively digested the flap from the 5' end prior to ligation. Nevertheless, together these observations are consistent with LP BER in mitochondrial extracts that do not stem from nuclear contaminants, and that an activity similar to FEN-1 is involved in mitochondrial LP-BER.

Shortly after publication, three independent papers confirmed our initial observation of LP-BER in extracts from protease-treated mitochondria [369,385,386]. Furthermore, they all detected FEN-1 in mitochondria by Western blot analysis, although the significance of FEN-1 in LP-BER varied between the studies. One group isolated a multi-protein complex that was proficient in LP-BER, but which did not appear to contain FEN-1. Moreover, siRNA-mediated downregulation of *FEN1*-mRNA and protein had little effect on flap endonuclease activity, and the product of a flap endonuclease assay yielded a product that was smaller than expected, although the possibility of exonucleic digestion of the released flap could not be ruled out. Thus, Szczesny et al argued that while FEN-1 was present in mitochondria, it was not involved in LP-BER [385]. On the other hand, Liu et al demonstrated that FEN-1 immunodepletion from mitochondrial extracts significantly reduced both flap endonuclease-activity and LP-BER. Furthermore, they demonstrated that FEN-1 downregulation by siRNA resulted in delayed repair of oxidative damage in mitochondrial, but not nuclear DNA [386]. Finally, Zheng et al detected mitochondrial

FEN-1, but also identified another flap endonuclease in mitochondria, the DNA replication helicase 2 homolog (yeast), abbreviated DNA2. This protein has ATPase, helicase and nuclease domains, the latter active on 5' flap structures. In yeast, it is involved in the resolution of certain secondary structures during replication, as well as the removal of Okazaki fragments. In human cells, DNA2 appears to be localised in mitochondria as well as the nucleus [387]. Moreover, Zheng et al reported a physical interaction between DNA2 and POLγ. Both DNA2 and FEN-1 were apparently involved in the removal of a 5' flap by slightly different mechanisms. FEN-1 cleaved the flap at the ssDNA/dsDNA junction, whereas DNA2 cleaved one to ten nucleotides at the 5' end of the flap. Importantly in the context of LP-BER, the immunodepletion of either protein lead to a lower ligation efficiency, and simultaneous immunodepletion of both abolished ligation completely. The addition of purified DNA2 or FEN-1 reversed these effects. Furthermore, reconstituted LP-BER with purified APE1, POLγ and LIG3α displayed only very low ligation efficiency when either DNA2 or FEN-1 was present and a high efficiency when both were present [369].

Taken together, the available evidence suggests that LP-BER takes place in human mitochondria. DNA2 and FEN-1 are both able to create ligatable ends during strand displacement synthesis, perhaps synergistically. Additionally, one or both enzymes are probably involved in the replication of mtDNA, an issue that is far from settled mechanistically [388,389]. Thus, several models are open for speculation. DNA2 and FEN-1 may be specific for either LP-BER or replication, with little or no functional overlap or they may operate synergistically in one or both pathways. The generation of mutants that are not imported into mitochondria would probably be an important step towards elucidating the roles of DNA2 and FEN-1 in mammalian mitochondria. It is, however, not likely that additional flap endonucleases are present in human mitochondria, since immunodepletion of both endonucleases abolished all detectable flap endonuclease activity in the mitochondrial extract [369].

4.2 Paper II: The rate of base excision repair of uracil is controlled by the initiating glycosylase.

Visnes T, Akbari M, Hagen L, Slupphaug G and Krokan HE

UDGs catalyse the first step in BER, in which a minimum of four enzymes cooperates to replace a damaged with a normal base. Interestingly, the intermediates in the process are strand-breaks that could be far more dangerous to the cell than the original base lesion. Thus, as suggested by the extensive network of protein-protein interactions documented for BER proteins, the process must be well coordinated and orchestrated to avoid accumulation of BER intermediates. However, the identity of the rate-limiting step of mammalian BER has so far remained elusive, every step of the pathway have been suggested to be rate limiting, with the exception of nucleotide insertion. Uracil-repair seems to be limited by the expression of the initiating glycosylase in male germ cells derived from young mice, while in cells from older mice the limiting factor seems to be Ape1 [381]. In reconstitution experiments, the rate of BER is apparently determined by the rate of the catalytically slowest process, i.e. the removal of the dRP-fragment by POLβ [301]. Finally, the ligation step has also been suggested to be rate-limiting [365]. If, however, the rate of the pathway were determined at any of the intermediate steps, then one would expect large amounts of base damage to be converted to the intermediate that precedes the rate-limiting step. Thus, if the lyase activity of POLβ were rate limiting, most of the base damage would be converted into a strand-break containing a newly inserted base at the 5' side, and a dRP-fragment at the 3' side. Such processes seem to be responsible for the cytotoxicity conferred by induced overexpression of OGG1, NTHL1 and MPG to agents that produce their substrates in DNA [203,257,258].

There is a substantial variation in uracil-excision activity among different tissues and individuals [146,147,148]. This variation is apparently not caused by variation in genotype, as a screen of 62 cell lines from human sources revealed no polymorphisms in the coding region of the *UNG*-gene [149]. However, extracts prepared from these cell

lines varied several-fold in the ability to remove uracil [149]. With all this in mind, how would the large variations in UDG-activity documented in human cell lines affect BER?

For this purpose, we prepared nuclear extracts from eight cell lines of human origin. Of these cell lines there were seven cancerous and one untransformed fibroblast. We did not detect any mitochondrial UNG1, which indicates that the nuclear extracts were essentially free of mitochondrial contaminants. We found great variation in UDG-activity among the extracts, as measured on a substrate containing [^{3}H]U:A base pairs. The measured UDG-activities corresponded roughly to those measured by Kvaløy et al (R^2=0.45, P=0.051) [149]. Furthermore, we found large variations in the content of other BER proteins according to Western blot analysis, with the exception of PCNA and POLδ, which were rather similar in all extracts. Furthermore, we observed a highly significant correlation between content of UNG2 in the extracts and measured UDG-activity. Only two of the proteins showed significant correlation with each other, namely POLδ and LIG1. No correlation, neither positive nor negative, was observed between TDG and UNG2, in spite of their inverse regulation through the cell cycle [124]. This suggests that UNG2 and TDG are truly differentially regulated from cell line to cell line, and the variations observed here are not only consequences of different cell cycle profiles at the time of harvest.

We furthermore subjected the eight nuclear extracts to *in vitro* BER-analysis, using cccDNA containing a single uracil opposite adenine or guanine. We also generated the AP-site and nicked AP-site (nAP) intermediates, by pre-treating the substrates with the purified catalytic domain of UNG, or UNG and APE1, respectively. The efficiency of repair was Uracil < AP < nAP in all eight extracts, and the repair of U:A and U:G showed a striking highly significant correlation with the level of UNG2 and UDG-activity, especially for the U:A substrate. These observations indicate that the rate of U:A repair – and to some extent the U:G substrate - was determined by the protein content and activity of the initiating glycosylase UNG2, and that overall rate of repair is determined at the first step of the pathway.

The lower correlation between UDG-activity and U:G repair was essentially caused by one outlier, the AGS extract, which repaired U:G substrate with disproportionately high efficiency. If data from this extract were excluded from the data set, the correlation between U:G repair and UNG2 was as good as that between U:A and UNG2. This suggests that UNG2 was the major initiator of U:G repair in the other cell lines, and that the rate of U:G BER was controlled at the first step for this lesion as well. Furthermore, the AGS extract contained the second lowest level of UNG2 in our panel and the highest content of TDG. We therefore decided to investigate the relative contribution of uracil-DNA glycosylases to U:G repair in three extracts. Using inhibitory antibodies against UNG2, SMUG1 and TDG we were able to silence >95% of U:G repair in AGS, SW480 and T-47D extracts, indicating that the fourth known UDG in human cells, MBD4, was of quantitative minor importance, and that the other three were quantitatively dominant. By omitting one of the three inhibitory antibodies, we estimated the contribution from each glycosylase to U:G repair. This revealed that UNG2 still accounted for most of the U:G repair in the AGS extract, but also that TDG was able to repair U:G very efficiently. In the two other cell lines, which contained more UNG2 and less TDG than the AGS extract, UNG2 was responsible for ~90% of repair while TDG only accounted for ~5%. SMUG1 appeared to be of low importance in all extracts, and UNG2 initiated >95% of U:A repair in all three extracts. Thus, the disproportionate efficient U:G repair in the AGS extract is most likely explained by a high expression level of TDG. This was a rather unexpected finding, as previous studies indicated that all detectable U:G excision activity in mammalian cells can be quenched by inhibiting UNG and SMUG1 [116,132]. However, TDG depends on SUMOylation to alleviate the extremely tight product inhibition by the AP-site. This process, in turn, requires Mg^{2+} and ATP, factors that are not included in oligonucleotide cleavage assays. Thus, using a BER assay on plasmid DNA allowed us to observe a hitherto unsuspected high activity of TDG in nuclear extracts of human origin. TDG is highly expressed in G1 and subsequently degraded in S-phase, and vice versa for UNG2. TDG could therefore be a major contributor to U:G repair outside of S-phase. UNG2, on the other hand, is probably the major activity on uracil in all contexts during the S-phase.

Intriguingly, we found the content of LIG3α to correlate with U:G repair equally well as UNG2. However, if we hypothesise that LIG3α is the rate-limiting factor for U:G repair, then one would expect it to be rate-limiting for AP-site and nAP-substrates as well. That was, however, not the case. Furthermore, ligation efficiency of U:G substrate was rather similar in all extracts. We found no other significant correlations between repair of the intermediate substrates and any of the BER factors studied. This raises the question of imbalanced repair of AP-sites, as some of the extracts that contained the most APE1 repaired AP-sites with low efficiency. One should keep in mind that APE1 has cellular functions that are independent of BER. Discrepancy between APE1 content and AP-site repair in extracts suggests that the expression level of APE1 in nuclei of human cell lines may be dictated by other factors than cellular repair capacity. However, another possible explanation for the discrepancy between APE1-level and AP-site repair is that Western blot analysis may not necessarily reflect the AP-site incision activity in the extracts. APE1 activity is likely to be affected by inhibitory proteins such as BCL-2 [289], post-translational modifications and complex formation.

We furthermore observed that uracil and AP-site substrates were repaired with higher efficiency when the lesion was positioned opposite guanine, rather than adenine. In contrast, the repair efficiency of nicked AP-site substrate was similar in both contexts. Therefore, specificity for the opposite base is likely to reside at the AP-site incision stage of the pathway, although purified recombinant human APE1 displayed a rather similar activity for both AP:A and AP:G, as also observed by others [390]. These observations suggest that there are other factors not considered in this paper that may direct AP-site incision in different contexts. A speculative notion is that DNA glycosylases, which display specificity to the opposite base and bind to AP-sites [158,180,196,210,227,251,275] can stimulate or inhibit the access of APE1 to the AP-sites. These interactions have been postulated to recruit APE1 to the AP-site and "protect" it [391], but in the few cases where this has been studied, the presence of a glycosylase bound to the AP-site inhibits incision activity of APE1 [158,275].

4.3 Paper III: Cytotoxicity of 5-fluoropyrimidines is mainly through RNA incorporation and thymidylate synthase inhibition rather than DNA fragmentation from DNA excision repair

Pettersen HS, Visnes T, Vågbø CB, Doseth B, Kavli B and Krokan HE

The exact mechanism causing 5-fluoroyracil (5-FU) cytotoxicity is still a matter of debate, even though it has been in clinical use for half a century. Already from the very start it was clear that 5-FU was internally metabolised and incorporated into RNA and DNA [392]. Incorporation into RNA perturbs the general metabolism of RNA in several different ways. It disturbs processing of rRNAs [33,393], post-transcriptional modification of tRNAs [30,31] as well as snRNA-protein complexes, thus inhibiting splicing of pre-mRNA [32,34], as well as post-transcriptional conversion of uridine to pseudouridine present in rRNA, tRNA and snRNA [35]. Another metabolite, 5-FdUMP, strongly inhibits the action of Thymidylate synthase (TS), which methylates dUMP at the 5' position of the base, thus generating dTMP. During inhibition of TS, less dTTP is generated, leading to an inhibition of DNA synthesis. When the level of dTMP is reduced, the levels of dAMP, dCMP and dGMP are perturbed as well, since the individual dNTP levels in the cell are regulated through various feedback mechanisms. Consequently, the DNA synthesis that does go on may be expected to be error prone, generating mismatches at a much higher frequency than under normal conditions [15]. The replicative polymerases may also incorporate analogues of dTTP into DNA. Incorporation of dUTP results in a U:A base pair, which may be repaired by UNG2 or SMUG1 [116], while incorporation of 5-FdUTP results in 5-FU:A or 5-FU:G base pairs [29,394]. Purified recombinant UNG2, SMUG1 and TDG are all able to recognise 5-FU:A in DNA, while 5-FU:G may be recognised by UNG2, SMUG1, TDG and MBD4 [116,151,155,157,382,383]. Furthermore, as 5-FU:G is a mismatch it can be repaired through the MMR pathway [384]. It has also been suggested that MMR may recognise and repair 5-FU:A [384].

While at least five different mechanisms may be involved in the repair of 5-FU from DNA, their relative significance in repair and cytotoxicity has so far not been

determined. Nevertheless, the role of DNA repair in 5-FU cytotoxicity has recently received considerable attention. Cells derived from knockout mice deficient in various DNA repair genes are generally less sensitive to fluoropyrimidines compared to wild type. This includes the MMR genes *Msh2* and *Mlh1* [89,187], as well as the BER genes *Tdg*, *Mbd4* and *Polb* [171,186,395]. Cells from gene-targeted mice deficient for these genes are all less sensitive to fluoropyrimidines. However, cells from *Ung*^{-/-} mice are as sensitive to fluoropyrimidines as wild type [130,151]. Finally, MEFs that express siRNA against *Smug1*-mRNA constitutively are more sensitive to 5-FU than control cells [151]. Human cells deficient in MMR reflect these findings, as they are less sensitive to fluoropyrimidines [89,90,91]. Less evidence is available for BER deficiency in human cells, as neither downregulation of POLβ by siRNA [311] nor expression of the UNG-specific inhibitor Ugi [396] has any apparent effect on fluoropyrimidine cytotoxicity. However, silencing of human *UNG2* by siRNA are reported to increase resistance towards 5-F(dU) [397].

Primarily, we wanted to clarify the relative significance of DNA glycosylases and MMR to 5-FU-DNA repair. Using nuclear extracts from several human cancer cell lines, we found that the excision of 5-FU opposite adenine and in single-strand context depended entirely on UNG2, as the addition of Ugi abolished these activities completely. However, when 5-FU was opposite guanine, the relative contribution from UNG2, SMUG1 and TDG were all rather similar and varied from extract to extract. A similar analysis using BER-incorporation assays mirrored the oligonucleotide cleavage assays in that 5-FU:A repair was completely inhibited by Ugi, while endogenous levels of UNG2, SMUG1 and TDG were all able to initiate repair of 5-FU:G. However, there was an apparent discrepancy between oligonucleotide cleavage and BER assays, in that TDG seemed a lot more active in the latter assays. One should keep in mind that TDG binds with strong affinity to the product AP-site, an interaction that abrogates enzymatic turnover [157,158]. This is alleviated by SUMOylation, which releases TDG from the AP-site [161,162]. SUMOylation *in vitro* requires ATP and Mg^{2+}, factors that were absent from the oligonucleotide cleavage assays. While ATP could potentially be included in the oligonucleotide cleavage assays, the presence of Mg^{2+} is not possible as it activates potent exonucleases in the extracts. TDG activity is therefore not favoured in

oligonucleotide cleavage assays, and its more dominating presence in BER assays could perhaps be expected.

We also wanted to clarify the roles of BER and MMR in the repair of 5-FU, as a recent paper suggested involvement of MMR in both 5-FU:A and 5-FU:G repair [384]. MMR assay strategies are usually depended on restriction enzymes that specifically differentiate between damaged/mismatched DNA and normal base pairs [79]. Because a 5-FU:A base pair is so similar to T:A that no known restriction enzyme can distinguish between them, the study of 5-FU:A using restriction enzymes alone is not possible [384]. Nevertheless, an MMR proficient extract incorporated more radioactivity into plasmid DNA containing 5-FU:A than an MMR-deficient control extract. The authors therefore suggested that MMR could be involved in the repair of 5-FU:A, in spite of the inability of the MutSα heterodimer to bind 5-FU:A base pairs in electrophoresis mobility shift-assays [384]. We were, however, able to monitor 5-FU:A-repair using the restriction enzyme HincII to distinguish between repaired and un-repaired DNA. The repaired DNA contained normal base pairs, which was recognisable for HincII. Unrepaired substrates contained 5-FU, which was removed by the catalytic domain of UNG after the reaction, thus generating an AP-site. This AP-site was further adducted with MX, which HincII could not recognise. Using this strategy, we were able to show that 5-FU:A was repaired exclusively by BER in HeLa and SW480 nuclear extracts. 5-FU:G could also be repaired by MMR, as we observed a nick-dependent conversion of 5-FU:G to C:G when UNG2, SMUG1 and TDG were inhibited and/or immunodepleted. The process was, however, rather slow compared to BER, which repaired >85% of 5-FU:G within 30 minutes of the reaction. This indicates that BER, initiated by UNG2, SMUG1 or TDG, is the predominant mode of repair in HeLa and SW480. However, the role of MMR is most likely underestimated using this assay, since it depends on the presence of nick in the DNA substrate, which is likely to be sealed directly by ligase activity in the nuclear extracts.

Having established that the majority of 5-FU-repair *in vitro* was performed by UNG2, SMUG1 and TDG we performed siRNA-mediated knockdown of these glycosylases in HeLa and SW480. Using specific siRNAs we reduced the protein levels of UNG2 and

TDG by >75% for several days. SMUG1 was reduced with at least 60% and this conferred tolerance to the drug 5-hydroxymethyl-2'-deoxyuridine (5-hm(dU)) in both cell lines. 5-hm(dU) is incorporated into DNA and the excision of this erroneous (but still rather innocent) base by SMUG1 mediates cytotoxicity through the generation of strand-breaks and probably DNA damage signalling. [398,399,400,401,402]. Thus, 5-hm(dU) cytotoxicity is rather similar to the DNA repair-mediated cytotoxicity proposed for 5-FU. However, it is not incorporated into RNA, and its metabolites does not inhibit TS [403], so it serves as a good control for BER-mediated cytotoxicity. However, all knockdown cells were as sensitive to varying concentrations of 5-FU, 5-F(dU) as 5-F(rU) as control cells in both cell lines. Thus, fluoropyrimidine cytotoxicity was apparently of a different nature than the DNA repair mediated cytotoxicity of 5-hm(dU), suggesting that other mechanisms than DNA repair mediated fluoropyrimidine cytotoxicity. Furthermore, downregulation of SMUG1 in cells exposed to 5-hm(dU) shifted the cells from G1/S arrest to G2-arrest, while downregulation of UNG, SMUG1 or TDG in cells exposed to 5-FU and 5-F(dU) had no such effect. Adding BER inhibitors MX and the PARP-1 inhibitor 4-amino-1,8-naphthalimide (4-AN) modulated the cytotoxicity of 5-hm(dU), but not of 5-F(dU). Again, this suggest that BER is not involved in 5-F(dU) cytotoxicity.

If DNA repair did not mediate the cytotoxicity of fluoropyrimidines, then what did? We observed that 5-FU, 5-F(dU) and 5-F(rU) inhibited TS with similar efficiencies in HeLa and SW480. Since the two cell lines displayed large variations in the sensitivity to these compounds, especially so for 5-F(dU), this indicated that other factors, in addition to TS-inhibition, modulated cytotoxicity in these cell lines. Using quantitative LC-MS/MS we found that cells exposed to 5-FU preferentially incorporated 5-FU into RNA rather than DNA, at a ~3000:1 ratio. For cells exposed to 5-F(dU), however, the RNA/DNA ratio was about ~6:1. Furthermore, we attempted to rescue the cells by adding increasing amounts of the nucleosides that presumably were in short supply during fluoropyrimidine exposure. The addition of uridine to 5-F(rU)-exposed cells had the greatest effect, and this treatment rescued both cell lines from the toxic effects of 5-F(rU). Concurrently, 5-FU incorporation into RNA was greatly reduced. This indicates

that incorporation into RNA mediated the cytotoxicity of 5-F(rU). Conversely, 5-F(dU) cytotoxicity was alleviated by the addition of thymidine and deoxyuridine, indicating that a lack of these DNA precursors is vital for the toxic effects of this drug. Finally, cytotoxicity of the clinically relevant 5-FU was partially reversed by high concentrations of uridine, but not thymidine or deoxyuridine. This indicates that incorporation of 5-FU into RNA plays at the very least some part in cytotoxicity, although addition of increasing levels of uridine did not reduce incorporation of 5-FU into RNA notably. The lack of uridine reversal for 5-FU is most likely explained by the fact that 5-FU may take two slightly different pathways into RNA. One is the sequential addition of ribose- and phosphate-groups by uridine phosphorylase (UP) and uridine kinase (UK), respectively, generating 5-F(rU) as intermediate product. The other pathway is the direct addition of both these groups by orotate phosphoribosyltransferase (OPRT). Both pathways generate 5-FUMP, but only the former pathway could be expected to be affected by addition of uridine to the medium.

Taken together, our *in vitro* data suggests that genomic 5-FU is primarily repaired via the BER pathway, initiated by UNG2, SMUG1 and TDG. However, the specific downregulation of these glycosylases had little effect on fluoropyrimidine cytotoxicity. Rather, the cytotoxicity of 5-FU in HeLa and SW480 is most likely mediated by TS-inhibition and incorporation into RNA, and not excessive DNA repair. This is based on the following lines of evidence: 1) Knockdown of the three quantitatively dominant 5-FU glycosylases had no effect of fluoropyrimidine cytotoxicity or cell cycle arrest. This was in contrast to the cytotoxicity and cell cycle arrest seen with 5-hm(dU), which both were affected by *SMUG1* downregulation. 2) Cells exposed to 5-FU incorporated 5-FU into RNA rather than into DNA. 3) Inhibition of BER using MX or 4-AN did not affect the cytotoxicity of 5-F(dU). 4) Rescue experiments using nucleotide precursors suggested that 5-FU cytotoxicity was mediated through TS inhibition and incorporation into RNA.

These results are apparently in conflict with recent publications that implicate BER in fluoropyrimidine cytotoxicity. $Ung^{-/-}$ MEFs are as sensitive to fluoropyrimidines as the wild type [130,151], while downregulation of $UNG2$ in HeLa by siRNA conferred a marked tolerance to 5-F(dU) [397]. However, transfecting HeLa with the UNG-specific inhibitor Ugi did not affect fluoropyrimidine response [396]. Downregulation of SMUG1 in MEFs sensitises cells, while induced overexpression increases the tolerance to 5-FU [151]. $Tdg^{-/-}$ MEF and ES cells are more tolerant to 5-FU than wild type, whereas downregulation of endogenous TDG-levels in HeLa has a marginally protective effect. However, induced overexpression of human TDG sensitises HeLa cells in an apparent dose-dependent manner [171], although matters may be complicated by the dual role of TDG as both a DNA repair protein and transcription regulator [173]. $Mbd4^{-/-}$ MEFs are less sensitive to 5-FU compared to wild type, but also to a range of other cytotoxic agents that induces lesions that are not suspected to be substrates for MBD4 [186,187]. This indicates that this glycosylase may function as a general apoptosis-promoting factor rather than being directly involved in the repair of 5-FU. Later steps of the BER pathway are not as well characterised, although $Polb^{-/-}$ MEFs are ~8-fold more resistant to 5-F(dU) than wild type [395]. On the other hand, downregulation of POLβ in human cancer cell lines has no apparent effect on the response to 5-FU [311]. Overexpression of a catalytically inactive mutant of human APE1, which binds to AP-sites and blocks subsequent repair steps, confers hypersensitivity to fluoropyrimidines in CHO-cells [404]. Finally, CHO-cells without functional $XRCC1$-genes are as sensitive as the wild type [405]. A coherent synthesis of these results is not easily achieved, although the results do suggest that 5-FU cytotoxicity, especially in MEFs, may very well take place by a mechanism that is partly mediated by DNA repair. Yet, there are probably quite a few differences in 5-FU metabolism and DNA repair between murine embryonic fibroblasts and human cancer cell lines. One example may be the very high preferential incorporation of 5-FU into RNA, compared to DNA observed for human cancer cell lines (~3000:1 in human cancer cell line, ~11:1 in MEFs [151]). Another example is provided by the relative contributions of glycosylases to 5-FU excision. Here, SMUG1 appeared to have a dominant role in MEFs, but not in human cell lines, in which UNG2 was the dominant enzyme. These species and/or cell type differences indicates that the DNA repair

64

response to 5-FU could be different in these systems. Nevertheless, the report from Caradonnas group clearly suggests that downregulation of UNG2 in HeLa increases the tolerance to 5-F(dU) [397]. Apart from technical differences (cell culture, incubation times), we find it hard to reconcile this result with ours, although it is conceivable that a cell could harbour functional mitochondrial succinate dehydrogenase (the enzyme responsible for colourimetric change in MTT assays) but still be unable to form colonies following trypsination.

MMR has been proposed to mediate 5-FU cytotoxicity in human cells, although our *in vitro* data suggest that the contribution of MMR to repair of 5-FU incorporated into DNA is rather modest. How, then, might a minor role for MMR in 5-FU repair be reconciled with its role as an apparent mediator of 5-FU cytotoxicity? MMR is also involved in the repair of mismatches between ordinary bases, which are generated as a result of imbalanced nucleotide pools during TS inhibition [15]. The synthesis of long repair patches under these conditions could also generate novel mismatches, thus initiating an iterative futile repair cycle. MMR is also involved in DNA damage checkpoint signalling, which is likely to affect 5-FU cytotoxicity [91]. Thus, in spite of its modest contribution to 5-FU repair *in vitro*, the MMR pathway could well mediate fluoropyrimidine cytotoxicity through mechanisms that are independent of 5-FU DNA repair.

If 5-FU-DNA glycosylases were important mediators of 5-FU cytotoxicity, then one might expect their expression (or at least the expression of some other downstream BER gene) to be altered in 5-FU resistant cells. This is, however, not the case in large scale microarray profiling of a variety of 5-FU resistant and sensitive cells, where BER genes tend not to be differentially regulated [406,407,408,409,410,411]. Even when we consider the obvious problem of false positives in these large-scale studies, the large variation in gene sets that characterise resistant or sensitive cells indicates that there may be several different mechanisms of 5-FU cytotoxicity in human cancer cells. As the different studies generally find different data sets, this suggests that resistance to 5-FU

can be achieved through several different strategies. Even so, the conspicuous absence of BER and DNA repair factors in these data sets indicates that modulation of DNA repair is not one of them. The current evidence points to TS-inhibition and RNA incorporation, as the main mechanisms of 5-FU cytotoxicity in human cancer cells [411,412,413,414,415].

5 REFERENCES

1. Lindahl T (1993) Instability and decay of the primary structure of DNA. Nature 362: 709-715.
2. Lindahl T, Nyberg B (1972) Rate of depurination of native deoxyribonucleic acid. Biochemistry 11: 3610-3618.
3. Nakamura J, Walker VE, Upton PB, Chiang SY, Kow YW, et al. (1998) Highly sensitive apurinic/apyrimidinic site assay can detect spontaneous and chemically induced depurination under physiological conditions. Cancer Res 58: 222-225.
4. Kavli B, Otterlei M, Slupphaug G, Krokan HE (2007) Uracil in DNA--general mutagen, but normal intermediate in acquired immunity. DNA Repair (Amst) 6: 505-516.
5. St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD (2002) Topology of Superoxide Production from Different Sites in the Mitochondrial Electron Transport Chain. J Biol Chem 277: 44784-44790.
6. Jackson AL, Loeb LA (2001) The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. Mutat Res 477: 7-21.
7. Bjelland S, Seeberg E (2003) Mutagenicity, toxicity and repair of DNA base damage induced by oxidation. Mutat Res 531: 37-80.
8. Blair IA (2008) DNA Adducts with Lipid Peroxidation Products. J Biol Chem 283: 15545-15549.
9. Marnett LJ (1999) Chemistry and biology of DNA damage by malondialdehyde. IARC Sci Publ: 17-27.
10. Friedberg EC (2006) DNA repair and mutagenesis. Washington, D.C.: ASM Press. xxix, 1118 p., [1111] p. of plates p.
11. Drablos F, Feyzi E, Aas PA, Vaagbo CB, Kavli B, et al. (2004) Alkylation damage in DNA and RNA--repair mechanisms and medical significance. DNA Repair (Amst) 3: 1389-1407.
12. Rydberg B, Lindahl T (1982) Nonenzymatic methylation of DNA by the intracellular methyl group donor S-adenosyl-L-methionine is a potentially mutagenic reaction. EMBO J 1: 211-216.
13. De Bont R, van Larebeke N (2004) Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis 19: 169-185.
14. Hecht SS, Hoffmann D (1988) Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. Carcinogenesis 9: 875-884.
15. Mathews CK (2006) DNA precursor metabolism and genomic stability. FASEB J 20: 1300-1314.
16. Furuichi M, Yoshida MC, Oda H, Tajiri T, Nakabeppu Y, et al. (1994) Genomic structure and chromosome location of the human mutT homologue gene MTH1 encoding 8-oxo-dGTPase for prevention of A:T to C:G transversion. Genomics 24: 485-490.
17. Akbari M, Pena-Diaz J, Andersen S, Liabakk NB, Otterlei M, et al. (2009) Extracts of proliferating and non-proliferating human cells display different base excision pathways and repair fidelity. DNA Repair (Amst).
18. Verri A, Mazzarello P, Biamonti G, Spadari S, Focher F (1990) The specific binding of nuclear protein(s) to the cAMP responsive element (CRE) sequence (TGACGTCA) is reduced by the misincorporation of U and increased by the deamination of C. Nucleic Acids Res 18: 5775-5780.
19. el-Hajj HH, Wang L, Weiss B (1992) Multiple mutant of Escherichia coli synthesizing virtually thymineless DNA during limited growth. J Bacteriol 174: 4450-4456.
20. Gadsden MH, McIntosh EM, Game JC, Wilson PJ, Haynes RH (1993) dUTP pyrophosphatase is an essential enzyme in Saccharomyces cerevisiae. EMBO J 12: 4425-4431.
21. Guillet M, Van Der Kemp PA, Boiteux S (2006) dUTPase activity is critical to maintain genetic stability in Saccharomyces cerevisiae. Nucleic Acids Res 34: 2056-2066.
22. Goulian M, Bleile B, Tseng BY (1980) Methotrexate-induced misincorporation of uracil into DNA. Proc Natl Acad Sci U S A 77: 1956-1960.
23. Blount BC, Mack MM, Wehr CM, MacGregor JT, Hiatt RA, et al. (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci U S A 94: 3290-3295.
24. Duthie SJ, Narayanan S, Sharp L, Little J, Basten G, et al. (2004) Folate, DNA stability and colo-rectal neoplasia. Proc Nutr Soc 63: 571-578.
25. Wyatt MD, Wilson DM, 3rd (2009) Participation of DNA repair in the response to 5-fluorouracil. Cell Mol Life Sci 66: 788-799.
26. Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3: 330-338.
27. Meyers M, Hwang A, Wagner MW, Bruening AJ, Veigl ML, et al. (2003) A role for DNA mismatch repair in sensing and responding to fluoropyrimidine damage. Oncogene 22: 7376-7388.
28. Noordhuis P, Holwerda U, Van der Wilt CL, Van Groeningen CJ, Smid K, et al. (2004) 5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibition of human colorectal cancers. Ann Oncol 15: 1025-1032.

29. Sowers LC, Eritja R, Kaplan B, Goodman MF, Fazakerly GV (1988) Equilibrium between a wobble and ionized base pair formed between fluorouracil and guanine in DNA as studied by proton and fluorine NMR. J Biol Chem 263: 14794-14801.

30. Santi DV, Hardy LW (1987) Catalytic mechanism and inhibition of tRNA (uracil-5-)methyltransferase: evidence for covalent catalysis. Biochemistry 26: 8599-8606.

31. Randerath K, Tseng WC, Harris JS, Lu LJ (1983) Specific effects of 5-fluoropyrimidines and 5-azapyrimidines on modification of the 5 position of pyrimidines, in particular the synthesis of 5-methyluracil and 5-methylcytosine in nucleic acids. Recent Results Cancer Res 84: 283-297.

32. Patton JR (1993) Ribonucleoprotein particle assembly and modification of U2 small nuclear RNA containing 5-fluorouridine. Biochemistry 32: 8939-8944.

33. Ghoshal K, Jacob ST (1994) Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil. Cancer Res 54: 632-636.

34. Doong SL, Dolnick BJ (1988) 5-Fluorouracil substitution alters pre-mRNA splicing in vitro. J Biol Chem 263: 4467-4473.

35. Samuelsson T (1991) Interactions of transfer RNA pseudouridine synthases with RNAs substituted with fluorouracil. Nucleic Acids Res 19: 6139-6144.

36. Harris RS, Petersen-Mahrt SK, Neuberger MS (2002) RNA Editing Enzyme APOBEC1 and Some of Its Homologs Can Act as DNA Mutators. Molecular Cell 10: 1247-1253.

37. Honjo T, Kinoshita K, Muramatsu M (2002) Molecular mechanism of class switch recombination: linkage with somatic hypermutation. Annu Rev Immunol 20: 165-196.

38. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, et al. (2000) Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell 102: 553-563.

39. Harris RS, Liddament MT (2004) Retroviral restriction by APOBEC proteins. Nat Rev Immunol 4: 868-877.

40. Drake JW, Charlesworth B, Charlesworth D, Crow JF (1998) Rates of spontaneous mutation. Genetics 148: 1667-1686.

41. Duesberg P, Li R, Fabarius A, Hehlmann R (2005) The chromosomal basis of cancer. Cell Oncol 27: 293-318.

42. Mitelman F (2000) Recurrent chromosome aberrations in cancer. Mutat Res 462: 247-253.

43. Fishel R (2001) The selection for mismatch repair defects in hereditary nonpolyposis colorectal cancer: revising the mutator hypothesis. Cancer Res 61: 7369-7374.

44. Bielas JH, Loeb KR, Rubin BP, True LD, Loeb LA (2006) Human cancers express a mutator phenotype. Proc Natl Acad Sci U S A 103: 18238-18242.

45. Venkatesan RN, Bielas JH, Loeb LA (2006) Generation of mutator mutants during carcinogenesis. DNA Repair (Amst) 5: 294-302.

46. Loeb LA, Bielas JH, Beckman RA (2008) Cancers exhibit a mutator phenotype: clinical implications. Cancer Res 68: 3551-3557; discussion 3557.

47. Veigl ML, Kasturi L, Olechnowicz J, Ma AH, Lutterbaugh JD, et al. (1998) Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci U S A 95: 8698-8702.

48. Jackson AL, Loeb LA (1998) On the origin of multiple mutations in human cancers. Semin Cancer Biol 8: 421-429.

49. Marx J (2002) Debate surges over the origins of genomic defects in cancer. Science 297: 544-546.

50. Bodmer W, Bielas JH, Beckman RA (2008) Genetic instability is not a requirement for tumor development. Cancer Res 68: 3558-3560; discussion 3560-3551.

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

52. Wood RD, Mitchell M, Lindahl T (2005) Human DNA repair genes, 2005. Mutat Res 577: 275-283.

53. Kurowski MA, Bhagwat AS, Papaj G, Bujnicki JM (2003) Phylogenomic identification of five new human homologs of the DNA repair enzyme AlkB. BMC Genomics 4: 48.

54. Gerken T, Girard CA, Tung YC, Webby CJ, Saudek V, et al. (2007) The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase. Science 318: 1469-1472.

55. Trewick SC, Henshaw TF, Hausinger RP, Lindahl T, Sedgwick B (2002) Oxidative demethylation by Escherichia coli AlkB directly reverts DNA base damage. Nature 419: 174-178.

56. Falnes PO, Klungland A, Alseth I (2007) Repair of methyl lesions in DNA and RNA by oxidative demethylation. Neuroscience 145: 1222-1232.

57. Tsujikawa K, Koike K, Kitae K, Shinkawa A, Arima H, et al. (2007) Expression and sub-cellular localization of human ABH family molecules. J Cell Mol Med 11: 1105-1116.

58. Aas PA, Otterlei M, Falnes PO, Vagbo CB, Skorpen F, et al. (2003) Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. Nature 421: 859-863.

59. Falnes PO, Bjoras M, Aas PA, Sundheim O, Seeberg E (2004) Substrate specificities of bacterial and human AlkB proteins. Nucleic Acids Res 32: 3456-3461.

60. Westbye MP, Feyzi E, Aas PA, Vagbo CB, Talstad VA, et al. (2008) Human AlkB homolog 1 is a mitochondrial protein that demethylates 3-methylcytosine in DNA and RNA. J Biol Chem 283: 25046-25056.

61. Saffhill R, Hall JA (1985) The incorporation of O6-methyldeoxyguanosine monophosphate and O4-methyldeoxythymidine monophosphate into polynucleotide templates leads to errors during subsequent in vitro DNA synthesis. Chem Biol Interact 56: 363-370.

62. Hall JA, Saffhill R (1983) The incorporation of O6-methyldeoxyguanosine and O4-methyldeoxythymidine monophosphates into DNA by DNA polymerases I and alpha. Nucleic Acids Res 11: 4185-4193.

63. Srivenugopal KS, Yuan XH, Friedman HS, Ali-Osman F (1996) Ubiquitination-Dependent Proteolysis of O6-Methylguanine-DNA Methyltransferase in Human and Murine Tumor Cells following Inactivation with O6-Benzylguanine or 1,3-Bis(2-chloroethyl)-1-nitrosourea. Biochemistry 35: 1328-1334.

64. Xu-Welliver M, Pegg AE (2002) Degradation of the alkylated form of the DNA repair protein, O6-alkylguanine-DNA alkyltransferase. Carcinogenesis 23: 823-830.

65. Kaina B, Fritz G, Mitra S, Coquerelle T (1991) Transfection and expression of human O6-methylguanine-DNA methyltransferase (MGMT) cDNA in Chinese hamster cells: the role of MGMT in protection against the genotoxic effects of alkylating agents. Carcinogenesis 12: 1857-1867.

66. Reese JS, Qin X, Ballas CB, Sekiguchi M, Gerson SL (2001) MGMT expression in murine bone marrow is a major determinant of animal survival after alkylating agent exposure. J Hematother Stem Cell Res 10: 115-123.

67. Shiraishi A, Sakumi K, Sekiguchi M (2000) Increased susceptibility to chemotherapeutic alkylating agents of mice deficient in DNA repair methyltransferase. Carcinogenesis 21: 1879-1883.

68. Kuo CC, Liu JF, Chang JY (2006) DNA repair enzyme, O6-methylguanine DNA methyltransferase, modulates cytotoxicity of camptothecin-derived topoisomerase I inhibitors. J Pharmacol Exp Ther 316: 946-954.

69. Gerson SL (2004) MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 4: 296-307.

70. Helleday T, Lo J, van Gent DC, Engelward BP (2007) DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair (Amst) 6: 923-935.

71. Pastink A, Eeken JC, Lohman PH (2001) Genomic integrity and the repair of double-strand DNA breaks. Mutat Res 480-481: 37-50.

72. Shrivastav M, De Haro LP, Nickoloff JA (2008) Regulation of DNA double-strand break repair pathway choice. Cell Res 18: 134-147.

73. Paull TT, Gellert M (1998) The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks. Mol Cell 1: 969-979.

74. Baumann P, Benson FE, West SC (1996) Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. Cell 87: 757-766.

75. Sigurdsson S, Van Komen S, Bussen W, Schild D, Albala JS, et al. (2001) Mediator function of the human Rad51B-Rad51C complex in Rad51/RPA-catalyzed DNA strand exchange. Genes Dev 15: 3308-3318.

76. Ip SC, Rass U, Blanco MG, Flynn HR, Skehel JM, et al. (2008) Identification of Holliday junction resolvases from humans and yeast. Nature 456: 357-361.

77. Watson JD, Crick FH (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature 171: 737-738.

78. Li GM (2008) Mechanisms and functions of DNA mismatch repair. Cell Res 18: 85-98.

79. Baerenfaller K, Fischer F, Jiricny J (2006) Characterization of the "mismatch repairosome" and its role in the processing of modified nucleosides in vitro. Methods Enzymol 408: 285-303.

80. Fang WH, Modrich P (1993) Human strand-specific mismatch repair occurs by a bidirectional mechanism similar to that of the bacterial reaction. J Biol Chem 268: 11838-11844.

81. Dzantiev L, Constantin N, Genschel J, Iyer RR, Burgers PM, et al. (2004) A Defined Human System That Supports Bidirectional Mismatch-Provoked Excision. Molecular Cell 15: 31-41.

82. Zhang Y, Yuan F, Presnell SR, Tian K, Gao Y, et al. (2005) Reconstitution of 5'-Directed Human Mismatch Repair in a Purified System. Cell 122: 693-705.

83. Kadyrov FA, Dzantiev L, Constantin N, Modrich P (2006) Endonucleolytic Function of MutL[alpha] in Human Mismatch Repair. Cell 126: 297-308.

84. Cannavo E, Gerrits B, Marra G, Schlapbach R, Jiricny J (2007) Characterization of the Interactome of the Human MutL Homologues MLH1, PMS1, and PMS2. J Biol Chem 282: 2976-2986.

85. Papouli E, Cejka P, Jiricny J (2004) Dependence of the cytotoxicity of DNA-damaging agents on the mismatch repair status of human cells. Cancer Res 64: 3391-3394.

86. Aebi S, Kurdi-Haidar B, Gordon R, Cenni B, Zheng H, et al. (1996) Loss of DNA mismatch repair in acquired resistance to cisplatin. Cancer Res 56: 3087-3090.

87. Waters TR, Swann PF (1997) Cytotoxic mechanism of 6-thioguanine: hMutSalpha, the human mismatch binding heterodimer, binds to DNA containing S6-methylthioguanine. Biochemistry 36: 2501-2506.

88. Swann PF, Waters TR, Moulton DC, Xu YZ, Zheng Q, et al. (1996) Role of postreplicative DNA mismatch repair in the cytotoxic action of thioguanine. Science 273: 1109-1111.

89. Meyers M, Wagner MW, Mazurek A, Schmutte C, Fishel R, et al. (2005) DNA mismatch repair-dependent response to fluoropyrimidine-generated damage. J Biol Chem 280: 5516-5526.

90. Meyers M, Wagner MW, Hwang HS, Kinsella TJ, Boothman DA (2001) Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. Cancer Res 61: 5193-5201.

91. Liu A, Yoshioka K, Salerno V, Hsieh P (2008) The mismatch repair-mediated cell cycle checkpoint response to fluorodeoxyuridine. J Cell Biochem 105: 245-254.
92. Carethers JM, Chauhan DP, Fink D, Nebel S, Bresalier RS, et al. (1999) Mismatch repair proficiency and in vitro response to 5-fluorouracil. Gastroenterology 117: 123-131.
93. Yamane K, Taylor K, Kinsella TJ (2004) Mismatch repair-mediated G2/M arrest by 6-thioguanine involves the ATR-Chk1 pathway. Biochem Biophys Res Commun 318: 297-302.
94. Caporali S, Falcinelli S, Starace G, Russo MT, Bonmassar E, et al. (2004) DNA damage induced by temozolomide signals to both ATM and ATR: role of the mismatch repair system. Mol Pharmacol 66: 478-491.
95. Li LS, Morales JC, Hwang A, Wagner MW, Boothman DA (2008) DNA mismatch repair-dependent activation of c-Abl/p73alpha/GADD45alpha-mediated apoptosis. J Biol Chem 283: 21394-21403.
96. York SJ, Modrich P (2006) Mismatch Repair-dependent Iterative Excision at Irreparable O6-Methylguanine Lesions in Human Nuclear Extracts. J Biol Chem 281: 22674-22683.
97. Kraemer KH, Patronas NJ, Schiffmann R, Brooks BP, Tamura D, et al. (2007) Xeroderma pigmentosum, trichothiodystrophy and Cockayne syndrome: a complex genotype-phenotype relationship. Neuroscience 145: 1388-1396.
98. Wood RD (1999) DNA damage recognition during nucleotide excision repair in mammalian cells. Biochimie 81: 39-44.
99. Leibeling D, Laspe P, Emmert S (2006) Nucleotide excision repair and cancer. J Mol Histol 37: 225-238.
100. Araki M, Masutani C, Takemura M, Uchida A, Sugasawa K, et al. (2001) Centrosome protein centrin 2/caltractin 1 is part of the xeroderma pigmentosum group C complex that initiates global genome nucleotide excision repair. J Biol Chem 276: 18665-18672.
101. Nishi R, Okuda Y, Watanabe E, Mori T, Iwai S, et al. (2005) Centrin 2 stimulates nucleotide excision repair by interacting with xeroderma pigmentosum group C protein. Mol Cell Biol 25: 5664-5674.
102. Fousteri M, Mullenders LH (2008) Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. Cell Res 18: 73-84.
103. Aboussekhra A, Biggerstaff M, Shivji MK, Vilpo JA, Moncollin V, et al. (1995) Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell 80: 859-868.
104. Araujo SJ, Tirode F, Coin F, Pospiech H, Syvaoja JE, et al. (2000) Nucleotide excision repair of DNA with recombinant human proteins: definition of the minimal set of factors, active forms of TFIIH, and modulation by CAK. Genes Dev 14: 349-359.
105. Krokan HE, Standal R, Slupphaug G (1997) DNA glycosylases in the base excision repair of DNA. Biochem J 325 (Pt 1): 1-16.
106. Wiederhold L, Leppard JB, Kedar P, Karimi-Busheri F, Rasouli-Nia A, et al. (2004) AP endonuclease-independent DNA base excision repair in human cells. Mol Cell 15: 209-220.
107. Takahashi T, Tada M, Igarashi S, Koyama A, Date H, et al. (2007) Aprataxin, causative gene product for EAOH/AOA1, repairs DNA single-strand breaks with damaged 3'-phosphate and 3'-phosphoglycolate ends. Nucleic Acids Res 35: 3797-3809.
108. Kubota Y, Nash RA, Klungland A, Schar P, Barnes DE, et al. (1996) Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein. EMBO J 15: 6662-6670.
109. Bellacosa A, Cicchillitti L, Schepis F, Riccio A, Yeung AT, et al. (1999) MED1, a novel human methyl-CpG-binding endonuclease, interacts with DNA mismatch repair protein MLH1. Proc Natl Acad Sci U S A 96: 3969-3974.
110. Pascucci B, Stucki M, Jonsson ZO, Dogliotti E, Hubscher U (1999) Long Patch Base Excision Repair with Purified Human Proteins. DNA LIGASE I AS PATCH SIZE MEDIATOR FOR DNA POLYMERASES delta AND epsilon. J Biol Chem 274: 33696-33702.
111. Aravind L, Koonin EV (2000) The alpha/beta fold uracil DNA glycosylases: a common origin with diverse fates. Genome Biol 1: RESEARCH0007.
112. Nilsen H, Otterlei M, Haug T, Solum K, Nagelhus TA, et al. (1997) Nuclear and mitochondrial uracil-DNA glycosylases are generated by alternative splicing and transcription from different positions in the UNG gene. Nucl Acids Res 25: 750-755.
113. Otterlei M, Haug T, Nagelhus TA, Slupphaug G, Lindmo T, et al. (1998) Nuclear and mitochondrial splice forms of human uracil-DNA glycosylase contain a complex nuclear localisation signal and a strong classical mitochondrial localisation signal, respectively. Nucleic Acids Res 26: 4611-4617.
114. Stivers JT, Jiang YL (2003) A mechanistic perspective on the chemistry of DNA repair glycosylases. Chem Rev 103: 2729-2759.
115. Dizdaroglu M, Karakaya A, Jaruga P, Slupphaug G, Krokan HE (1996) Novel activities of human uracil DNA N-glycosylase for cytosine-derived products of oxidative DNA damage. Nucleic Acids Res 24: 418-422.
116. Kavli B, Sundheim O, Akbari M, Otterlei M, Nilsen H, et al. (2002) 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. J Biol Chem 277: 39926-39936.

70

117. Slupphaug G, Eftedal I, Kavli B, Bharati S, Helle NM, et al. (1995) Properties of a recombinant human uracil-DNA glycosylase from the UNG gene and evidence that UNG encodes the major uracil-DNA glycosylase. Biochemistry 34: 128-138.

118. Eftedal I, Guddal PH, Slupphaug G, Volden G, Krokan HE (1993) Consensus sequences for good and poor removal of uracil from double stranded DNA by uracil-DNA glycosylase. Nucleic Acids Res 21: 2095-2101.

119. Haug T, Skorpen F, Aas PA, Malm V, Skjelbred C, et al. (1998) Regulation of expression of nuclear and mitochondrial forms of human uracil-DNA glycosylase. Nucleic Acids Res 26: 1449-1457.

120. Slupphaug G, Olsen LC, Helland D, Aasland R, Krokan HE (1991) Cell cycle regulation and in vitro hybrid arrest analysis of the major human uracil-DNA glycosylase. Nucleic Acids Res 19: 5131-5137.

121. Bouziane M, Miao F, Bates SE, Somsouk L, Sang BC, et al. (2000) Promoter structure and cell cycle dependent expression of the human methylpurine-DNA glycosylase gene. Mutat Res 461: 15-29.

122. Otterlei M, Warbrick E, Nagelhus TA, Haug T, Slupphaug G, et al. (1999) Post-replicative base excision repair in replication foci. EMBO J 18: 3834-3844.

123. Hagen L, Kavli B, Sousa MM, Torseth K, Liabakk NB, et al. (2008) Cell cycle-specific UNG2 phosphorylations regulate protein turnover, activity and association with RPA. EMBO J 27: 51-61.

124. Hardeland U, Kunz C, Focke F, Szadkowski M, Schar P (2007) Cell cycle regulation as a mechanism for functional separation of the apparently redundant uracil DNA glycosylases TDG and UNG2. Nucleic Acids Res 35: 3859-3867.

125. Muller-Weeks S, Balzer RJ, Anderson R, Caradonna S (2005) Proliferation-dependent expression of nuclear uracil-DNA glycosylase is mediated in part by E2F-4. DNA Repair (Amst) 4: 183-190.

126. Fischer JA, Muller-Weeks S, Caradonna S (2004) Proteolytic degradation of the nuclear isoform of uracil-DNA glycosylase occurs during the S phase of the cell cycle. DNA Repair (Amst) 3: 505-513.

127. Akbari M, Otterlei M, Pena-Diaz J, Aas PA, Kavli B, et al. (2004) Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells. Nucleic Acids Res 32: 5486-5498.

128. Parlanti E, Locatelli G, Maga G, Dogliotti E (2007) Human base excision repair complex is physically associated to DNA replication and cell cycle regulatory proteins. Nucleic Acids Res 35: 1569-1577.

129. Akbari M, Visnes T, Krokan HE, Otterlei M (2008) Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. DNA Repair (Amst) 7: 605-616.

130. Andersen S, Heine T, Sneve R, Konig I, Krokan HE, et al. (2005) Incorporation of dUMP into DNA is a major source of spontaneous DNA damage, while excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts. Carcinogenesis 26: 547-555.

131. Nilsen H, Stamp G, Andersen S, Hrivnak G, Krokan HE, et al. (2003) Gene-targeted mice lacking the Ung uracil-DNA glycosylase develop B-cell lymphomas. Oncogene 22: 5381-5386.

132. Nilsen H, Haushalter KA, Robins P, Barnes DE, Verdine GL, et al. (2001) Excision of deaminated cytosine from the vertebrate genome: role of the SMUG1 uracil-DNA glycosylase. EMBO J 20: 4278-4286.

133. Endres M, Biniszkiewicz D, Sobol RW, Harms C, Ahmadi M, et al. (2004) Increased postischemic brain injury in mice deficient in uracil-DNA glycosylase. J Clin Invest 113: 1711-1721.

134. Nilsen H, An Q, Lindahl T (2005) Mutation frequencies and AID activation state in B-cell lymphomas from Ung-deficient mice. Oncogene 24: 3063-3066.

135. Kronenberg G, Harms C, Sobol RW, Cardozo-Pelaez F, Linhart H, et al. (2008) Folate deficiency induces neurodegeneration and brain dysfunction in mice lacking uracil DNA glycosylase. J Neurosci 28: 7219-7230.

136. An Q, Robins P, Lindahl T, Barnes DE (2005) C --> T mutagenesis and gamma-radiation sensitivity due to deficiency in the Smug1 and Ung DNA glycosylases. EMBO J 24: 2205-2213.

137. Linhart HG, Troen A, Bell GW, Cantu E, Chao WH, et al. (2009) Folate deficiency induces genomic uracil misincorporation and hypomethylation but does not increase DNA point mutations. Gastroenterology 136: 227-235 e223.

138. Endres M, Ahmadi M, Kruman I, Biniszkiewicz D, Meisel A, et al. (2005) Folate deficiency increases postischemic brain injury. Stroke 36: 321-325.

139. Rada C, Williams GT, Nilsen H, Barnes DE, Lindahl T, et al. (2002) Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in UNG-deficient mice. Curr Biol 12: 1748-1755.

140. Begum NA, Kinoshita K, Kakazu N, Muramatsu M, Nagaoka H, et al. (2004) Uracil DNA glycosylase activity is dispensable for immunoglobulin class switch. Science 305: 1160-1163.

141. Di Noia JM, Williams GT, Chan DT, Buerstedde JM, Baldwin GS, et al. (2007) Dependence of antibody gene diversification on uracil excision. J Exp Med 204: 3209-3219.

142. Begum NA, Izumi N, Nishikori M, Nagaoka H, Shinkura R, et al. (2007) Requirement of non-canonical activity of uracil DNA glycosylase for class switch recombination. J Biol Chem 282: 731-742.

143. Begum NA, Stanlie A, Doi T, Sasaki Y, Jin HW, et al. (2009) Further evidence for involvement of a noncanonical function of uracil DNA glycosylase in class switch recombination. Proc Natl Acad Sci U S A 106: 2752-2757.

144. Imai K, Slupphaug G, Lee WI, Revy P, Nonoyama S, et al. (2003) Human uracil-DNA glycosylase deficiency associated with profoundly impaired immunoglobulin class-switch recombination. Nat Immunol 4: 1023-1028.

145. Kavli B, Andersen S, Otterlei M, Liabakk NB, Imai K, et al. (2005) B cells from hyper-IgM patients carrying UNG mutations lack ability to remove uracil from ssDNA and have elevated genomic uracil. J Exp Med 201: 2011-2021.

146. Myrnes B, Giercksky KE, Krokan H (1983) Interindividual variation in the activity of O6-methyl guanine-DNA methyltransferase and uracil-DNA glycosylase in human organs. Carcinogenesis 4: 1565-1568.

147. Krokan H, Haugen A, Myrnes B, Guddal PH (1983) Repair of premutagenic DNA lesions in human fetal tissues: evidence for low levels of O6-methylguanine-DNA methyltransferase and uracil-DNA glycosylase activity in some tissues. Carcinogenesis 4: 1559-1564.

148. Slupphaug G, Lettrem I, Myrnes B, Krokan HE (1992) Expression of O6-methylguanine--DNA methyltransferase and uracil--DNA glycosylase in human placentae from smokers and non-smokers. Carcinogenesis 13: 1769-1773.

149. Kvaloy K, Nilsen H, Steinsbekk KS, Nedal A, Monterotti B, et al. (2001) Sequence variation in the human uracil-DNA glycosylase (UNG) gene. Mutat Res 461: 325-338.

150. Welsh SJ, Hobbs S, Aherne GW (2003) Expression of uracil DNA glycosylase (UDG) does not affect cellular sensitivity to thymidylate synthase (TS) inhibition. Eur J Cancer 39: 378-387.

151. An Q, Robins P, Lindahl T, Barnes DE (2007) 5-Fluorouracil incorporated into DNA is excised by the Smug1 DNA glycosylase to reduce drug cytotoxicity. Cancer Res 67: 940-945.

152. Braithwaite EK, Kedar PS, Lan L, Polosina YY, Asagoshi K, et al. (2005) DNA polymerase lambda protects mouse fibroblasts against oxidative DNA damage and is recruited to sites of DNA damage/repair. J Biol Chem 280: 31641-31647.

153. Neddermann P, Gallinari P, Lettieri T, Schmid D, Truong O, et al. (1996) Cloning and expression of human G/T mismatch-specific thymine-DNA glycosylase. J Biol Chem 271: 12767-12774.

154. Neddermann P, Jiricny J (1993) The purification of a mismatch-specific thymine-DNA glycosylase from HeLa cells. J Biol Chem 268: 21218-21224.

155. Morgan MT, Bennett MT, Drohat AC (2007) Excision of 5-halogenated uracils by human thymine DNA glycosylase. Robust activity for DNA contexts other than CpG. J Biol Chem 282: 27578-27586.

156. Hardeland U, Bentele M, Jiricny J, Schar P (2003) The versatile thymine DNA-glycosylase: a comparative characterization of the human, Drosophila and fission yeast orthologs. Nucleic Acids Res 31: 2261-2271.

157. Hardeland U, Bentele M, Jiricny J, Schar P (2000) Separating substrate recognition from base hydrolysis in human thymine DNA glycosylase by mutational analysis. J Biol Chem 275: 33449-33456.

158. Waters TR, Gallinari P, Jiricny J, Swann PF (1999) Human thymine DNA glycosylase binds to apurinic sites in DNA but is displaced by human apurinic endonuclease 1. J Biol Chem 274: 67-74.

159. Cortazar D, Kunz C, Saito Y, Steinacher R, Schar P (2007) The enigmatic thymine DNA glycosylase. DNA Repair (Amst) 6: 489-504.

160. Hay RT (2005) SUMO: a history of modification. Mol Cell 18: 1-12.

161. Hardeland U, Steinacher R, Jiricny J, Schar P (2002) Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. EMBO J 21: 1456-1464.

162. Steinacher R, Schar P (2005) Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. Curr Biol 15: 616-623.

163. Shimizu Y, Iwai S, Hanaoka F, Sugasawa K (2003) Xeroderma pigmentosum group C protein interacts physically and functionally with thymine DNA glycosylase. EMBO J 22: 164-173.

164. Guan X, Bai H, Shi G, Theriot CA, Hazra TK, et al. (2007) The human checkpoint sensor Rad9-Rad1-Hus1 interacts with and stimulates NEIL1 glycosylase. Nucleic Acids Res 35: 2463-2472.

165. Wang W, Brandt P, Rossi ML, Lindsey-Boltz L, Podust V, et al. (2004) The human Rad9-Rad1-Hus1 checkpoint complex stimulates flap endonuclease 1. Proc Natl Acad Sci U S A 101: 16762-16767.

166. Toueille M, El-Andaloussi N, Frouin I, Freire R, Funk D, et al. (2004) The human Rad9/Rad1/Hus1 damage sensor clamp interacts with DNA polymerase beta and increases its DNA substrate utilisation efficiency: implications for DNA repair. Nucleic Acids Res 32: 3316-3324.

167. Gembka A, Toueille M, Smirnova E, Poltz R, Ferrari E, et al. (2007) The checkpoint clamp, Rad9-Rad1-Hus1 complex, preferentially stimulates the activity of apurinic/apyrimidinic endonuclease 1 and DNA polymerase beta in long patch base excision repair. Nucleic Acids Res 35: 2596-2608.

168. Friedrich-Heineken E, Toueille M, Tannler B, Burki C, Ferrari E, et al. (2005) The two DNA clamps Rad9/Rad1/Hus1 complex and proliferating cell nuclear antigen differentially regulate flap endonuclease 1 activity. J Mol Biol 353: 980-989.

169. Smirnova E, Toueille M, Markkanen E, Hubscher U (2005) The human checkpoint sensor and alternative DNA clamp Rad9-Rad1-Hus1 modulates the activity of DNA ligase I, a component of the long-patch base excision repair machinery. Biochem J 389: 13-17.

170. Sard L, Tornielli S, Gallinari P, Minoletti F, Jiricny J, et al. (1997) Chromosomal localizations and molecular analysis of TDG gene-related sequences. Genomics 44: 222-226.

171. Kunz C, Focke F, Saito Y, Schuermann D, Lettieri T, et al. (2009) Base excision by thymine DNA glycosylase mediates DNA-directed cytotoxicity of 5-fluorouracil. PLoS Biol 7: e91.
172. Dianov G, Bischoff C, Piotrowski J, Bohr VA (1998) Repair pathways for processing of 8-oxoguanine in DNA by mammalian cell extracts. J Biol Chem 273: 33811-33816.
173. Kim EJ, Um SJ (2008) Thymine-DNA glycosylase interacts with and functions as a coactivator of p53 family proteins. Biochem Biophys Res Commun 377: 838-842.
174. Chen D, Lucey MJ, Phoenix F, Lopez-Garcia J, Hart SM, et al. (2003) T:G mismatch-specific thymine-DNA glycosylase potentiates transcription of estrogen-regulated genes through direct interaction with estrogen receptor alpha. J Biol Chem 278: 38586-38592.
175. Chevray PM, Nathans D (1992) Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. Proc Natl Acad Sci U S A 89: 5789-5793.
176. Boland MJ, Christman JK (2008) Characterization of Dnmt3b:thymine-DNA glycosylase interaction and stimulation of thymine glycosylase-mediated repair by DNA methyltransferase(s) and RNA. J Mol Biol 379: 492-504.
177. Hendrich B, Bird A (1998) Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol 18: 6538-6547.
178. Petronzelli F, Riccio A, Markham GD, Seeholzer SH, Genuardi M, et al. (2000) Investigation of the substrate spectrum of the human mismatch-specific DNA N-glycosylase MED1 (MBD4): fundamental role of the catalytic domain. J Cell Physiol 185: 473-480.
179. Bader S, Walker M, Hendrich B, Bird A, Bird C, et al. (1999) Somatic frameshift mutations in the MBD4 gene of sporadic colon cancers with mismatch repair deficiency. Oncogene 18: 8044-8047.
180. Petronzelli F, Riccio A, Markham GD, Seeholzer SH, Stoerker J, et al. (2000) Biphasic kinetics of the human DNA repair protein MED1 (MBD4), a mismatch-specific DNA N-glycosylase. J Biol Chem 275: 32422-32429.
181. Yamada T, Koyama T, Ohwada S, Tago K, Sakamoto I, et al. (2002) Frameshift mutations in the MBD4/MED1 gene in primary gastric cancer with high-frequency microsatellite instability. Cancer Lett 181: 115-120.
182. Bader S, Walker M, Harrison D (2000) Most microsatellite unstable sporadic colorectal carcinomas carry MBD4 mutations. Br J Cancer 83: 1646-1649.
183. Abdel-Rahman WM, Knuutila S, Peltomaki P, Harrison DJ, Bader SA (2008) Truncation of MBD4 predisposes to reciprocal chromosomal translocations and alters the response to therapeutic agents in colon cancer cells. DNA Repair (Amst) 7: 321-328.
184. Wong E, Yang K, Kuraguchi M, Werling U, Avdievich E, et al. (2002) Mbd4 inactivation increases Cright-arrowT transition mutations and promotes gastrointestinal tumor formation. Proc Natl Acad Sci U S A 99: 14937-14942.
185. Millar CB, Guy J, Sansom OJ, Selfridge J, MacDougall E, et al. (2002) Enhanced CpG mutability and tumorigenesis in MBD4-deficient mice. Science 297: 403-405.
186. Cortellino S, Turner D, Masciullo V, Schepis F, Albino D, et al. (2003) The base excision repair enzyme MED1 mediates DNA damage response to antitumor drugs and is associated with mismatch repair system integrity. Proc Natl Acad Sci U S A 100: 15071-15076.
187. Sansom OJ, Zabkiewicz J, Bishop SM, Guy J, Bird A, et al. (2003) MBD4 deficiency reduces the apoptotic response to DNA-damaging agents in the murine small intestine. Oncogene 22: 7130-7136.
188. Screaton RA, Kiessling S, Sansom OJ, Millar CB, Maddison K, et al. (2003) Fas-associated death domain protein interacts with methyl-CpG binding domain protein 4: a potential link between genome surveillance and apoptosis. Proc Natl Acad Sci U S A 100: 5211-5216.
189. Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, et al. (1999) Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. Proc Natl Acad Sci U S A 96: 13300-13305.
190. Michaels ML, Miller JH (1992) The GO system protects organisms from the mutagenic effect of the spontaneous lesion 8-hydroxyguanine (7,8-dihydro-8-oxoguanine). J Bacteriol 174: 6321-6325.
191. Thomas D, Scot AD, Barbey R, Padula M, Boiteux S (1997) Inactivation of OGG1 increases the incidence of G . C-->T . A transversions in Saccharomyces cerevisiae: evidence for endogenous oxidative damage to DNA in eukaryotic cells. Mol Gen Genet 254: 171-178.
192. Shinmura K, Kasai H, Sasaki A, Sugimura H, Yokota J (1997) 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) DNA glycosylase and AP lyase activities of hOGG1 protein and their substrate specificity. Mutat Res 385: 75-82.
193. Nishioka K, Ohtsubo T, Oda H, Fujiwara T, Kang D, et al. (1999) Expression and differential intracellular localization of two major forms of human 8-oxoguanine DNA glycosylase encoded by alternatively spliced OGG1 mRNAs. Mol Biol Cell 10: 1637-1652.
194. Dhenaut A, Boiteux S, Radicella JP (2000) Characterization of the hOGG1 promoter and its expression during the cell cycle. Mutat Res 461: 109-118.
195. Saitoh T, Shinmura K, Yamaguchi S, Tani M, Seki S, et al. (2001) Enhancement of OGG1 protein AP lyase activity by increase of APEX protein. Mutat Res 486: 31-40.

196. Hill JW, Hazra TK, Izumi T, Mitra S (2001) Stimulation of human 8-oxoguanine-DNA glycosylase by AP-endonuclease: potential coordination of the initial steps in base excision repair. Nucleic Acids Res 29: 430-438.
197. Vidal AE, Hickson ID, Boiteux S, Radicella JP (2001) Mechanism of stimulation of the DNA glycosylase activity of hOGG1 by the major human AP endonuclease: bypass of the AP lyase activity step. Nucleic Acids Res 29: 1285-1292.
198. Pascucci B, Maga G, Hubscher U, Bjoras M, Seeberg E, et al. (2002) Reconstitution of the base excision repair pathway for 7,8-dihydro-8-oxoguanine with purified human proteins. Nucleic Acids Res 30: 2124-2130.
199. Fortini P, Parlanti E, Sidorkina OM, Laval J, Dogliotti E (1999) The type of DNA glycosylase determines the base excision repair pathway in mammalian cells. J Biol Chem 274: 15230-15236.
200. Sakumi K, Tominaga Y, Furuichi M, Xu P, Tsuzuki T, et al. (2003) Ogg1 knockout-associated lung tumorigenesis and its suppression by Mth1 gene disruption. Cancer Res 63: 902-905.
201. Minowa O, Arai T, Hirano M, Monden Y, Nakai S, et al. (2000) Mmh/Ogg1 gene inactivation results in accumulation of 8-hydroxyguanine in mice. Proc Natl Acad Sci U S A 97: 4156-4161.
202. Fortini P, Pascucci B, Parlanti E, D'Errico M, Simonelli V, et al. (2003) 8-Oxoguanine DNA damage: at the crossroad of alternative repair pathways. Mutat Res 531: 127-139.
203. Yang N, Galick H, Wallace SS (2004) Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks. DNA Repair (Amst) 3: 1323-1334.
204. Yang N, Chaudhry MA, Wallace SS (2006) Base excision repair by hNTH1 and hOGG1: a two edged sword in the processing of DNA damage in gamma-irradiated human cells. DNA Repair (Amst) 5: 43-51.
205. Shibutani S, Takeshita M, Grollman AP (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 349: 431-434.
206. Slupska MM, Baikalov C, Luther WM, Chiang JH, Wei YF, et al. (1996) Cloning and sequencing a human homolog (hMYH) of the Escherichia coli mutY gene whose function is required for the repair of oxidative DNA damage. J Bacteriol 178: 3885-3892.
207. Slupska MM, Luther WM, Chiang JH, Yang H, Miller JH (1999) Functional expression of hMYH, a human homolog of the Escherichia coli MutY protein. J Bacteriol 181: 6210-6213.
208. Parlanti E, Fortini P, Macpherson P, Laval J, Dogliotti E (2002) Base excision repair of adenine/8-oxoguanine mispairs by an aphidicolin-sensitive DNA polymerase in human cell extracts. Oncogene 21: 5204-5212.
209. McGoldrick JP, Yeh YC, Solomon M, Essigmann JM, Lu AL (1995) Characterization of a mammalian homolog of the Escherichia coli MutY mismatch repair protein. Mol Cell Biol 15: 989-996.
210. Yang H, Clendenin WM, Wong D, Demple B, Slupska MM, et al. (2001) Enhanced activity of adenine-DNA glycosylase (Myh) by apurinic/apyrimidinic endonuclease (Ape1) in mammalian base excision repair of an A/GO mismatch. Nucleic Acids Res 29: 743-752.
211. Ohtsubo T, Nishioka K, Imaiso Y, Iwai S, Shimokawa H, et al. (2000) Identification of human MutY homolog (hMYH) as a repair enzyme for 2-hydroxyadenine in DNA and detection of multiple forms of hMYH located in nuclei and mitochondria. Nucleic Acids Res 28: 1355-1364.
212. Parker A, Gu Y, Mahoney W, Lee SH, Singh KK, et al. (2001) Human homolog of the MutY repair protein (hMYH) physically interacts with proteins involved in long patch DNA base excision repair. J Biol Chem 276: 5547-5555.
213. Boldogh I, Milligan D, Lee MS, Bassett H, Lloyd RS, et al. (2001) hMYH cell cycle-dependent expression, subcellular localization and association with replication foci: evidence suggesting replication-coupled repair of adenine:8-oxoguanine mispairs. Nucleic Acids Res 29: 2802-2809.
214. Hashimoto K, Tominaga Y, Nakabeppu Y, Moriya M (2004) Futile short-patch DNA base excision repair of adenine:8-oxoguanine mispair. Nucleic Acids Res 32: 5928-5934.
215. Maga G, Crespan E, Wimmer U, van Loon B, Amoroso A, et al. (2008) Replication protein A and proliferating cell nuclear antigen coordinate DNA polymerase selection in 8-oxo-guanine repair. Proc Natl Acad Sci U S A 105: 20689-20694.
216. Chou KM, Cheng YC (2002) An exonucleolytic activity of human apurinic/apyrimidinic endonuclease on 3' mispaired DNA. Nature 415: 655-659.
217. Xie Y, Yang H, Cunanan C, Okamoto K, Shibata D, et al. (2004) Deficiencies in mouse Myh and Ogg1 result in tumor predisposition and G to T mutations in codon 12 of the K-ras oncogene in lung tumors. Cancer Res 64: 3096-3102.
218. Russo MT, De Luca G, Degan P, Parlanti E, Dogliotti E, et al. (2004) Accumulation of the oxidative base lesion 8-hydroxyguanine in DNA of tumor-prone mice defective in both the Myh and Ogg1 DNA glycosylases. Cancer Res 64: 4411-4414.
219. Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, et al. (2002) Inherited variants of MYH associated with somatic G:C-->T:A mutations in colorectal tumors. Nat Genet 30: 227-232.
220. Cheadle JP, Sampson JR (2007) MUTYH-associated polyposis--from defect in base excision repair to clinical genetic testing. DNA Repair (Amst) 6: 274-279.
221. Farrington SM, Tenesa A, Barnetson R, Wiltshire A, Prendergast J, et al. (2005) Germline susceptibility to colorectal cancer due to base-excision repair gene defects. Am J Hum Genet 77: 112-119.

74

222. Dizdaroglu M, Karahalil B, Senturker S, Buckley TJ, Roldan-Arjona T (1999) Excision of products of oxidative DNA base damage by human NTH1 protein. Biochemistry 38: 243-246.
223. Dizdaroglu M (2005) Base-excision repair of oxidative DNA damage by DNA glycosylases. Mutat Res 591: 45-59.
224. Eide L, Luna L, Gustad EC, Henderson PT, Essigmann JM, et al. (2001) Human endonuclease III acts preferentially on DNA damage opposite guanine residues in DNA. Biochemistry 40: 6653-6659.
225. Ikeda S, Biswas T, Roy R, Izumi T, Boldogh I, et al. (1998) Purification and characterization of human NTH1, a homolog of Escherichia coli endonuclease III. Direct identification of Lys-212 as the active nucleophilic residue. J Biol Chem 273: 21585-21593.
226. Luna L, Bjoras M, Hoff E, Rognes T, Seeberg E (2000) Cell-cycle regulation, intracellular sorting and induced overexpression of the human NTH1 DNA glycosylase involved in removal of formamidopyrimidine residues from DNA. Mutat Res 460: 95-104.
227. Marenstein DR, Chan MK, Altamirano A, Basu AK, Boorstein RJ, et al. (2003) Substrate specificity of human endonuclease III (hNTH1). Effect of human APE1 on hNTH1 activity. J Biol Chem 278: 9005-9012.
228. Oyama M, Wakasugi M, Hama T, Hashidume H, Iwakami Y, et al. (2004) Human NTH1 physically interacts with p53 and proliferating cell nuclear antigen. Biochem Biophys Res Commun 321: 183-191.
229. Marenstein DR, Ocampo MT, Chan MK, Altamirano A, Basu AK, et al. (2001) Stimulation of human endonuclease III by Y box-binding protein 1 (DNA-binding protein B). Interaction between a base excision repair enzyme and a transcription factor. J Biol Chem 276: 21242-21249.
230. Liu X, Choudhury S, Roy R (2003) In vitro and in vivo dimerization of human endonuclease III stimulates its activity. J Biol Chem 278: 50061-50069.
231. Takao M, Aburatani H, Kobayashi K, Yasui A (1998) Mitochondrial targeting of human DNA glycosylases for repair of oxidative DNA damage. Nucleic Acids Res 26: 2917-2922.
232. Ocampo MT, Chaung W, Marenstein DR, Chan MK, Altamirano A, et al. (2002) Targeted deletion of mNth1 reveals a novel DNA repair enzyme activity. Mol Cell Biol 22: 6111-6121.
233. Takao M, Kanno S, Kobayashi K, Zhang QM, Yonei S, et al. (2002) A back-up glycosylase in Nth1 knock-out mice is a functional Nei (endonuclease VIII) homologue. J Biol Chem 277: 42205-42213.
234. Bandaru V, Sunkara S, Wallace SS, Bond JP (2002) A novel human DNA glycosylase that removes oxidative DNA damage and is homologous to Escherichia coli endonuclease VIII. DNA Repair (Amst) 1: 517-529.
235. Hazra TK, Izumi T, Boldogh I, Imhoff B, Kow YW, et al. (2002) Identification and characterization of a human DNA glycosylase for repair of modified bases in oxidatively damaged DNA. Proc Natl Acad Sci U S A 99: 3523-3528.
236. Hazra TK, Kow YW, Hatahet Z, Imhoff B, Boldogh I, et al. (2002) Identification and characterization of a novel human DNA glycosylase for repair of cytosine-derived lesions. J Biol Chem 277: 30417-30420.
237. Das A, Wiederhold L, Leppard JB, Kedar P, Prasad R, et al. (2006) NEIL2-initiated, APE-independent repair of oxidized bases in DNA: Evidence for a repair complex in human cells. DNA Repair (Amst) 5: 1439-1448.
238. Grin IR, Khodyreva SN, Nevinsky GA, Zharkov DO (2006) Deoxyribophosphate lyase activity of mammalian endonuclease VIII-like proteins. FEBS Lett 580: 4916-4922.
239. Hu J, de Souza-Pinto NC, Haraguchi K, Hogue BA, Jaruga P, et al. (2005) Repair of formamidopyrimidines in DNA involves different glycosylases: role of the OGG1, NTH1, and NEIL1 enzymes. J Biol Chem 280: 40544-40551.
240. Dou H, Mitra S, Hazra TK (2003) Repair of oxidized bases in DNA bubble structures by human DNA glycosylases NEIL1 and NEIL2. J Biol Chem 278: 49679-49684.
241. Dou H, Theriot CA, Das A, Hegde ML, Matsumoto Y, et al. (2008) Interaction of the human DNA glycosylase NEIL1 with proliferating cell nuclear antigen. The potential for replication-associated repair of oxidized bases in mammalian genomes. J Biol Chem 283: 3130-3140.
242. Das A, Boldogh I, Lee JW, Harrigan JA, Hegde ML, et al. (2007) The human Werner syndrome protein stimulates repair of oxidative DNA base damage by the DNA glycosylase NEIL1. J Biol Chem 282: 26591-26602.
243. Muftuoglu M, de Souza-Pinto NC, Dogan A, Aamann M, Stevnsner T, et al. (2009) Cockayne syndrome group B protein stimulates repair of formamidopyrimidines by NEIL1 DNA glycosylase. J Biol Chem 284: 9270-9279.
244. Maiti AK, Boldogh I, Spratt H, Mitra S, Hazra TK (2008) Mutator phenotype of mammalian cells due to deficiency of NEIL1 DNA glycosylase, an oxidized base-specific repair enzyme. DNA Repair (Amst) 7: 1213-1220.
245. Vartanian V, Lowell B, Minko IG, Wood TG, Ceci JD, et al. (2006) The metabolic syndrome resulting from a knockout of the NEIL1 DNA glycosylase. Proc Natl Acad Sci U S A 103: 1864-1869.
246. Chan MK, Ocampo-Hafalla MT, Vartanian V, Jaruga P, Kirkali G, et al. (2009) Targeted deletion of the genes encoding NTH1 and NEIL1 DNA N-glycosylases reveals the existence of novel carcinogenic oxidative damage to DNA. DNA Repair (Amst) 8: 786-794.
247. Das S, Chattopadhyay R, Bhakat KK, Boldogh I, Kohno K, et al. (2007) Stimulation of NEIL2-mediated oxidized base excision repair via YB-1 interaction during oxidative stress. J Biol Chem 282: 28474-28484.

248. Takao M, Oohata Y, Kitadokoro K, Kobayashi K, Iwai S, et al. (2009) Human Nei-like protein NEIL3 has AP lyase activity specific for single-stranded DNA and confers oxidative stress resistance in Escherichia coli mutant. Genes Cells 14: 261-270.

249. Wyatt MD, Allan JM, Lau AY, Ellenberger TE, Samson LD (1999) 3-methyladenine DNA glycosylases: structure, function, and biological importance. Bioessays 21: 668-676.

250. Miao F, Bouziane M, Dammann R, Masutani C, Hanaoka F, et al. (2000) 3-Methyladenine-DNA glycosylase (MPG protein) interacts with human RAD23 proteins. J Biol Chem 275: 28433-28438.

251. Xia L, Zheng L, Lee HW, Bates SE, Federico L, et al. (2005) Human 3-methyladenine-DNA glycosylase: effect of sequence context on excision, association with PCNA, and stimulation by AP endonuclease. J Mol Biol 346: 1259-1274.

252. Watanabe S, Ichimura T, Fujita N, Tsuruzoe S, Ohki I, et al. (2003) Methylated DNA-binding domain 1 and methylpurine-DNA glycosylase link transcriptional repression and DNA repair in chromatin. Proc Natl Acad Sci U S A 100: 12859-12864.

253. Campalans A, Marsin S, Nakabeppu Y, O'Connor T R, Boiteux S, et al. (2005) XRCC1 interactions with multiple DNA glycosylases: a model for its recruitment to base excision repair. DNA Repair (Amst) 4: 826-835.

254. Beranek DT (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat Res 231: 11-30.

255. Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C (1997) Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rev 23: 35-61.

256. Coquerelle T, Dosch J, Kaina B (1995) Overexpression of N-methylpurine-DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents--a case of imbalanced DNA repair. Mutat Res 336: 9-17.

257. Rinne M, Caldwell D, Kelley MR (2004) Transient adenoviral N-methylpurine DNA glycosylase overexpression imparts chemotherapeutic sensitivity to human breast cancer cells. Mol Cancer Ther 3: 955-967.

258. Rinne ML, He Y, Pachkowski BF, Nakamura J, Kelley MR (2005) N-methylpurine DNA glycosylase overexpression increases alkylation sensitivity by rapidly removing non-toxic 7-methylguanine adducts. Nucleic Acids Res 33: 2859-2867.

259. Horton JK, Wilson SH (2007) Hypersensitivity phenotypes associated with genetic and synthetic inhibitor-induced base excision repair deficiency. DNA Repair (Amst) 6: 530-543.

260. Trivedi RN, Almeida KH, Fornsaglio JL, Schamus S, Sobol RW (2005) The role of base excision repair in the sensitivity and resistance to temozolomide-mediated cell death. Cancer Res 65: 6394-6400.

261. Sobol RW, Kartalou M, Almeida KH, Joyce DF, Engelward BP, et al. (2003) Base excision repair intermediates induce p53-independent cytotoxic and genotoxic responses. J Biol Chem 278: 39951-39959.

262. Paquette Y, Crine P, Verly WG (1972) Properties of the endonuclease for depurinated DNA from Escherichia coli. Can J Biochem 50: 1199-1209.

263. Kane CM, Linn S (1981) Purification and characterization of an apurinic/apyrimidinic endonuclease from HeLa cells. J Biol Chem 256: 3405-3414.

264. Evans AR, Limp-Foster M, Kelley MR (2000) Going APE over ref-1. Mutat Res 461: 83-108.

265. Ischenko AA, Saparbaev MK (2002) Alternative nucleotide incision repair pathway for oxidative DNA damage. Nature 415: 183-187.

266. Gros L, Ishchenko AA, Ide H, Elder RH, Saparbaev MK (2004) The major human AP endonuclease (Ape1) is involved in the nucleotide incision repair pathway. Nucleic Acids Res 32: 73-81.

267. Fishel ML, Kelley MR (2007) The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. Mol Aspects Med 28: 375-395.

268. Xanthoudakis S, Smeyne RJ, Wallace JD, Curran T (1996) The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proc Natl Acad Sci U S A 93: 8919-8923.

269. Huamani J, McMahan CA, Herbert DC, Reddick R, McCarrey JR, et al. (2004) Spontaneous mutagenesis is enhanced in Apex heterozygous mice. Mol Cell Biol 24: 8145-8153.

270. Meira LB, Devaraj S, Kisby GE, Burns DK, Daniel RL, et al. (2001) Heterozygosity for the mouse Apex gene results in phenotypes associated with oxidative stress. Cancer Res 61: 5552-5557.

271. Walker LJ, Craig RB, Harris AL, Hickson ID (1994) A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress. Nucleic Acids Res 22: 4884-4889.

272. Fung H, Demple B (2005) A vital role for Ape1/Ref1 protein in repairing spontaneous DNA damage in human cells. Mol Cell 17: 463-470.

273. Robertson KA, Bullock HA, Xu Y, Tritt R, Zimmerman E, et al. (2001) Altered expression of Ape1/ref-1 in germ cell tumors and overexpression in NT2 cells confers resistance to bleomycin and radiation. Cancer Res 61: 2220-2225.

274. Fung H, Bennett RA, Demple B (2001) Key role of a downstream specificity protein 1 site in cell cycle-regulated transcription of the AP endonuclease gene APE1/APEX in NIH3T3 cells. J Biol Chem 276: 42011-42017.

275. Pettersen HS, Sundheim O, Gilljam KM, Slupphaug G, Krokan HE, et al. (2007) Uracil-DNA glycosylases SMUG1 and UNG2 coordinate the initial steps of base excision repair by distinct mechanisms. Nucleic Acids Res 35: 3879-3892.

276. Sidorenko VS, Nevinsky GA, Zharkov DO (2007) Mechanism of interaction between human 8-oxoguanine-DNA glycosylase and AP endonuclease. DNA Repair (Amst) 6: 317-328.

277. Parikh SS, Putnam CD, Tainer JA (2000) Lessons learned from structural results on uracil-DNA glycosylase. Mutat Res 460: 183-199.

278. Masuda Y, Bennett RA, Demple B (1998) Dynamics of the interaction of human apurinic endonuclease (Ape1) with its substrate and product. J Biol Chem 273: 30352-30359.

279. Mol CD, Izumi T, Mitra S, Tainer JA (2000) DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination [corrected]. Nature 403: 451-456.

280. Bennett RA, Wilson DM, 3rd, Wong D, Demple B (1997) Interaction of human apurinic endonuclease and DNA polymerase beta in the base excision repair pathway. Proc Natl Acad Sci U S A 94: 7166-7169.

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

282. Dianova, II, Bohr VA, Dianov GL (2001) Interaction of human AP endonuclease 1 with flap endonuclease 1 and proliferating cell nuclear antigen involved in long-patch base excision repair. Biochemistry 40: 12639-12644.

283. Ranalli TA, Tom S, Bambara RA (2002) AP endonuclease 1 coordinates flap endonuclease 1 and DNA ligase I activity in long patch base excision repair. J Biol Chem 277: 41715-41724.

284. Prasad R, Liu Y, Deterding LJ, Poltoratsky VP, Kedar PS, et al. (2007) HMGB1 is a cofactor in mammalian base excision repair. Mol Cell 27: 829-841.

285. Kenny MK, Mendez F, Sandigursky M, Kureekattil RP, Goldman JD, et al. (2001) Heat shock protein 70 binds to human apurinic/apyrimidinic endonuclease and stimulates endonuclease activity at abasic sites. J Biol Chem 276: 9532-9536.

286. Vidal AE, Boiteux S, Hickson ID, Radicella JP (2001) XRCC1 coordinates the initial and late stages of DNA abasic site repair through protein-protein interactions. EMBO J 20: 6530-6539.

287. Gaiddon C, Moorthy NC, Prives C (1999) Ref-1 regulates the transactivation and pro-apoptotic functions of p53 in vivo. EMBO J 18: 5609-5621.

288. Ahn B, Harrigan JA, Indig FE, Wilson DM, 3rd, Bohr VA (2004) Regulation of WRN helicase activity in human base excision repair. J Biol Chem 279: 53465-53474.

289. Zhao J, Gao F, Zhang Y, Wei K, Liu Y, et al. (2008) Bcl2 inhibits abasic site repair by down-regulating APE1 endonuclease activity. J Biol Chem 283: 9925-9932.

290. Fan Z, Beresford PJ, Zhang D, Xu Z, Novina CD, et al. (2003) Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. Nat Immunol 4: 145-153.

291. Xi T, Jones IM, Mohrenweiser HW (2004) Many amino acid substitution variants identified in DNA repair genes during human population screenings are predicted to impact protein function. Genomics 83: 970-979.

292. Hung RJ, Brennan P, Canzian F, Szeszenia-Dabrowska N, Zaridze D, et al. (2005) Large-scale investigation of base excision repair genetic polymorphisms and lung cancer risk in a multicenter study. J Natl Cancer Inst 97: 567-576.

293. Whitehouse CJ, Taylor RM, Thistlethwaite A, Zhang H, Karimi-Busheri F, et al. (2001) XRCC1 stimulates human polynucleotide kinase activity at damaged DNA termini and accelerates DNA single-strand break repair. Cell 104: 107-117.

294. Chappell C, Hanakahi LA, Karimi-Busheri F, Weinfeld M, West SC (2002) Involvement of human polynucleotide kinase in double-strand break repair by non-homologous end joining. EMBO J 21: 2827-2832.

295. Rasouli-Nia A, Karimi-Busheri F, Weinfeld M (2004) Stable down-regulation of human polynucleotide kinase enhances spontaneous mutation frequency and sensitizes cells to genotoxic agents. Proc Natl Acad Sci U S A 101: 6905-6910.

296. Sobol RW, Horton JK, Kuhn R, Gu H, Singhal RK, et al. (1996) Requirement of mammalian DNA polymerase-beta in base-excision repair. Nature 379: 183-186.

297. Dianov G, Price A, Lindahl T (1992) Generation of single-nucleotide repair patches following excision of uracil residues from DNA. Mol Cell Biol 12: 1605-1612.

298. Podlutsky AJ, Dianova, II, Wilson SH, Bohr VA, Dianov GL (2001) DNA synthesis and dRPase activities of polymerase beta are both essential for single-nucleotide patch base excision repair in mammalian cell extracts. Biochemistry 40: 809-813.

299. Podlutsky AJ, Dianova, II, Podust VN, Bohr VA, Dianov GL (2001) Human DNA polymerase beta initiates DNA synthesis during long-patch repair of reduced AP sites in DNA. EMBO J 20: 1477-1482.

300. Matsumoto Y, Kim K (1995) Excision of deoxyribose phosphate residues by DNA polymerase beta during DNA repair. Science 269: 699-702.

301. Srivastava DK, Berg BJ, Prasad R, Molina JT, Beard WA, et al. (1998) Mammalian abasic site base excision repair. Identification of the reaction sequence and rate-determining steps. J Biol Chem 273: 21203-21209.

302. Sobol RW, Prasad R, Evenski A, Baker A, Yang XP, et al. (2000) The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity. Nature 405: 807-810.

303. Allinson SL, Dianova, II, Dianov GL (2001) DNA polymerase beta is the major dRP lyase involved in repair of oxidative base lesions in DNA by mammalian cell extracts. EMBO J 20: 6919-6926.

304. Almeida KH, Sobol RW (2007) A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification. DNA Repair (Amst) 6: 695-711.

305. Harrigan JA, Opresko PL, von Kobbe C, Kedar PS, Prasad R, et al. (2003) The Werner syndrome protein stimulates DNA polymerase beta strand displacement synthesis via its helicase activity. J Biol Chem 278: 22686-22695.

306. Prasad R, Dianov GL, Bohr VA, Wilson SH (2000) FEN1 stimulation of DNA polymerase beta mediates an excision step in mammalian long patch base excision repair. J Biol Chem 275: 4460-4466.

307. Fan J, Wilson DM, 3rd (2005) Protein-protein interactions and posttranslational modifications in mammalian base excision repair. Free Radic Biol Med 38: 1121-1138.

308. Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K (1994) Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science 265: 103-106.

309. Cabelof DC, Guo Z, Raffoul JJ, Sobol RW, Wilson SH, et al. (2003) Base excision repair deficiency caused by polymerase beta haploinsufficiency: accelerated DNA damage and increased mutational response to carcinogens. Cancer Res 63: 5799-5807.

310. Cabelof DC, Ikeno Y, Nyska A, Busuttil RA, Anyangwe N, et al. (2006) Haploinsufficiency in DNA polymerase beta increases cancer risk with age and alters mortality rate. Cancer Res 66: 7460-7465.

311. Albertella MR, Lau A, O'Connor MJ (2005) The overexpression of specialized DNA polymerases in cancer. DNA Repair (Amst) 4: 583-593.

312. Canitrot Y, Cazaux C, Frechet M, Bouayadi K, Lesca C, et al. (1998) Overexpression of DNA polymerase beta in cell results in a mutator phenotype and a decreased sensitivity to anticancer drugs. Proc Natl Acad Sci U S A 95: 12586-12590.

313. Bergoglio V, Pillaire MJ, Lacroix-Triki M, Raynaud-Messina B, Canitrot Y, et al. (2002) Deregulated DNA polymerase beta induces chromosome instability and tumorigenesis. Cancer Res 62: 3511-3514.

314. Starcevic D, Dalal S, Sweasy JB (2004) Is there a link between DNA polymerase beta and cancer? Cell Cycle 3: 998-1001.

315. Dalal S, Chikova A, Jaeger J, Sweasy JB (2008) The Leu22Pro tumor-associated variant of DNA polymerase beta is dRP lyase deficient. Nucleic Acids Res 36: 411-422.

316. Lang T, Maitra M, Starcevic D, Li SX, Sweasy JB (2004) A DNA polymerase beta mutant from colon cancer cells induces mutations. Proc Natl Acad Sci U S A 101: 6074-6079.

317. Sweasy JB, Lang T, Starcevic D, Sun KW, Lai CC, et al. (2005) Expression of DNA polymerase {beta} cancer-associated variants in mouse cells results in cellular transformation. Proc Natl Acad Sci U S A 102: 14350-14355.

318. Bhattacharyya N, Banerjee S (1997) A variant of DNA polymerase beta acts as a dominant negative mutant. Proc Natl Acad Sci U S A 94: 10324-10329.

319. Stucki M, Pascucci B, Parlanti E, Fortini P, Wilson SH, et al. (1998) Mammalian base excision repair by DNA polymerases delta and epsilon. Oncogene 17: 835-843.

320. Fortini P, Pascucci B, Parlanti E, Sobol RW, Wilson SH, et al. (1998) Different DNA polymerases are involved in the short- and long-patch base excision repair in mammalian cells. Biochemistry 37: 3575-3580.

321. Matsumoto Y, Kim K, Hurwitz J, Gary R, Levin DS, et al. (1999) Reconstitution of proliferating cell nuclear antigen-dependent repair of apurinic/apyrimidinic sites with purified human proteins. J Biol Chem 274: 33703-33708.

322. Harrington JJ, Lieber MR (1994) The characterization of a mammalian DNA structure-specific endonuclease. EMBO J 13: 1235-1246.

323. Liu Y, Kao HI, Bambara RA (2004) Flap endonuclease 1: a central component of DNA metabolism. Annu Rev Biochem 73: 589-615.

324. Zheng L, Zhou M, Chai Q, Parrish J, Xue D, et al. (2005) Novel function of the flap endonuclease 1 complex in processing stalled DNA replication forks. EMBO Rep 6: 83-89.

325. Nakamura J, La DK, Swenberg JA (2000) 5'-nicked apurinic/apyrimidinic sites are resistant to beta-elimination by beta-polymerase and are persistent in human cultured cells after oxidative stress. J Biol Chem 275: 5323-5328.

326. Klungland A, Lindahl T (1997) Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). EMBO J 16: 3341-3348.

327. Prasad R, Lavrik OI, Kim SJ, Kedar P, Yang XP, et al. (2001) DNA polymerase beta -mediated long patch base excision repair. Poly(ADP-ribose)polymerase-1 stimulates strand displacement DNA synthesis. J Biol Chem 276: 32411-32414.

328. Li X, Li J, Harrington J, Lieber MR, Burgers PM (1995) Lagging strand DNA synthesis at the eukaryotic replication fork involves binding and stimulation of FEN-1 by proliferating cell nuclear antigen. J Biol Chem 270: 22109-22112.

329. Tom S, Ranalli TA, Podust VN, Bambara RA (2001) Regulatory roles of p21 and apurinic/apyrimidinic endonuclease 1 in base excision repair. J Biol Chem 276: 48781-48789.

330. Brosh RM, Jr., von Kobbe C, Sommers JA, Karmakar P, Opresko PL, et al. (2001) Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity. EMBO J 20: 5791-5801.
331. Kucherlapati M, Yang K, Kuraguchi M, Zhao J, Lia M, et al. (2002) Haploinsufficiency of Flap endonuclease (Fen1) leads to rapid tumor progression. Proc Natl Acad Sci U S A 99: 9924-9929.
332. Singh P, Yang M, Dai H, Yu D, Huang Q, et al. (2008) Overexpression and hypomethylation of flap endonuclease 1 gene in breast and other cancers. Mol Cancer Res 6: 1710-1717.
333. Zheng L, Dai H, Zhou M, Li M, Singh P, et al. (2007) Fen1 mutations result in autoimmunity, chronic inflammation and cancers. Nat Med 13: 812-819.
334. Grawunder U, Zimmer D, Fugmann S, Schwarz K, Lieber MR (1998) DNA ligase IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. Mol Cell 2: 477-484.
335. Montecucco A, Rossi R, Levin DS, Gary R, Park MS, et al. (1998) DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories. EMBO J 17: 3786-3795.
336. Montecucco A, Biamonti G, Savini E, Focher F, Spadari S, et al. (1992) DNA ligase I gene expression during differentiation and cell proliferation. Nucleic Acids Res 20: 6209-6214.
337. Prasad R, Singhal RK, Srivastava DK, Molina JT, Tomkinson AE, et al. (1996) Specific interaction of DNA polymerase beta and DNA ligase I in a multiprotein base excision repair complex from bovine testis. J Biol Chem 271: 16000-16007.
338. Levin DS, McKenna AE, Motycka TA, Matsumoto Y, Tomkinson AE (2000) Interaction between PCNA and DNA ligase I is critical for joining of Okazaki fragments and long-patch base-excision repair. Curr Biol 10: 919-922.
339. Wang W, Lindsey-Boltz LA, Sancar A, Bambara RA (2006) Mechanism of stimulation of human DNA ligase I by the Rad9-rad1-Hus1 checkpoint complex. J Biol Chem 281: 20865-20872.
340. Mackey ZB, Ramos W, Levin DS, Walter CA, McCarrey JR, et al. (1997) An alternative splicing event which occurs in mouse pachytene spermatocytes generates a form of DNA ligase III with distinct biochemical properties that may function in meiotic recombination. Mol Cell Biol 17: 989-998.
341. Lakshmipathy U, Campbell C (1999) The human DNA ligase III gene encodes nuclear and mitochondrial proteins. Mol Cell Biol 19: 3869-3876.
342. Caldecott KW, McKeown CK, Tucker JD, Ljungquist S, Thompson LH (1994) An interaction between the mammalian DNA repair protein XRCC1 and DNA ligase III. Mol Cell Biol 14: 68-76.
343. Caldecott KW (2003) XRCC1 and DNA strand break repair. DNA Repair (Amst) 2: 955-969.
344. Leppard JB, Dong Z, Mackey ZB, Tomkinson AE (2003) Physical and functional interaction between DNA ligase IIIalpha and poly(ADP-Ribose) polymerase 1 in DNA single-strand break repair. Mol Cell Biol 23: 5919-5927.
345. Parsons JL, Dianova, II, Allinson SL, Dianov GL (2005) DNA polymerase beta promotes recruitment of DNA ligase III alpha-XRCC1 to sites of base excision repair. Biochemistry 44: 10613-10619.
346. Horton JK, Watson M, Stefanick DF, Shaughnessy DT, Taylor JA, et al. (2008) XRCC1 and DNA polymerase beta in cellular protection against cytotoxic DNA single-strand breaks. Cell Res 18: 48-63.
347. Lan L, Nakajima S, Oohata Y, Takao M, Okano S, et al. (2004) In situ analysis of repair processes for oxidative DNA damage in mammalian cells. Proc Natl Acad Sci U S A 101: 13738-13743.
348. Mani RS, Karimi-Busheri F, Fanta M, Caldecott KW, Cass CE, et al. (2004) Biophysical characterization of human XRCC1 and its binding to damaged and undamaged DNA. Biochemistry 43: 16505-16514.
349. Nazarkina ZK, Khodyreva SN, Marsin S, Lavrik OI, Radicella JP (2007) XRCC1 interactions with base excision repair DNA intermediates. DNA Repair (Amst) 6: 254-264.
350. Wong HK, Kim D, Hogue BA, McNeill DR, Wilson DM, 3rd (2005) DNA damage levels and biochemical repair capacities associated with XRCC1 deficiency. Biochemistry 44: 14335-14343.
351. Marsin S, Vidal AE, Sossou M, Menissier-de Murcia J, Le Page F, et al. (2003) Role of XRCC1 in the coordination and stimulation of oxidative DNA damage repair initiated by the DNA glycosylase hOGG1. J Biol Chem 278: 44068-44074.
352. Masson M, Niedergang C, Schreiber V, Muller S, Menissier-de Murcia J, et al. (1998) XRCC1 is specifically associated with poly(ADP-ribose) polymerase and negatively regulates its activity following DNA damage. Mol Cell Biol 18: 3563-3571.
353. Schreiber V, Ame JC, Dolle P, Schultz I, Rinaldi B, et al. (2002) Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. J Biol Chem 277: 23028-23036.
354. Fan J, Otterlei M, Wong HK, Tomkinson AE, Wilson DM, 3rd (2004) XRCC1 co-localizes and physically interacts with PCNA. Nucleic Acids Res 32: 2193-2201.
355. Luo H, Chan DW, Yang T, Rodriguez M, Chen BP, et al. (2004) A new XRCC1-containing complex and its role in cellular survival of methyl methanesulfonate treatment. Mol Cell Biol 24: 8356-8365.
356. Tebbs RS, Flannery ML, Meneses JJ, Hartmann A, Tucker JD, et al. (1999) Requirement for the Xrcc1 DNA base excision repair gene during early mouse development. Dev Biol 208: 513-529.
357. Ladiges WC (2006) Mouse models of XRCC1 DNA repair polymorphisms and cancer. Oncogene 25: 1612-1619.

358. Hu Z, Ma H, Chen F, Wei Q, Shen H (2005) XRCC1 polymorphisms and cancer risk: a meta-analysis of 38 case-control studies. Cancer Epidemiol Biomarkers Prev 14: 1810-1818.

359. Frouin I, Maga G, Denegri M, Riva F, Savio M, et al. (2003) Human proliferating cell nuclear antigen, poly(ADP-ribose) polymerase-1, and p21waf1/cip1. A dynamic exchange of partners. J Biol Chem 278: 39265-39268.

360. Benjamin RC, Gill DM (1980) ADP-ribosylation in mammalian cell ghosts. Dependence of poly(ADP-ribose) synthesis on strand breakage in DNA. J Biol Chem 255: 10493-10501.

361. Ferro AM, Olivera BM (1982) Poly(ADP-ribosylation) in vitro. Reaction parameters and enzyme mechanism. J Biol Chem 257: 7808-7813.

362. Allinson SL, Sleeth KM, Matthewman GE, Dianov GL (2004) Orchestration of base excision repair by controlling the rates of enzymatic activities. DNA Repair (Amst) 3: 23-31.

363. El-Khamisy SF, Masutani M, Suzuki H, Caldecott KW (2003) A requirement for PARP-1 for the assembly or stability of XRCC1 nuclear foci at sites of oxidative DNA damage. Nucleic Acids Res 31: 5526-5533.

364. Kim MY, Zhang T, Kraus WL (2005) Poly(ADP-ribosyl)ation by PARP-1: 'PAR-laying' NAD+ into a nuclear signal. Genes Dev 19: 1951-1967.

365. Oei SL, Ziegler M (2000) ATP for the DNA ligation step in base excision repair is generated from poly(ADP-ribose). J Biol Chem 275: 23234-23239.

366. Shieh WM, Ame JC, Wilson MV, Wang ZQ, Koh DW, et al. (1998) Poly(ADP-ribose) polymerase null mouse cells synthesize ADP-ribose polymers. J Biol Chem 273: 30069-30072.

367. Ame JC, Rolli V, Schreiber V, Niedergang C, Apiou F, et al. (1999) PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. J Biol Chem 274: 17860-17868.

368. Menissier de Murcia J, Ricoul M, Tartier L, Niedergang C, Huber A, et al. (2003) Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. EMBO J 22: 2255-2263.

369. Zheng L, Zhou M, Guo Z, Lu H, Qian L, et al. (2008) Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. Mol Cell 32: 325-336.

370. Clayton DA, Doda JN, Friedberg EC (1974) The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. Proc Natl Acad Sci U S A 71: 2777-2781.

371. Clayton DA, Doda JN, Friedberg EC (1975) Absence of a pyrimidine dimer repair mechanism for mitochondrial DNA in mouse and human cells. Basic Life Sci 5B: 589-591.

372. Pascucci B, Versteegh A, van Hoffen A, van Zeeland AA, Mullenders LH, et al. (1997) DNA repair of UV photoproducts and mutagenesis in human mitochondrial DNA. J Mol Biol 273: 417-427.

373. Thyagarajan B, Padua RA, Campbell C (1996) Mammalian mitochondria possess homologous DNA recombination activity. J Biol Chem 271: 27536-27543.

374. Kajander OA, Karhunen PJ, Holt IJ, Jacobs HT (2001) Prominent mitochondrial DNA recombination intermediates in human heart muscle. EMBO Rep 2: 1007-1012.

375. Lakshmipathy U, Campbell C (1999) Double strand break rejoining by mammalian mitochondrial extracts. Nucleic Acids Res 27: 1198-1204.

376. Larsen NB, Rasmussen M, Rasmussen LJ (2005) Nuclear and mitochondrial DNA repair: similar pathways? Mitochondrion 5: 89-108.

377. Mason PA, Matheson EC, Hall AG, Lightowlers RN (2003) Mismatch repair activity in mammalian mitochondria. Nucleic Acids Res 31: 1052-1058.

378. de Souza-Pinto NC, Mason PA, Hashiguchi K, Weissman L, Tian J, et al. (2009) Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. DNA Repair (Amst).

379. Pinz KG, Bogenhagen DF (1998) Efficient repair of abasic sites in DNA by mitochondrial enzymes. Mol Cell Biol 18: 1257-1265.

380. Stierum RH, Dianov GL, Bohr VA (1999) Single-nucleotide patch base excision repair of uracil in DNA by mitochondrial protein extracts. Nucleic Acids Res 27: 3712-3719.

381. Intano GW, McMahan CA, McCarrey JR, Walter RB, McKenna AE, et al. (2002) Base excision repair is limited by different proteins in male germ cell nuclear extracts prepared from young and old mice. Mol Cell Biol 22: 2410-2418.

382. Mauro DJ, De Riel JK, Tallarida RJ, Sirover MA (1993) Mechanisms of excision of 5-fluorouracil by uracil DNA glycosylase in normal human cells. Mol Pharmacol 43: 854-857.

383. Turner DP, Cortellino S, Schupp JE, Caretti E, Loh T, et al. (2006) The DNA N-glycosylase MED1 exhibits preference for halogenated pyrimidines and is involved in the cytotoxicity of 5-iododeoxyuridine. Cancer Res 66: 7686-7693.

384. Fischer F, Baerenfaller K, Jiricny J (2007) 5-Fluorouracil is efficiently removed from DNA by the base excision and mismatch repair systems. Gastroenterology 133: 1858-1868.

385. Szczesny B, Tann AW, Longley MJ, Copeland WC, Mitra S (2008) Long patch base excision repair in mammalian mitochondrial genomes. J Biol Chem 283: 26349-26356.

386. Liu P, Qian L, Sung JS, de Souza-Pinto NC, Zheng L, et al. (2008) Removal of oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria. Mol Cell Biol 28: 4975-4987.

80

387. Duxin JP, Dao B, Martinsson P, Rajala N, Guittat L, et al. (2009) Human dna2 is a nuclear and mitochondrial DNA maintenance protein. Mol Cell Biol 29: 4274-4282.

388. Bowmaker M, Yang MY, Yasukawa T, Reyes A, Jacobs HT, et al. (2003) Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. J Biol Chem 278: 50961-50969.

389. Brown TA, Cecconi C, Tkachuk AN, Bustamante C, Clayton DA (2005) Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. Genes Dev 19: 2466-2476.

390. Wilson DM, 3rd, Takeshita M, Grollman AP, Demple B (1995) Incision activity of human apurinic endonuclease (Ape) at abasic site analogs in DNA. J Biol Chem 270: 16002-16007.

391. Hang B, Singer B (2003) Protein-protein interactions involving DNA glycosylases. Chem Res Toxicol 16: 1181-1195.

392. Chaudhuri NK, Montag BJ, Heidelberger C (1958) Studies on fluorinated pyrimidines. III. The metabolism of 5-fluorouracil-2-C14 and 5-fluoroorotic-2-C14 acid in vivo. Cancer Res 18: 318-328.

393. Kanamaru R, Kakuta H, Sato T, Ishioka C, Wakui A (1986) The inhibitory effects of 5-fluorouracil on the metabolism of preribosomal and ribosomal RNA in L-1210 cells in vitro. Cancer Chemother Pharmacol 17: 43-46.

394. Yu H, Eritja R, Bloom LB, Goodman MF (1993) Ionization of bromouracil and fluorouracil stimulates base mispairing frequencies with guanine. J Biol Chem 268: 15935-15943.

395. Li L, Berger SH, Wyatt MD (2004) Involvement of base excision repair in response to therapy targeted at thymidylate synthase. Mol Cancer Ther 3: 747-753.

396. Luo Y, Walla M, Wyatt MD (2008) Uracil incorporation into genomic DNA does not predict toxicity caused by chemotherapeutic inhibition of thymidylate synthase. DNA Repair (Amst) 7: 162-169.

397. Fischer JA, Muller-Weeks S, Caradonna SJ (2006) Fluorodeoxyuridine modulates cellular expression of the DNA base excision repair enzyme uracil-DNA glycosylase. Cancer Res 66: 8829-8837.

398. Kahilainen LI, Bergstrom DE, Vilpo JA (1985) 5-Hydroxymethyl-2'-deoxyuridine. Cytotoxicity and DNA incorporation studied by using a novel [2-14C]-derivative with normal and leukemic human hematopoietic cells. Acta Chem Scand B 39: 477-484.

399. Rogstad DK, Darwanto A, Herring JL, Rogstad KN, Burdzy A, et al. (2007) Measurement of the incorporation and repair of exogenous 5-hydroxymethyl-2'-deoxyuridine in human cells in culture using gas chromatography-negative chemical ionization-mass spectrometry. Chem Res Toxicol 20: 1787-1796.

400. Vilpo JA, Suvanto E, Kangas L (1987) Antileukemic activity against L1210 leukemia, pharmacokinetics and hematological side effects of 5-hydroxymethyl-2'-deoxyuridine. Leuk Res 11: 877-880.

401. Boorstein RJ, Cummings A, Jr., Marenstein DR, Chan MK, Ma Y, et al. (2001) Definitive identification of mammalian 5-hydroxymethyluracil DNA N-glycosylase activity as SMUG1. J Biol Chem 276: 41991-41997.

402. Horton JK, Stefanick DF, Naron JM, Kedar PS, Wilson SH (2005) Poly(ADP-ribose) polymerase activity prevents signaling pathways for cell cycle arrest after DNA methylating agent exposure. J Biol Chem 280: 15773-15785.

403. Wataya Y, Santi DV, Hansch C (1977) Inhibition of Lactobacillus casei thymidylate synthetase by 5-substituted 2'-deoxyuridylates. Preliminary quantitative structure-activity relationship. J Med Chem 20: 1469-1473.

404. McNeill DR, Lam W, DeWeese TL, Cheng YC, Wilson DM, 3rd (2009) Impairment of APE1 function enhances cellular sensitivity to clinically relevant alkylators and antimetabolites. Mol Cancer Res 7: 897-906.

405. Haveman J, Castro Kreder N, Rodermond HM, van Bree C, Franken NA, et al. (2004) Cellular response of X-ray sensitive hamster mutant cell lines to gemcitabine, cisplatin and 5-fluorouracil. Oncol Rep 12: 187-192.

406. Schmidt WM, Kalipciyan M, Dornstauder E, Rizovski B, Steger GG, et al. (2004) Dissecting progressive stages of 5-fluorouracil resistance in vitro using RNA expression profiling. Int J Cancer 112: 200-212.

407. Park JS, Young Yoon S, Kim JM, Yeom YI, Kim YS, et al. (2004) Identification of novel genes associated with the response to 5-FU treatment in gastric cancer cell lines using a cDNA microarray. Cancer Lett 214: 19-33.

408. Kang HC, Kim IJ, Park JH, Shin Y, Ku JL, et al. (2004) Identification of genes with differential expression in acquired drug-resistant gastric cancer cells using high-density oligonucleotide microarrays. Clin Cancer Res 10: 272-284.

409. de Angelis PM, Fjell B, Kravik KL, Haug T, Tunheim SH, et al. (2004) Molecular characterizations of derivatives of HCT116 colorectal cancer cells that are resistant to the chemotherapeutic agent 5-fluorouracil. Int J Oncol 24: 1279-1288.

410. Mariadason JM, Arango D, Shi Q, Wilson AJ, Corner GA, et al. (2003) Gene expression profiling-based prediction of response of colon carcinoma cells to 5-fluorouracil and camptothecin. Cancer Res 63: 8791-8812.

411. Zembutsu H, Ohnishi Y, Tsunoda T, Furukawa Y, Katagiri T, et al. (2002) Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. Cancer Res 62: 518-527.

412. Peters GJ, Backus HH, Freemantle S, van Triest B, Codacci-Pisanelli G, et al. (2002) Induction of thymidylate synthase as a 5-fluorouracil resistance mechanism. Biochim Biophys Acta 1587: 194-205.

413. Pullarkat ST, Stoehlmacher J, Ghaderi V, Xiong YP, Ingles SA, et al. (2001) Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy. Pharmacogenomics J 1: 65-70.

414. Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, et al. (2000) A gene expression database for the molecular pharmacology of cancer. Nat Genet 24: 236-244.

415. Brody JR, Hucl T, Costantino CL, Eshleman JR, Gallmeier E, et al. (2009) Limits to thymidylate synthase and TP53 genes as predictive determinants for fluoropyrimidine sensitivity and further evidence for RNA-based toxicity as a major influence. Cancer Res 69: 984-991.

6 PAPERS I-III

Paper I:

Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis.

(Akbari M, Visnes T, Krokan HE and Otterlei M).

Paper II:

The rate of base excision repair of uracil is controlled by the initiating glycosylase.

(Visnes T, Akbari M, Hagen L, Slupphaug G and Krokan HE)

Paper III:

Cytotoxicity of 5-fluoropyrimidines is mainly through RNA incorporation and thymidylate synthase inhibition rather than DNA fragmentation from DNA excision repair

(Pettersen HS, Visnes T, Vågbø CB, Doseth B, Kavli B, and Krokan HE)

Paper I

Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis

Mansour Akbari, Torkild Visnes, Hans E. Krokan, Marit Otterlei *

*Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University
of Science and Technology, N-7006 Trondheim, Norway*

ABSTRACT

Base excision repair (BER) corrects a variety of small base lesions in DNA. The *UNG* gene encodes both the nuclear (UNG2) and the mitochondrial (UNG1) forms of the human uracil-DNA glycosylase (UDG). We prepared mitochondrial extracts free of nuclear BER proteins from human cell lines. Using these extracts we show that UNG is the only detectable UDG in mitochondria, and mitochondrial BER (mtBER) of uracil and AP sites occur by both single-nucleotide insertion and long-patch repair DNA synthesis. Importantly, extracts of mitochondria carry out repair of modified AP sites which in nuclei occurs through long-patch BER. Such lesions may be rather prevalent in mitochondrial DNA because of its proximity to the electron transport chain, the primary site of production of reactive oxygen species. Furthermore, mitochondrial extracts remove 5′ protruding flaps from DNA which can be formed during long-patch BER, by a "flap endonuclease like" activity, although flap endonuclease (FEN1) is not present in mitochondria. In conclusion, combined short- and long-patch BER activities enable mitochondria to repair a broader range of lesions in mtDNA than previously known.

1. Introduction

Human mitochondrial DNA (mtDNA) is a closed-circular molecule of approximately 16,600 basepairs containing 37 genes which code for 13 polypeptides, 22 tRNAs and 2 rRNAs. All polypeptides are subunits of mitochondrial respiratory complexes of the inner membrane. Although mtDNA only encodes 13 of the ∼90 different proteins present in the respiratory chain, it is important for normal cellular function because cells depleted of mtDNA (ρ^0 cells) do not respire normally [1].

Genetically engineered mutator mice that accumulated a substantial number of mutations in mtDNA showed early aging phenotypes and reduced lifespan underlining the significance of mtDNA maintenance [2].

The electron flow during mitochondrial respiration can give rise to reactive oxygen species (ROS) [3]. ROS can cause DNA base lesions and strand breaks, which if left unrepaired may result in mutations and genomic instability [4]. The mutation rate in some regions of human mtDNA, including rRNA and tRNA sequences, is 20–100-fold higher than that of nuclear

DNA [5]. Somatic and hereditary mutations of mtDNA are associated with a variety of diseases including diabetes and deafness [6,7] cancer [8] and neurodegenerative disorders [9].

DNA polymerase γ (POLγ) is the only DNA polymerase identified in human mitochondria [10]. POLγ is a processive DNA polymerase which consists of two subunits, a large 140 kDa catalytic subunit, POLγA [11] and an accessory factor, POLγB [12]. The large subunit contains a $3'$–$5'$ exonuclease (proof-reading) as well as a dRP lyase activity that removes $5'$-deoxyribosephosphate (dRP) moiety during BER [13]. The accessory subunit stimulates the DNA synthesis activity and processivity of POLγ [12,14].

BER is apparently the main mechanism for repair of ROS-generated base lesions in DNA [4]. BER of several oxidative base lesions and uracil have been detected in mitochondria [reviewed in 15]. Nuclear BER in human cells occurs by replacement of a single nucleotide or short-patch repair (SP) or several nucleotides; the so-called long-patch (LP) repair [16]. It is known that the dRP lyase activity of POLβ is unable to cleave modified (oxidized/reduced) moieties [17], the repair of which requires flap endonuclease and LP BER. Given the high rate of ROS production in mitochondria, it is likely that oxidized moieties are continuously formed in mtDNA. How mitochondria deal with DNA damage that requires LP BER in nuclei is not known. BER by enzymes purified from *Xenopus laevis* mitochondria, or by extract from rat liver mitochondria apparently occurs as single-nucleotide insertion [18,19].

Our main aim in conducting this study was to examine the capacity of mitochondria for repair of lesions that in the nucleus require LP BER. First we established an improved method for purification of mitochondria that enabled us to prepare mitochondrial extracts free of detectable nuclear BER proteins. Using these extracts we examined the role of UNG in removal of uracil from mtDNA and carried out mitochondrial BER analysis including, patch-size analysis, and repair of modified AP sites. We found that UNG is the predominant uracil-DNA glycosylase in mitochondria and BER of uracil and AP sites by mitochondrial extracts is in form of SP as well as LP BER.

2. Materials and methods

2.1. Chemicals and antibodies

Synthetic oligonucleotides were purchased from MedProbe (Oslo, Norway). [α-^{33}P]dTTP, [α-^{33}P]dCTP, and [γ-^{33}P]ATP (3000 Ci/mmol) were from Amersham Biosciences. Proteinase K, aphidicolin, N-ethylmaleimide (NEM), and Percoll® were from Sigma–Aldrich. Complete® protease inhibitor and T4 DNA ligase were from Roche Inc. Restriction enzymes and T4 polynucleotide kinase were from New England Biolabs. Primary antibodies; APE1 (ab194), APE2 (ab13691), VDAC1 (ab15895), COX IV (ab16056), lamin A + C (ab8984), FEN-1 (ab 462) were all from Abcam Ltd., UK. Antibody to PCNA (PC10, sc-56) was from Santa Cruz Biotechnology, Inc., USA, polyclonal FEN-1 antibody was from Bethyl (BL587), and POLδ (D73020) was from Transduction Laboratories. Neutralizing antibody against the catalytic domain of UNG has been described previously [20]. Paramagnetic protein-A beads were from Dynal, Norway.

2.2. Cell culture

HeLa and HaCaT cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 0.03% glutamine, and 0.1 mg/ml gentamicin in 5% CO_2.

2.3. Isolation of crude mitochondria

We harvested the cells by trypsination and washed the cells once with cold PBS and once with an isotonic buffer (20 mM HEPES-KOH pH 7.4, 5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, and 0.25 M sucrose), resuspended the cells in a hypotonic buffer (20 mM HEPES-KOH pH 7.4, 5 mM $MgCl_2$, 5 mM KCl, and 1 mM DTT) and incubated them on ice for 5–10 min before disruption of the cells by a Dounce homogenizer (5–10 strokes). We immediately added (1:1, v/v) 2$\times$ MSH buffer (20 mM HEPES-KOH pH 7.4, 4 mM EDTA, 2 mM EGTA, 5 mM DTT, 0.42 M mannitol, 0.14 M sucrose) to the homogenate to stabilize the mitochondrial membrane as described previously [21]. We centrifuged the homogenate three times at 2000 $\times$ g, each time for 5 min to separate cell debris and nuclei (the pellet) from mitochondria (the supernatant), and then pelleted the mitochondria at 3000 $\times$ g for 30 min. The mitochondrial pellet was then resuspended in 1 ml 1$\times$ MSH/50% Percoll, the suspension loaded on top of a 1$\times$ MSH/50% Percoll gradient (12 ml) and centrifuged at 50,000 $\times$ g for 1 h at 4 °C. The mitochondria were removed from the gradient and washed once with 1$\times$ MSH buffer to remove Percoll, once with 1 ml buffer B (10 mM HEPES-KOH pH 7.4, 0.21 M mannitol, 0.7 M sucrose, and 2.5 mM DTT), resuspended in buffer B containing 1 mg/ml proteinase K in a final volume of 1 ml (unless otherwise is indicated) and incubated at 37 °C for 30 min. The mitochondria were pelleted at 10,000 $\times$ g for 5 min and washed twice with 0.5 ml of a protease inhibitor mix (0.5 ml protease inhibitor cocktail (1 Complete® tablet dissolved in 1 ml water), 0.5 ml 2$\times$ MSH, and 5 mM phenylmethylsulphonyl fluoride (PMSF)). We routinely isolated mitochondria from 30 dishes (150 mm) at 85–90% confluence which after proteinase K treatment yielded on average 0.6–0.8 mg mitochondrial protein.

2.4. Western blot analysis of intact mitochondria

We isolated mitochondria from 30 dishes (150 mm) by Percoll gradient as described above. The crude mitochondrial pellet was resuspended in 0.35 ml buffer B and the suspension divided in seven tubes (0.05 ml each). Proteinase K was added to the samples at the indicated concentrations followed by incubation at 37 °C for 30 min. Proteinase K was inactivated by adding 5 mM PMSF and Complete® protease inhibitor to the samples followed by addition of loading buffer (NuPage) and heating of the samples at 85 °C for 10 min. We separated proteins in 10% denaturing SDS-polyacrylamide gel (NuPage), and transferred them to a PVDF membrane (Immobilon^{TM}, Millipore). The membrane was incubated with the primary antibodies at 4 °C overnight, followed by incubation for 1 h at room temperature with either peroxidase-labeled polyclonal rabbit anti-mouse IgG/HRP or peroxidase-labeled polyclonal swine anti-rabbit IgG/HRP (DakoCytomation, Denmark). We incubated the membrane with chemiluminescence reagent (SuperSignal® West Femto Maximum, PIERCE, USA), and visu-

alized the bands in Image Station 2000R (Eastmann Kodak Company, USA).

2.5. Preparation of nuclear and mitochondrial extracts

We used a modification of the procedure described previously [22]. Mitochondria (pretreated with 1 mg/ml proteinase K) or nuclei were resuspended at 1× packed pellet volume (PPV) in buffer I (10 mM HEPES-KOH pH 7.9, and 150 mM KCl) and 1× PPV of buffer II (10 mM HEPES-KOH pH 7.9, 150 mM KCl, 2 mM EDTA, 2 mM DTT, 40% glycerol, 1% Nonidet P-40, 1% Triton X-100, 5 mM PMSF, Complete® protease inhibitor, and phosphatase inhibitor cocktails). The samples were then gently rotated at 4 °C for 1 h, followed by centrifugation at 22,000 × g at 4 °C for 15 min, and the supernatants recovered. The protein concentration was measured using the Bio-Rad protein assay. We always examined the purity of mitochondrial extracts by Western blot analysis for PCNA, POLδ, VDAC-1, and COX IV before using the extracts in biochemical tests. Failure to inactivate proteinase K would be detected as degraded COX IV.

2.6. DNA substrates, BER assay, flap-removal assay, immunoprecipitation, and uracil-DNA glycosylase (UDG) activity assay

We prepared DNA substrates for BER assay containing uracil or a synthetic analog of an AP site, 3-hydroxy-2-hydroxymethyltetrahydrofuran (THF) at a single position as described before [23,24]. Normal AP site was generated by incubating the uracil-containing DNA substrate with purified catalytic domain of UNG [20]. For nick-DNA, the AP site containing DNA was incubated with recombinant APE1. BER assays using nuclear or mitochondrial extracts were carried out as described before [23–25].

For flap-DNA substrates, we annealed oligonucleotides containing none, one, two, or five non-complementary adenines at 5′ end (Fig. 5A, underlined nucleotides) as well as a 5′ end-labeled oligonucleotide upstream to the flap-containing oligo (shown in bold) to single-stranded circular plasmid (pGEM®-3Zf(+)) and carried out DNA synthesis with T4 gene 32 ssDNA-binding protein, T4 DNA polymerase, T4 DNA ligase, and dNTPs. The DNA was purified using a PCR Purification Kit (Qiagen). Unless otherwise is indicated, the flap-removal assay was carried out in 0.020 mg mitochondrial extract, 50 mM HEPES-KOH pH 7.4, 2 mM DTT, 5 mM MgCl₂, 75 mM KCl, 1 mM ATP, 0.36 mg/ml BSA, T4 DNA ligase and 2 pmol of the indicated DNA-substrates at 37 °C for 15 min in 20 μl reaction. The reaction was stopped by adding EDTA, SDS and proteinase K and further incubation for 30 min. The flap-removal activity of the immunoprecipitates was carried out in the presence of T4 DNA ligase.

For immunoprecipitation we covalently attached 0.02 mg polyclonal FEN-1 antibody to 0.2 ml premagnetic beads as described by the manufacturer. We incubated 0.04 ml of the beads with 0.2 mg HeLa nuclear or mitochondrial extract at 4 °C for 4 h under constant rotation. The beads were washed four times with wash buffer (10 mM Tris–HCl pH 7.5, 100 mM KCl). The beads were then either used directly in flap-removal assay or boiled in loading buffer and used for Western blot

analysis. UDG activity assay was performed as described earlier [25].

3. Results

3.1. Proteinase K treatment clears isolated mitochondria of nuclear protein contaminants

A prerequisite for analysis of mtBER *in vitro* is the preparation of mitochondrial extracts free of nuclear BER proteins. During the early stage of this study we found it difficult to isolate mitochondria completely free of nuclear proteins. In an attempt to clear mitochondria of nuclear proteins we treated intact mitochondria with proteinase K, a serine protease that exhibits broad cleavage specificity. Fig. 1A shows results of Western blot analysis of mitochondria that had been incubated with different concentrations of proteinase K at 37 °C for 30 min. The absence of detectable nuclear lamin in mitochondrial extract has frequently been used to test the purity of mitochondria. Notably, the sample not treated with proteinase K was free of lamin A + C, while we detected nuclear proteins including UNG2, POLδ, and PCNA (Fig. 1A, lane 1). Traces of nuclear proteins were still detectable in the samples treated with 0.5 mg/ml proteinase K (Fig. 1A, lane 2), but at 1 and 1.5 mg/ml proteinase K, the samples were cleared of detectable nuclear proteins (lanes 3 and 4). Proteinase K treatment degraded a fraction of the outer mitochondrial membrane (OMM) protein, voltage-dependent anion channel 1 (VDAC-1) at concentrations of 0.5 mg/ml and higher, while the inner membrane (IMM) proteins remained seemingly intact at proteinase K concentrations up to 2.5 mg/ml as demonstrated by the presence of full length COX IV (Fig. 1A). The amount of APE1 was reduced considerably at 0.5 mg/ml proteinase K compared with the untreated sample (Fig. 1A, lanes 2 and 1, respectively), but a fraction remained unchanged at concentrations of proteinase K of 0.5–2.5 mg/ml (lanes 2–6). This supports that APE1 is both a nuclear and a mitochondrial protein [26,27]. The amount of the second human endonuclease, APE2, did not change in the samples treated with proteinase K, indicating that APE2 is a true mitochondrial protein [27,28]. The additional band seen above the major APE2 band after proteinase K treatment is likely caused by cross-reaction of the antibody with a degraded protein. As expected UNG2 but not UNG1 was degraded by proteinase K treatment.

The ability to detect a protein by Western blot analysis depends on the sensitivity of the antibodies. We compared the sensitivity of antibodies against the nuclear proteins lamin A + C and PCNA by Western blot analysis of a serial dilution of total HeLa extract. PCNA was detected in fourfold more diluted extracts compared with lamin A + C (Fig. 1B). Next, we carried out Western blot analysis of a serially diluted purified recombinant PCNA and found that the PCNA antibody was able to detect as low as 1.8 ng protein (Fig. 1C). These results together with those shown in Fig. 1A support the use of this particular PCNA antibody as a suitable marker for detection of nuclear protein contaminants in mitochondrial extract. In conclusion, the results of Western blot analysis suggest that treatment of intact mitochondria with proteinase K enables us to prepare mitochondria that are free of nuclear BER proteins. We
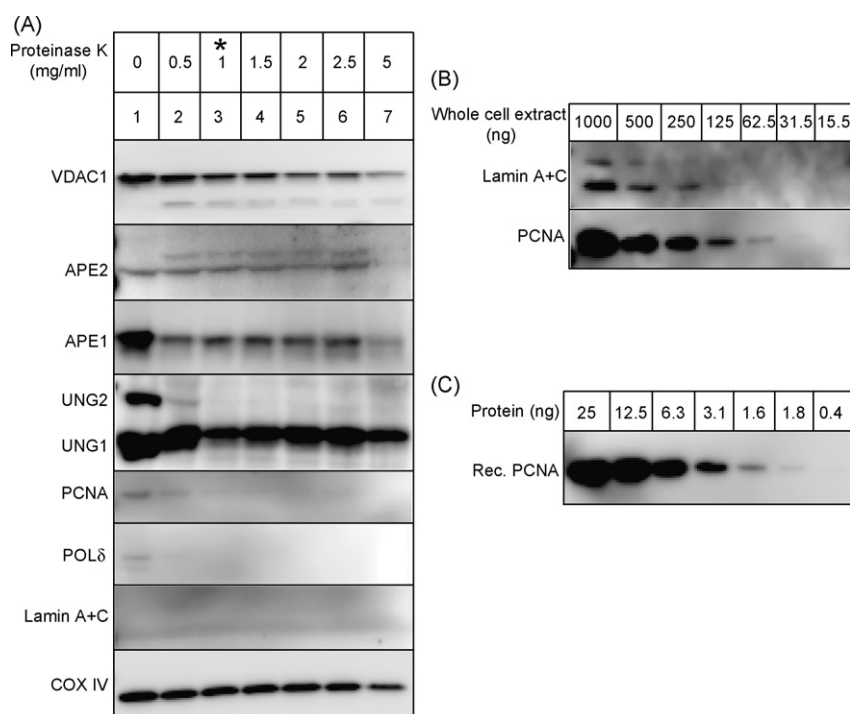
Fig. 1 – (A) Western blot analysis of mitochondria isolated from HeLa cells and treated with the indicated concentrations of proteinase K at 37 °C for 30 min. (B) Western blot analysis of serially diluted total HeLa extract for lamin A + C and PCNA. (C) Western blot analysis of a serial dilution of purified recombinant PCNA.

therefore routinely treated intact mitochondria with 1 mg/ml proteinase K prior to preparation of extract that we used in the following experiments.

3.2.   Extracts prepared from proteinase K treated mitochondria display POLγ specific DNA synthesis activity

Mammalian DNA polymerases show different sensitivity for aphidicolin and N-ethylmaleimide (NEM). Thus, aphidicolin inhibits DNA polymerases α, δ, and ε at 0.06 mM but not polymerases γ and β, while NEM inhibits DNA polymerases γ, α, δ, and ε at 2 mM but not POLβ even at 10 mM [29–31]. POLγ is the only DNA polymerase identified in human mitochondria [10]. We incubated mitochondrial extract with DNA substrate containing a nick at a defined position (Fig. 2A) with or without aphidicolin or NEM in the repair reaction. We used nicked-DNA to exclude possible inhibition of repair reactions before the DNA synthesis step by aphidicolin or NEM. The control sample shows that the extract is capable of carrying out DNA synthesis and subsequent ligation of newly synthesized DNA (Fig. 2B, lane 1). Addition of 0.1 or 0.3 mM aphidicolin to the reaction had no detectable inhibitory effect on DNA synthesis activity of the extract, while NEM at 5 mM dramatically inhibited this activity (Fig. 2B, lanes 2–4). This experiment indicates that our mitochondrial extract was not contaminated with the nuclear DNA polymerases α, δ, ε (which are inhibited by aphidicolin) or β (which is not inhibited by NEM), and that the extract displayed DNA polymerase activity comparable with POLγ. Altogether, the results shown in Figs. 1 and 2 demonstrate that the method used allowed us to prepare a pure mitochondrial extract.

3.3.   Pure mitochondrial extracts retain BER activity and UNG is the only detectable uracil-DNA glycosylase in HeLa mitochondria

In human cells, four uracil-DNA glycosylases (UDG) have been identified [32]. Among these, UNG is the only known UDG targeted to both nucleus (UNG2) and mitochondria (UNG1) [33,34].

Nuclear BER in human cells has been extensively studied and shown to occur via SP and LP BER [16]. BER carried out by enzymes purified from *Xenopus laevis* mitochondria and extract from rat liver mitochondria was reported to occur via single-nucleotide insertion [18,19]. We tested mitochondrial uracil-BER using [α-³³P]dTTP or [α-³³P]dCTP and DNA substrate containing uracil at a specific position (Fig. 3A, U:A). We carried out the repair reaction in the absence or presence of neutralizing antibody against the catalytic domain of UNG which is identical in UNG1 and UNG2. Notably, repair of uracil was in form of several-nucleotide insertion (Fig. 3B, lanes 1 and 3). Neutralizing UNG by antibody prevented uracil-dependent repair DNA synthesis activity by mitochondrial extract (lanes 2 and 4, respectively).

Next, we used a more sensitive assay and verified the ability of mitochondrial extract to remove uracil from single-stranded as well as double-stranded DNA oligos containing U:A or U:G pairs. We found that inhibition of UNG in the reaction abolished all the uracil releasing activity of the extract (Fig. 3C, lanes 10–12). These results strongly suggest that UNG is the only DNA glycosylase responsible for removal of uracil in human mtDNA. Notably, because U:G is also a substrate for TDG and SMUG1 DNA glycosylases [32] the complete inhibition of removal of uracil from U:G substrate by neutralizing

(A)

pGEM®-3Zf(+)
3199 bp

XbaI   HindIII

5′ TCTAGAGXCGAC..............AAGCTT 3′
3′ AGATCTCAGCTG...............TTCGAA 5′

[α-³³P]dTTP, XbaI/HindIII (24-mer)

(B)
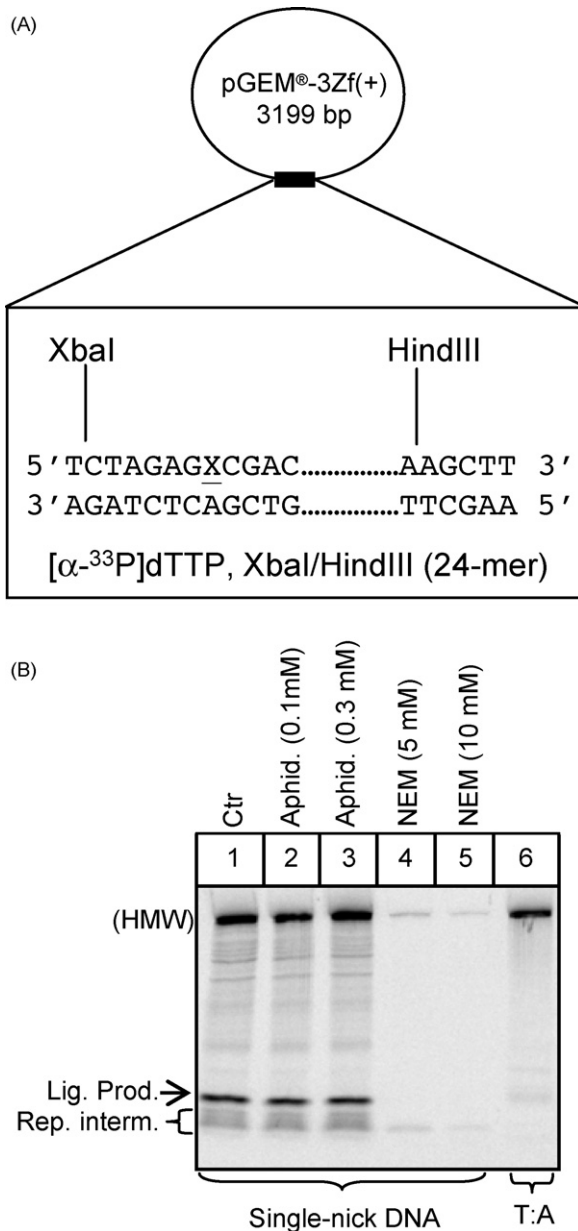
Fig. 2 – Effect of inhibitors of DNA polymerases on mitochondrial DNA synthesis activity. (A) Schematic illustration of substrate for analysis of DNA synthesis activity. X represents the site of nick in DNA. (B) We incubated mitochondrial extract with nick-containing DNA substrate and [α-³³P]dTTP in the absence or presence of aphidicolin or NEM at the indicated concentrations at 32 °C for 60 min. Purified DNA was digested with XbaI and HindIII and resolved in 12% denaturing polyacrylamide gel. As undamaged substrate we used DNA containing T in place of nick (T:A). High molecular weight (HMW) bands represent nucleotide incorporation outside the short fragments released after digestion of DNA with the indicated restriction enzymes.

UNG antibody indicates that our mitochondrial extract is free of the nuclear DNA glycosylases TDG and SMUG1, further supporting that the mitochondrial extract was essentially free of nuclear proteins.

3.4. BER DNA synthesis during repair of both uracil and AP sites by mitochondrial extract occurs through incorporation of several nucleotides

Next we carried out patch-size analysis of AP site BER. AP sites were produced by incubation of uracil-containing DNA substrates with recombinant UDG. A fraction of AP site repair was apparently via LP BER (Fig. 4A, lanes 2–4). Human DNA ligases need ATP for activity. We carried out BER in the presence or absence of additional ATP and ATP-generating agents in the reaction. In the absence of ATP, repair intermediates of different sizes were readily detected (Fig. 4B, lane 2) demonstrating the ability of the mitochondrial DNA polymerase, likely POLγ, to incorporate more than one nucleotide during BER DNA synthesis. Incubation of the purified DNA with T4 DNA ligase at 16 °C overnight resulted in close to complete ligation of repair intermediates (Fig. 4B, lanes 3 and 4). This indicates that most repair intermediates observed in lane 2 did not contain unprocessed 5′ deoxyribosephosphate (dRP) or 5′ flaps. Moreover, the results show that the indicated repaired fragments released by the digestion of DNA with XbaI and HindIII (see Fig. 3A) represent short and long-patch products and are not merely products of resynthesis of DNA past the HindIII recognition site.

The 3′–5′ exonuclease activity of POLγ may result in DNA synthesis 5′ upstream to the damage. We tested this using U:G DNA substrate (Fig. 3A) in combination with [α-³³P]dTTP (to detect possible incorporation of radioactivity 5′ upstream to the damage) or [α-³³P]dCTP (to detect incorporation of radioactivity at the site of the damage). We did not detect DNA synthesis activity 5′ upstream to the damage above the background (not shown). In conclusion, under our experimental conditions, DNA synthesis activity 5′ upstream to the damage either does not occur or takes place at very low frequency.

To test if the observed LP BER also takes place in cells other than HeLa cells, we carried out patch-size analysis of mitochondrial extract prepared from HaCaT cells. As shown in Fig. 4C, the isolation and purification of HaCaT mitochondria with our procedure cleared residues of PCNA and POLδ from mitochondria. BER analysis of the extract showed that, like HeLa mitochondrial extract, a fraction of the repair DNA synthesis product was between four to eight nucleotides long (Fig. 4D, lane 2). In conclusion, under our BER assay conditions, we found that BER by HeLa and HaCaT mitochondrial extracts takes place through both SP and LP DNA synthesis.

3.5. Mitochondrial extract removes 5′ protruding flaps from DNA

The observed LP BER by mitochondrial extract suggests strand-displacement during repair DNA synthesis resulting in the formation of 5′ single-strand flaps. Such flaps must be removed from DNA in order for ligation of DNA ends to take place. To search for a possible 5′ flap removal activity in mitochondrial extract we used the DNA substrate strategy
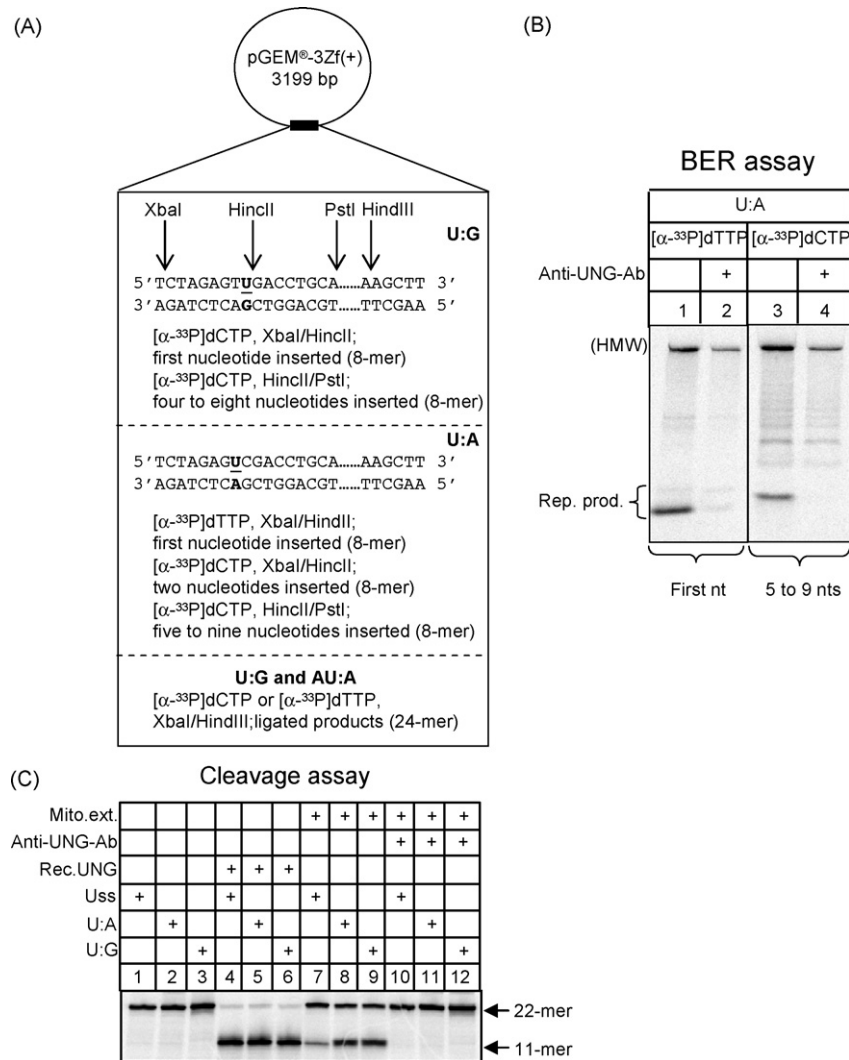
Fig. 3 – Role of UNG in mitochondrial uracil-BER. (A) Schematic presentation of the strategy for BER analysis of mitochondrial extract. (B) We carried out uracil-BER analysis of mitochondrial extract in the absence or presence of a specific neutralizing UNG antibody (anti-UNG-Ab) using U:A DNA substrate and [α-³³P]dCTP or [α-³³P]dTTP as indicated. The repaired products (rep. prod.) were digested with XbaI/HincII (lanes 1 and 2) or HincII/PstI (lanes 3 and 4) to examine the uracil-BER patch-size as described in (A). (C) We incubated mitochondrial extract with 5′ end-labeled single- or double-strand olignucleotides (22-mer) containing a centrally located uracil in the absence or presence of neutralizing UNG antibody as indicated (lanes 7–12). The reaction was carried out at 37 °C for 60 min. As control we incubated the substrates with purified catalytic domain of UNG (rec.UNG, lanes 4–6). The full length and the cleaved oligos are shown as 22-mer and 11-mer, respectively.

outlined in Fig. 5A. A schematic illustration of DNA fragments released after digestion of DNA with EcoRI and HindIII is provided in Fig. 5B, and the control digestion of the substrates is shown in Fig. 5C. To facilitate the identification of repaired DNA fragments (fragments II and IV), we prepared DNA substrate using an oligo that does not form flap upon annealing to template DNA. This "flap" oligo was either phosphorylated at 5′ position (F0-P) or not (F0). During the preparation of F0-P substrate, the ligation of fragments I and F0-P oligo will give rise to fragment IV. Fragment II represents restriction digested plasmid where the *in vitro* DNA synthesis has been incomplete (Fig. 5C, lane 1). The weak bands in fragments II and IV (lanes 2–5) represent synthesis extension of ³³P end-labeled oligo on single-strand circular DNA templates lacking the downstream flap-oligo, because of incomplete annealing of these oligos to the template DNA. Upon removal of the flap and subsequent ligation of DNA, the intensity of the bands corresponding to fragments II and IV will increase relative to the intensity of the initial DNA substrate concomitantly with a reduction in the intensity of the bands corresponding to fragments I and III. For repair analysis, DNA substrates were incubated with mitochondrial extract in the absence (Fig. 5D, lanes 1–3 and 7–9) or presence of EDTA (Fig. 5D, lanes 4–6 and 10–12). As can be seen in Fig. 5D, the intensity of the bands corresponding to fragments II and IV is higher in the absence than in the presence of EDTA (compare fragments II and IV, lanes 1–3 to lanes 4–6, respectively). Single digestion of DNA with EcoRI (Fig. 5D, lanes 7–12), further confirmed the indicated migration pattern
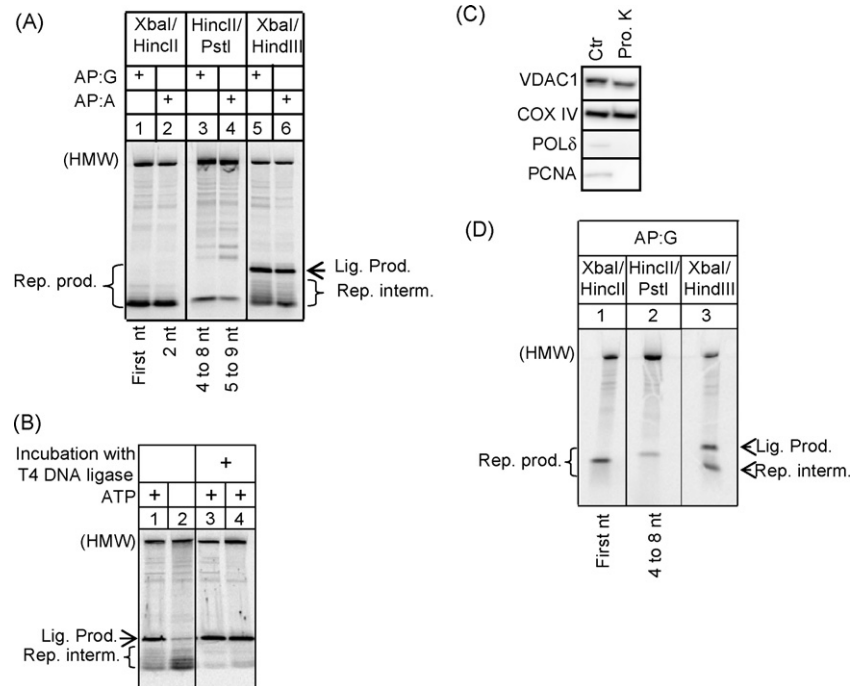
Fig. 4 – Patch-size analysis of mitochondrial AP-BER. (A) We incubated mitochondrial extract with [α-^{33}P]dCTP and the indicated AP site containing DNA substrates at 32 °C for 60 min. Then the purified DNA was digested with the indicated restriction enzymes to examine the size of repair DNA synthesis as well as the amount of ligated (total repair products) and unligated repair intermediates. (B) BER assay was carried out in the presence or absence of ATP and ATP generating agents (lanes 1 and 2, respectively). Half part of the purified DNA was further incubated with T4 DNA ligase at 16 °C overnight (lanes 3 and 4). (C) Western blot analysis of HaCaT mitochondrial extract before (lane 1) and after (lane 2) treatment of intact mitochondria with 1 mg/ml proteinase K. (D) BER analysis of HaCaT mitochondrial extract from proteinase K treated mitochondria.

of DNA fragments in Fig. 5B and C. Note that the repaired (ligated) DNA fragments were too long to be resolved in the gel after single digestion with EcoRI (Fig. 5D, the fragments over the dotted line). These results indicate that 5′ protruding flaps that may be formed during LP are cleaved from DNA by mitochondrial extract.

In the nuclei, single-stranded DNA flaps that can be formed during LP BER are cleaved by the nuclear protein FEN-1. Notably, using Western blot analysis we did not detect FEN-1 in our mitochondrial extract (Fig. 5E, lane 3). However, the ability of Western blot analysis to detect target proteins depends on the sensitivity of antibodies used. To further assure that the mitochondrial extract was not contaminated with FEN-1, we carried out immunoprecipitation of possible FEN-1 from mitochondrial extract as described in Section 2, using nuclear extract as control. Western blot analysis showed that immunoprecipitation removed a substantial fraction of FEN-1 from the nuclear extract (Fig. 5E, compare lane 1 with 2). FEN-1 was neither detected in mitochondrial extract, nor in immunoprecipitates from mitochondrial extract (Fig. 5E, lane 6) while it was detected in immunoprecipitates from nuclear extract (Fig. 5E, lane 5).

Next, we used the immunoprecipitated materials (anti-FEN-1) from nuclei and mitochondrial extracts in our flap-removal assay. The pattern of DNA fragments in the sample incubated with immunoprecipitated material from

mitochondrial extract was identical to that of DNA substrate alone (Fig. 5F, lanes 1, 2, 4, and 5). However, a substantial increase in the intensity of the fragments II and IV was observed in the sample incubated with the immunoprecipitated FEN-1 from the nuclear extract (Fig. 5F, lane 3) similar to what was observed with the mitochondrial extract (Fig. 5D).

We next examined whether the 5′ protruding DNA was incised as a flap or digested exonucleolytically. For this purpose we end-labeled the oligo that contains five non-complementary adenines and prepared double-strand DNA substrates as above but in the absence of T4 DNA ligase (Fig. 5A, F5, the underlined DNA sequence). Incubation of the flap containing substrate with mitochondrial and nuclear extracts resulted in the release of 5-mer DNA (Fig. 5G, lanes 4 and 5, respectively). The reaction was carried out at 37 °C for 2 min. A fraction of the released DNA was degraded by the extract. Longer incubation (5 min) resulted in even more degradation of DNA (not shown). To further test if the observed fragment corresponded to 5-mer flap and to avoid degradation of the released flap by the extract we used with immunoprecipitated FEN-1 from nuclear extract in the reaction (Fig. 5G, lanes 6). The migration pattern of the released oligo (lane 6) corresponded to those of the extracts (lanes 4 and 5) and as expected not degraded. The end-labeled "flap-less" oligo (Fig. 5A, F0, the underlined DNA fragment) was used as controls (Fig. 5G, lanes 8–11). Addition of EDTA to the reaction
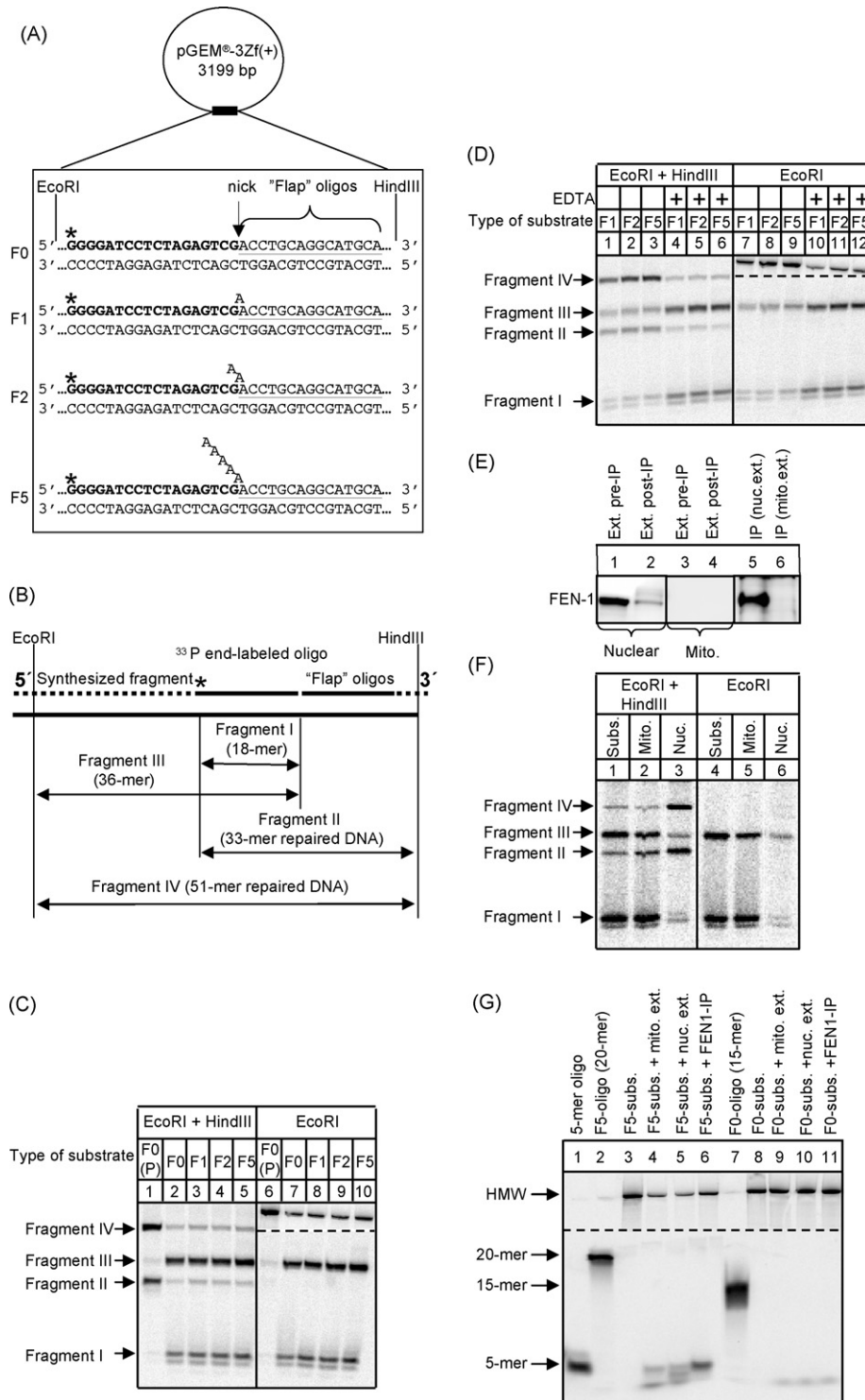
Fig. 5 – Analysis of 5′ flap-removal activity by HeLa mitochondrial extract. (A) Schematic illustration of the strategy for analysis of removal of 5′ protruding flaps by mitochondrial extract. The ^{33}P end-labeled common oligo is shown in bold and marked with * at the 5′ end position. (B) Schematic presentation of DNA fragments released after digestion of DNA substrates with EcoRI and HindIII. Incomplete DNA synthesis (shown as dotted line) during the preparation of substrate together with failure to ligate ^{33}P end-labeled oligo at 3′ end position to the downstream "flap" oligo results in the release of fragment I. Fragment II corresponds to ^{33}P end-labeled oligo ligated at 3′ end position to the downstream oligo, but not ligated at the 5′ end position. Fragment III corresponds to ^{33}P end-labeled oligo that is joined at the 5′ end to the synthesized DNA (shown as dotted line). Fragment IV represents ^{33}P end-labeled oligo that has become ligated at both ends. (C) Lane 1, F0 (P) is the 5′ end phosphorylated form of F0 (flap-less oligo). Lanes 1–10 show the migration pattern of DNA substrates either digested with both EcoRI and HindIII or EcoRI alone as indicated. (D) The flap-containing DNA substrates
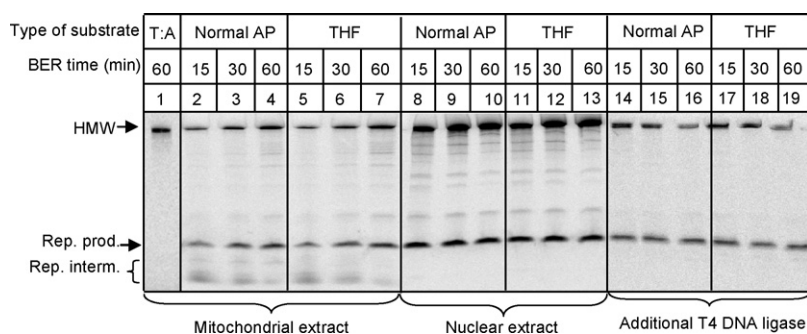
Fig. 6 – BER of normal and synthetic (THF) AP sites by HeLa mitochondrial and nuclear extracts. The strategy for BER analysis is shown in Fig. 2A only in this experiment we used DNA substrates containing normal AP sites (normal AP) or THF in place of nick. As control we used DNA substrate with normal nucleotides (lane 1). The reactions were carried out at 32 °C for the indicated times. At zero time point no signal was detected (not shown). After termination of the reaction, half part of the purified DNA was incubated with XbaI and HindIII and analyzed for ligated products (total BER) and unligated repair intermediates. The remaining DNA from samples corresponding to lanes 2–7 were further incubated with T4 DNA ligase at 16 °C overnight followed by digestion with XbaI and HindIII (lanes 14–19).

completely inhibited the flap cleavage activity in the samples indicating that it is MgCl$_2$-dependent (not shown). Altogether, these results show that (a) mitochondrial extract is able to incise 5′ protruding flaps from DNA and (b) this activity is not a result of contamination of the mitochondrial extract with FEN-1.

3.6. Mitochondrial extract carries out repair of AP site analog tetrahydrofuran (THF)

DNA polymerases γ and β have lyase activity [13,35] enabling them to cleave the blocking 5′ terminal dRP moiety from DNA. The lyase activity of POLβ and POLγ implicates formation of Schiff's base intermediate in an β-elimination reaction mechanism [13]. The lyase activity of POLβ is, similar to reduced/oxidized AP sites, unable to remove the 5′ THF residue [17,35], because these lesions are resistant to β-elimination. Repair of these lesions requires the action of a flap-endonuclease and implicates LP-BER [36]. Therefore, we used THF as a model to test the ability of mitochondrial extract to repair modified AP sites compared with nuclear extract. For comparison we included repair of normal AP sites (normal AP) in the experiment. We found that mitochondrial extract repaired THF and normal AP sites with comparable efficiency (Fig. 6, lanes 2–7). An increasing amount of ligated products was detected with prolonged incubation indicating that (a) the

proteinase K was inactivated and (b) strand-displacement cannot account for the observed long-patch shown in Figs. 3 and 4. An equal amount of nuclear extracts measured as total protein concentration was used in a parallel experiment for comparison. Like THF, NaHB$_4$-reduced AP sites are not susceptible to β-elimination, making them resistant to dRP lyase activity of POLβ, thus implicating LP for repair [37]. We prepared NaHB$_4$-reduced AP sites as describe previously [37] using circular DNA shown in Fig. 3A. Mitochondrial extract was able to repair NaHB$_4$-reduced AP sites, further indicating the ability of mtBER to repair different types of modified AP sites (not shown).

A fraction of repair products of both lesions (normal AP site and THF) by mitochondrial extract, but not by nuclear extract, was in the form of repair intermediates. To test if the presence of the repair intermediates was a result of unprocessed 5′ dRPs or 5′ flaps in DNA we further incubated half of the DNA from samples corresponding to lanes 2–7 with T4 DNA ligase at 16 °C overnight. This treatment resulted in ligation of repair intermediates (compare lanes 2–7 with lanes 14–19, respectively). This indicates that the 5′ dRP ends and possibly also 5′ flaps that could have been formed during LP BER were processed during repair.

In summary our results show that (a) our mitochondrial extract is free of nuclear BER proteins, (b) UNG is the only uracil-DNA glycosylase present in HeLa mitochondria, and (c)

were incubated with mitochondrial extract in the absence or presence of EDTA as shown. After purification of DNA from the extract, half part of DNA was digested with both EcoRI and HindIII (lanes 1–6), and the other half was digested with EcoRI (lanes 7–12). (E) Western blot analysis of the nuclear (lanes 1 and 2) and the mitochondrial (lanes 3 and 4) extracts before (Ext. pre-IP) and after (Ext. post-IP) the FEN1 immunoprecipitation. And Western blot analysis of immunoprecipitated material from the nuclear (lane 5) and the mitochondrial (lane 6) extracts. (F) Flap-removal activity of the immunoprecipitated FEN-1 from nuclear extract (lanes 3 and 6) and possible FEN-1 contaminant from the mitochondrial extract (lanes 2 and 5). We used 5F DNA substrate and carried out the reaction in the presence of additional T4 DNA ligase. Lanes 1 and 4 show the untreated flap-DNA substrate included as control. (G) Flap-endonuclease activity of mitochondrial and the nuclear extracts as well as FEN1 immunoprecipitated from nuclear extract was assayed using circular DNA substrate containing 5′ end-labeled 5-mer flap (F5-subs.). As control we used circular DNA containing 5′-end labeled nick (F0-subs.). The broken line (- - -) indicates that a part of the gel has been deleted to shorten the image.

the mitochondrial extract is able to carry out SP and LP BER and displays flap-endonuclease specific activity.

4. Discussion

A prerequisite for analysis of mtBER *in vitro* is the preparation of mitochondrial extract free of nuclear BER proteins. During the present study we tested several procedures for isolation of mitochondria. Using Western blot analysis we found that a fraction of nuclear proteins including PCNA, POLδ, and UNG2 associated with mitochondria, possibly by attaching to mitochondria upon disruption of the cells. In addition to the Percoll gradient step that we routinely used in the present study, we also applied a discontinuous density gradient method which was reported to efficiently separate mitochondria from other organelles [38]. Thus, the gradient step enables us to separate organelles, but was insufficient for removing nuclear proteins attached to mitochondria. Incubation of intact mitochondria with trypsin has been used to clear mitochondria from nuclear proteins [27,39]. However, we found that some nuclear proteins including PCNA and UNG2 were particularly difficult to completely digest by this treatment. Thus, an additional or alternative step was necessary to clear mitochondria of nuclear proteins involved in long-patch BER in order to examine potential long-patch BER in mitochondria. Using proteinase K enabled preparation of a mitochondrial fraction devoid of detectable nuclear proteins. Biochemical analysis clearly demonstrated that the mitochondrial extracts prepared in this way are proficient in BER and therefore suitable for this line of analysis.

Our results indicate that UNG is the only DNA glycosylase in mitochondria for removal of uracil from mtDNA. Several reports support a role for UNG also in repair of oxidative DNA damage [27,40–42]. $Ung^{-/-}$ mice showed increase infarct size after focal-brain ischemia compared to control animals and experiments indicated a role for mitochondrial Ung in brain protection [41]. Recently, expression of UNG1 was shown to increase twofold after oxidative stress [27]. In addition to uracil, UNG removes isodialuric acid, alloxan and 5-hydroxyuracil [43] although relatively inefficiently. These are cytosine-derived products of oxidative base damage. Experimental demonstration of these lesions in mtDNA, and a role of UNG1 for their removal, remains to be examined.

Nuclear BER has been extensively studied and found to take place as both SP and LP BER [16]. By comparison, mtBER patch-size has been far less studied. To our knowledge two reports on this subject are available [18,19]. In the first report a reconstituted BER with POLγ, AP-endonuclease, and DNA ligase, all purified from *Xenopus laevis* mitochondria, was in form of single-nucleotide insertion [18]. It is possible that factors contributing to the processivity of POLγ and LP BER were absent in the purified fractions. In the second study [19], difference in reaction conditions including sensitivity and type of DNA substrate used may explain the discrepancy in conclusions.

During nuclear LP BER, short 5′ single-stranded DNA (flaps) can be formed that is subsequently cleaved by flap endonuclease-1 (FEN-1) [44]. In *Escherichia coli*, the 5′ to 3′ nuclease activity of PolI carries out this action [44,45]. To our knowledge a specific 5′ flap endonuclease in human

mitochondria has not been identified. However, the results presented in Fig. 5 show that 5′ protruding flaps of length between 1 and 5 nucleotides were removed from DNA by a mitochondrial extract free of FEN-1. We are working on identifying the enzyme(s) responsible for this activity.

Exposure of DNA to ROS results in the formation of a variety of lesions, including oxidized AP sites [46]. Because of the close proximity of mtDNA to the inner membrane, which is the main site of ROS production in mitochondria, it is likely that oxidative modification of AP sites in mtDNA is a rather frequent event. We tested the ability of mitochondrial extract to repair modified AP sites using a DNA substrate containing THF which is resistant to lyase activity of POLβ [17] and that requires LP pathway for repair [36,47]. We found that THF and normal AP sites were repaired with equal efficiency by mitochondrial extract. Whether a 5′–3′ exonuclease activity or the lyase activity of POLγ is responsible for the removal 5′ THF or a hitherto unidentified mitochondrial-specific 5′ flap endonuclease carries out this action remains to be examined.

In conclusion in this study we show that UNG is the predominant uracil-DNA glycosylase in mitochondria. Furthermore, we show that mitochondrial extract is able to carry out repair of modified AP sites, displays LP BER and specific flap-endonuclease activity. These data suggest that mitochondria repair a broad repertoire of DNA lesions that are expected to occur frequently in mtDNA.

Acknowledgements

REFERENCES

[1] J. Shen, N. Khan, L.D. Lewis, R. Armand, O. Grinberg, E. Demidenko, H. Swartz, Oxygen consumption rates and oxygen concentration in molt-4 cells and their mtDNA depleted (ρ^0) mutants, Biophys. J. 84 (2003) 1291–1298.

[2] A. Trifunovic, A. Wredenberg, M. Falkenberg, J.N. Spelbrink, A.T. Rovio, C.E. Bruder, Y.M. Bohlooly, S. Gidlof, A. Oldfors, R. Wibom, J. Tornell, H.T. Jacobs, N.G. Larsson, Premature ageing in mice expressing defective mitochondrial DNA polymerase, Nature 429 (2004) 417–423.

[3] R.G. Hansford, B.A. Hogue, V. Mildaziene, Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age, J. Bioenergy Biomembr. 29 (1997) 89–95.

[4] G. Slupphaug, B. Kavli, H.E. Krokan, The interacting pathways for prevention and repair of oxidative DNA damage, Mutat. Res. 531 (2003) 231–251.

[5] G. Pesole, C. Gissi, A. De Chirico, C. Saccone, Nucleotide substitution rate of mammalian mitochondrial genomes, J. Mol. Evol. 48 (1999) 427–434.

[6] M. Akbari, C. Skjelbred, I. Følling, J. Sagen, H.E. Krokan, A gel electrophoresis method for detection of mitochondrial DNA mutation (3243 tRNA(Leu (UUR))) applied to a Norwegian family with diabetes mellitus and hearing loss, Scand. J. Clin. Lab. Invest. 64 (2004) 86–92.

[7] J.M. van den Ouweland, H.H. Lemkes, W. Ruitenbeek, L.A. Sandkuijl, M.F. de Vijlder, P.A. Struyvenberg, J.J. van de Kamp, J.A. Maassen, Mutation in mitochondrial tRNA(Leu)(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness, Nat. Genet. 1 (1992) 368–371.

[8] M. Brandon, P. Baldi, D.C. Wallace, Mitochondrial mutations in cancer, Oncogene 25 (2006) 4647–4662.

[9] D.C. Wallace, A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine, Annu. Rev. Genet. 39 (2005) 359–407.

[10] L.S. Kaguni, DNA polymerase gamma, the mitochondrial replicase, Annu. Rev. Biochem. 73 (2004) 293–320.

[11] F. Ye, J.A. Carrodeguas, D.F. Bogenhagen, The gamma subfamily of DNA polymerases: cloning of a developmentally regulated cDNA encoding *Xenopus laevis* mitochondrial DNA polymerase gamma, Nucleic Acids Res. 24 (1996) 1481–1488.

[12] L. Fan, P.C. Sanschagrin, L.S. Kaguni, L.A. Kuhn, The accessory subunit of mtDNA polymerase shares structural homology with aminoacyl-tRNA synthetases: implications for a dual role as a primer recognition factor and processivity clamp, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 9527–9532.

[13] M.J. Longley, R. Prasad, D.K. Srivastava, S.H. Wilson, W.C. Copeland, Identification of 5′-deoxyribose phosphate lyase activity in human DNA polymerase gamma and its role in mitochondrial base excision repair *in vitro*, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 12244–12248.

[14] L. Fan, S. Kim, C.L. Farr, K.T. Schaefer, K.M. Randolph, J.A. Tainer, L.S. Kaguni, A novel processive mechanism for DNA synthesis revealed by structure, modeling and mutagenesis of the accessory subunit of human mitochondrial DNA polymerase, J. Mol. Biol. 358 (2006) 1229–1243.

[15] N.B. Larsen, M. Rasmussen, L.J. Rasmussen, Nuclear and mitochondrial DNA repair: similar pathways? Mitochondrion 5 (2005) 89–108.

[16] P. Fortini, E. Dogliotti, Base damage and single-strand break repair: mechanisms and functional significance of short- and long-patch repair subpathways, DNA Repair (Amst.) 6 (2007) 398–409.

[17] Y. Matsumoto, K. Kim, D.F. Bogenhagen, Proliferating cell nuclear antigen-dependent abasic site repair in *Xenopus laevis* oocytes: an alternative pathway of base excision DNA repair, Mol. Cell Biol. 14 (1994) 6187–6197.

[18] K.G. Pinz, D.F. Bogenhagen, Efficient repair of abasic sites in DNA by mitochondrial enzymes, Mol. Cell Biol. 18 (1998) 1257–1265.

[19] R.H. Stierum, G.L. Dianov, V.A. Bohr, Single-nucleotide patch base excision repair of uracil in DNA by mitochondrial protein extracts, Nucleic Acids Res. 27 (1999) 3712–3719.

[20] G. Slupphaug, I. Eftedal, B. Kavli, S. Bharati, N.M. Helle, T. Haug, D.W. Levine, H.E. Krokan, Properties of a recombinant human uracil-DNA glycosylase from the UNG gene and evidence that UNG encodes the major uracil-DNA glycosylase, Biochemistry 34 (1995) 128–138.

[21] D.L. Croteau, C.M. ap Rhys, E.K. Hudson, G.L. Dianov, R.G. Hansford, V.A. Bohr, An oxidative damage-specific endonuclease from rat liver mitochondria, J. Biol. Chem. 272 (1997) 27338–27344.

[22] M. Tanaka, J.S. Lai, W. Herr, Promoter-selective activation domains in Oct-1 and Oct-2 direct differential activation of an snRNA and mRNA promoter, Cell 68 (1992) 755–767.

[23] M. Akbari, M. Otterlei, J. Pena-Diaz, P.A. Aas, B. Kavli, N.B. Liabakk, L. Hagen, K. Imai, A. Durandy, G. Slupphaug, H.E. Krokan, Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells, Nucleic Acids Res. 32 (2004) 5486–5498.

[24] G. Frosina, E. Cappelli, P. Fortini, E. Dogliotti, *In vitro* base excision repair assay using mammalian cell extracts, Methods Mol. Biol. 113 (1999) 301–315.

[25] B. Kavli, O. Sundheim, M. Akbari, M. Otterlei, H. Nilsen, F. Skorpen, P.A. Aas, L. Hagen, H.E. Krokan, G. Slupphaug, 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, J. Biol. Chem. 277 (2002) 39926–39936.

[26] G. Tell, E. Crivellato, A. Pines, I. Paron, C. Pucillo, G. Manzini, A. Bandiera, M.R. Kelley, C. Di Loreto, G. Damante, Mitochondrial localization of APE/Ref-1 in thyroid cells, Mutat. Res. 485 (2001) 143–152.

[27] M. Akbari, M. Otterlei, J. Pena-Diaz, H.E. Krokan, Different organization of base excision repair of uracil in DNA in nuclei and mitochondria and selective upregulation of mitochondrial uracil-DNA glycosylase after oxidative stress, Neuroscience 145 (2007) 1201–1212.

[28] D. Tsuchimoto, Y. Sakai, K. Sakumi, K. Nishioka, M. Sasaki, T. Fujiwara, Y. Nakabeppu, Human APE2 protein is mostly localized in the nuclei and to some extent in the mitochondria, while nuclear APE2 is partly associated with proliferating cell nuclear antigen, Nucleic Acids Res. 29 (2001) 2349–2360.

[29] S. Ikegami, T. Taguchi, M. Ohashi, M. Oguro, H. Nagano, Y. Mano, Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-alpha, Nature 275 (1978) 458–460.

[30] H. Krokan, P. Schaffer, M.L. DePamphilis, Involvement of eucaryotic deoxyribonucleic acid polymerases alpha and gamma in the replication of cellular and viral deoxyribonucleic acid, Biochemistry 18 (1979) 4431–4443.

[31] M.Y. Lee, C.K. Tan, K.M. Downey, A.G. So, Structural and functional properties of calf thymus DNA polymerase delta, Prog. Nucleic Acid Res. Mol. Biol. 26 (1981) 83–96.

[32] H.E. Krokan, F. Drablos, G. Slupphaug, Uracil in DNA—occurrence, consequences and repair, Oncogene 21 (2002) 8935–8948.

[33] H. Nilsen, M. Otterlei, T. Haug, K. Solum, T.A. Nagelhus, F. Skorpen, H.E. Krokan, Nuclear and mitochondrial uracil-DNA glycosylases are generated by alternative splicing and transcription from different positions in the UNG gene, Nucleic Acids Res. 25 (1997) 750–755.

[34] M. Otterlei, T. Haug, T.A. Nagelhus, G. Slupphaug, T. Lindmo, H.E. Krokan, Nuclear and mitochondrial splice forms of human uracil-DNA glycosylase contain a complex nuclear localisation signal and a strong classical mitochondrial localisation signal, respectively, Nucleic Acids Res. 26 (1998) 4611–4617.

[35] Y. Matsumoto, K. Kim, Excision of deoxyribose phosphate residues by DNA polymerase beta during DNA repair, Science 269 (1995) 699–702.

[36] K. Kim, S. Biade, Y. Matsumoto, Involvement of flap endonuclease 1 in base excision DNA repair, J. Biol. Chem. 273 (1998) 8842–8848.

[37] A. Klungland, T. Lindahl, Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1), EMBO J. 16 (1997) 3341–3348.

[38] A.B. Hansen, N.B. Griner, J.P. Anderson, G.C. Kujoth, T.A. Prolla, L.A. Loeb, E. Glick, Mitochondrial DNA integrity is not dependent on DNA polymerase-beta activity, DNA Repair (Amst.) 5 (2006) 71–79.

[39] B. Szczesny, T.K. Hazra, J. Papaconstantinou, S. Mitra, I. Boldogh, Age-dependent deficiency in import of mitochondrial DNA glycosylases required for repair of oxidatively damaged bases, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 10670–10675.

[40] Q. An, P. Robins, T. Lindahl, D.E. Barnes, C → T mutagenesis and gamma-radiation sensitivity due to deficiency in the Smug1 and Ung DNA glycosylases, EMBO J. 24 (2005) 2205–2213.

[41] M. Endres, D. Biniszkiewicz, R.W. Sobol, C. Harms, M. Ahmadi, A. Lipski, J. Katchanov, P. Mergenthaler, U. Dirnagl, S.H. Wilson, A. Meisel, R. Jaenisch, Increased postischemic brain injury in mice deficient in uracil-DNA glycosylase, J. Clin. Invest. 113 (2004) 1711–1721.

[42] K.J. Kyng, A. May, R.M. Brosh Jr., W.H. Cheng, C. Chen, K.G. Becker, V.A. Bohr, The transcriptional response after oxidative stress is defective in Cockayne syndrome group B cells, Oncogene 22 (2003) 1135–1149.

[43] M. Dizdaroglu, A. Karakaya, P. Jaruga, G. Slupphaug, H.E. Krokan, Novel activities of human uracil DNA N-glycosylase for cytosine-derived products of oxidative DNA damage, Nucleic Acids Res. 24 (1996) 418–422.

[44] Y. Liu, H.I. Kao, R.A. Bambara, Flap endonuclease 1: a central component of DNA metabolism, Annu. Rev. Biochem. 73 (2004) 589–615.

[45] R.C. Lundquist, B.M. Olivera, Transient generation of displaced single-stranded DNA during nick translation, Cell 31 (1982) 53–60.

[46] B. Demple, L. Harrison, Repair of oxidative damage to DNA: enzymology and biology, Annu. Rev. Biochem. 63 (1994) 915–948.

[47] Y. Liu, W.A. Beard, D.D. Shock, R. Prasad, E.W. Hou, S.H. Wilson, DNA polymerase beta and flap endonuclease 1 enzymatic specificities sustain DNA synthesis for long patch base excision repair, J. Biol. Chem. 280 (2005) 3665–3674.

Paper II

The rate of base excision repair of uracil is controlled by the initiating glycosylase

*Torkild Visnes, Mansour Akbari, Lars Hagen, Geir Slupphaug, Hans E. Krokan**

Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Erling Skjalgssons gate 1, N-7006, Trondheim, Norway

ABSTRACT

Uracil in DNA is repaired by base excision repair (BER) initiated by a DNA glycosylase, followed by strand incision, trimming of ends, gap filling and ligation. Uracil in DNA comes in two distinct forms; U:A pairs, typically resulting from replication errors, and mutagenic U:G mismatches, arising from cytosine deamination. To identify proteins critical to the rate of repair of these lesions, we quantified overall repair of U:A pairs, U:G mismatches and repair intermediates (abasic sites and nicked abasic sites) *in vitro*. For this purpose we used circular DNA substrates and nuclear extracts of eight human cell lines with wide variation in the content of BER proteins. We identified the initiating uracil–DNA glycosylase UNG2 as the major overall rate-limiting factor. UNG2 is apparently the sole glycosylase initiating BER of U:A pairs and generally initiated repair of almost 90% of the U:G mismatches. Surprisingly, TDG contributed at least as much as single-strand selective monofunctional uracil–DNA glycosylase 1 (SMUG1) to BER of U:G mismatches. Furthermore, in a cell line that expressed unusually high amounts of TDG, this glycosylase contributed to initiation of as much as ~30% of U:G repair. Repair of U:G mismatches was generally faster than that of U:A pairs, which agrees with the known substrate preference of UNG-type glycosylases. Unexpectedly, repair of abasic sites opposite G was also generally faster than when opposite A, and this could not be explained by the properties of the purified APE1 protein. It may rather reflect differences in substrate recognition or repair by different complex(es). Lig III is apparently a minor rate-regulator for U:G repair. APE1, Pol β, Pol δ, PCNA, XRCC1 and Lig I did not seem to be rate-limiting for overall repair of any of the substrates. These results identify damaged base removal as the major rate-limiting step in BER of uracil in human cells.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Uracil is a frequently occurring lesion in DNA and the largest contribution probably comes from incorporation of dUMP instead of dTMP during replication, resulting in U:A pairs [1,2]. The amount of incorporated uracil in DNA may be enhanced by cytostatics that increase the dUTP/dTTP ratio [1,3]. Although incorporated dUMP is thought to be non-mutagenic, it may

perturb DNA metabolism by interfering with the sequence specific binding of transcription factors [4]. Uracil in DNA may also result from spontaneous hydrolytic deamination of cytosine, generating mutagenic U:G mismatches at a rate of 70–200 events per day in each cell. In the absence of repair, this would clearly result in an unacceptably high mutation rate, given the perfect pairing of U with A [5,6].

In general, base excision repair (BER) of uracil in DNA is initiated by a uracil–DNA glycosylase (UDG) that cleaves the N-glycosidic bond between the base and deoxyribose, leaving an abasic site (AP-site). In humans, four enzymes displaying UDG-activity are known, among which nuclear UNG2 in the conserved UNG-family and single-strand selective monofunctional uracil–DNA glycosylase 1 (SMUG1) have been considered the most important in BER. SMUG1 was reported to be central in repair of U:G mismatches in mouse cells [7], but this is not necessarily the case for human cells, where UNG2 may be more important [8,9]. In fact, roles in BER of SMUG1 and particularly thymine–DNA glycosylase (TDG) which actually prefers uracil, are unsettled. Essentially nothing is known about the functional significance in BER of the fourth mammalian uracil–DNA glycosylases, methyl binding domain protein 4 (MBD4) [10]. Subsequent to base excision, AP-endonuclease 1 (APE1) cleaves DNA 5′ to the AP-site. BER may then follow two distinct routes characterised by the insertion of either one (short patch, SP) or several (long patch, LP) nucleotides. Both pathways have been reconstituted *in vitro* with purified enzymes. The SP pathway requires as a minimum a DNA glycosylase, APE1, Pol β, which removes 5′-deoxyribosephosphate (dRP) and inserts a single nucleotide, and finally ligation by DNA ligase III, usually in complex with XRCC1 [11]. If the AP-site is modified to become resistant to the AP-lyase activity of Pol β, an alternative polymerase may displace a segment of single-stranded DNA containing the lesion. The displaced strand is then cleaved by the structure specific enzyme flap endonuclease 1 (FEN1) and the resulting nick is ligated, most likely by DNA ligase I. The LP pathway has been reconstituted with APE1, DNA polymerase δ/ε, replication factor C (RFC), proliferating cell nuclear antigen (PCNA), FEN1 and DNA ligase I [12,13]. *In vivo* the mechanism is almost certainly more complex due to apparent redundancy of proteins carrying out different steps, different expression of the proteins during the cell cycle, compartmentalization of proteins, extensive protein–protein interactions, as well as post-translational modifications [2,14–18]. In addition, BER may under some conditions involve several other proteins, such as poly-(ADP-ribose) polymerase (PARP) [19], p53 [20] and WRN [21].

The ability to excise uracil among human cell lines was previously found to vary several-fold; a variation which was not caused by polymorphisms in the coding region of the human *UNG*-gene [22]. This variation is not limited to cancer cell lines, since substantial inter-individual variation in UDG-activity has also been reported in human tumour tissues and normal tissues [23,24]. The purpose of the present study was to examine which of the known nuclear BER proteins, if any, are bottle necks in the repair of uracil in DNA in human cell lines. Previous studies have proposed removal of 5′dRP by Pol β [25] or ligation [26] as rate-limiting step in mammalian BER. We report here that UNG2, in spite of a very considerable variation in the level of several of the other proteins known to

be required for BER, is the major rate-limiting factor in repair of U:A and U:G in nuclear DNA in human cell lines. However, for U:G repair TDG and SMUG1 also contribute to the initiation of BER. Surprisingly, TDG was at least as important for U:G repair as SMUG1. Except for a possible rate-limiting effect of low DNA ligase III content, we found no significant correlation between BER capacity and the content of several other BER proteins, indicating that no single factor can be identified as rate-limiting in human cancer cell lines.

2. Materials and methods

2.1. Cell culture and nuclear extracts

All cell lines were cultured in Dulbeccos modified Eagle medium (4500 mg/l glucose), with 10% fetal calf serum, 0.03% glutamine and 0.1 mg/ml gentamicin in 5% CO_2. The cell lines were harvested at 50–70% confluence by trypsination, followed by washing in ice-cold phosphate-buffered saline (PBS). Nuclear extracts were prepared by swelling the cells in hypotonic buffer (20 mM HEPES-KOH pH 7.8, 1 mM $MgCl_2$, 5 mM KCl, 1 mM DTT and 1× Complete® EDTA-free protease inhibitor cocktail (Roche)) for 45 min followed by lysis of the cells using a Dounce homogenizer with a tight fitting pestle. Nuclei were centrifuged at 180 g and resuspended in 10× packed nuclear volume (PNV) of buffer I (10 mM Tris–HCl pH 8.0, 200 mM KCl, 2 mM EDTA, 1 mM DTT and 1× Complete® EDTA-free protease inhibitor cocktail (Roche)), recentrifuged and resuspended in 2× PNV buffer II (same as buffer I, but also containing 0.5% (v/v) NP-40 and 40% (v/v) glycerol). Protein was extracted at 4 °C for 2 h and cell debris removed by centrifugation at 13,000 × g for 15 min. Supernatants were aliquoted, snap frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were measured using the Bradford method (BioRad).

2.2. Standard UDG-assays on nick-translated DNA

Standard UDG-assays were performed essentially as described [9]. The standard substrate in UDG-assays was calf thymus DNA nick-translated in the presence of [³H]dUTP and unlabelled dNTPs. Thus, the substrate contains labelled uracil in a U:A context and the assay essentially measures activity encoded by the *UNG*-gene [27]. UDG-activity is given as units/mg protein in nuclear extract, where one unit is the amount of enzyme required to release 1 nmol of uracil from the UDG-substrate per minute at 30 °C [16]. The 20 μl reaction mixtures contained final concentrations of 40 mM HEPES-KOH pH 7.8, 70 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1 μg/μl BSA, 1.8 μM [³H]dUMP-containing calf thymus DNA (specific activity 0.5 mCi/μmol) and diluted nuclear extracts.

2.3. Western blot analysis

50 μg protein from each nuclear extract were separated by electrophoresis on NuPAGE® 4–12% Bis–Tris gradient gels (Invitrogen) and blotted onto PVDF membranes (Immobilon™, Millipore) by standard procedure, followed by blocking in 5% fat-free dry milk in PBS containing 0.1% Tween®-20 and hybridisation with primary and secondary antibodies, the lat-

ter conjugated with horseradish peroxidase (DAKO, Denmark). For Western blot analysis of UNG we used an antibody recognising the catalytic domain [27] at 0.5 μg/ml. TDG was detected with anti-murine TDG serum at a 1:500 dilution (a kind gift from Primo Schär). Antibodies against PCNA (ab29), DNA polymerase β (ab2856), XRCC1 (ab1838), DNA ligase I (ab615) and DNA ligase III (ab587) were supplied by Abcam Ltd., UK. The antibody against APE1 (NB100–504) was from Novus Biologicals Inc., Littleton, CO, USA and the antibody against DNA polymerase δ (D73020–050) from Transduction Laboratories, Lexington, KY, USA and used as recommended by the supplier. The membrane used for the visualisation of TDG had previously been used for the visualisation of DNA ligase III (~100 kDa), then stripped using 0.2 M NaOH for 5 min at room temperature, washed in water, reblocked and reprobed. All other BER proteins were visualised on separate membranes. We quantified the content of individual BER proteins in the extracts by luminometry using the SuperSignal West Femto Substrate (Pierce) a Kodak ImageStation 2000R and Kodak Molecular Imaging Software v4.0.1.

2.4. *In vitro BER-assays*

Substrates for the BER assay were prepared as described [16,28]. Briefly, an uracil-containing oligonucleotide (5′-GAT CCT CTA GAG TUG ACC TGC A-3′) was annealed to ssDNAs derived from the pGEM-3Zf(+) plasmid, containing either A or G opposite uracil. The lesions were positioned in otherwise identical sequence contexts in order to rule out any differences due to the sequence-dependency of uracil excision [29]. Following strand elongation and ligation, covalently closed circular DNA (cccDNA) was collected from a CsCl/ethidium bromide gradient, ethanol precipitated, washed and resuspended. These substrates will be referred to as U:A and U:G substrates, respectively. Substrates containing an AP-site were prepared by treating U:A and U:G cccDNA with the purified recombinant catalytic domain of human UNG [27], while substrates with nicked AP-sites (nAP) were prepared by additional treatment with recombinant purified human APE1. The AP-site substrate and the nAP-site substrate are therefore identical to natural intermediates in the BER process. Unless otherwise indicated, 250 ng cccDNA substrate was incubated at 30 °C for 30 min with 10 μg protein in final concentrations of 40 mM HEPES-KOH, 70 mM KCl, 5 mM MgCl$_2$, 0.5 mM DTT, 2 mM ATP, 20 μM dATP, 20 μM dGTP, 8 μM dCTP or dTTP depending on the radioactive isotope used, 4.4 mM phosphocreatine, 62.5 ng/μl creatine kinase and 50 nCi/μl [α-^{32}P]dCTP or [α-^{32}P]dTTP in a volume of 40 μl. The reactions were stopped by the addition of EDTA (to 18 mM) and 6 μg RNase A and incubated at 37 °C for 10 min followed by the addition of SDS (to 0.5%) and 12 μg proteinase K. After a further incubation for 30 min at 37 °C, DNA was purified by phenol/chloroform-extraction and ethanol precipitation and unless otherwise indicated digested with XbaI and HincII (New England Biolabs). This generated an 8-mer fragment labelled with a single incorporated nucleotide at the position of the original lesion. Provided that the ligation step was fairly efficient, this fragment was a quantitative measure of uracil-repair by both short patch and long patch pathways. Following 12% PAGE, bands were visualised and quantified with arbitrary units

using ImageQuant software (Fujifilm). We investigated relative contribution of SMUG1, TDG and UNG2 to the initiation of uracil repair by pre-incubating extracts with a neutralising antibody to SMUG1 (0.11 μg/μl final concentration) [9], UNG (0.3 μg/μl final concentration), and/or neutralising anti-serum towards TDG at (1:50 dilution) [30] on ice for 30 min prior to the reaction.

2.5. *AP-site incision assay*

APE1 activity was measured by monitoring the incision of an oligonucleotide containing an AP-site opposite A or G. Briefly, a 22-mer oligonucleotide (5′-GAT CCT CTA GAG TUG ACC TGC A-3′) was 5′ end-labelled using T4 polynucleotide kinase and [γ-^{33}P]ATP and annealed to a complementary 22-mer (5′-TGC AGG TCX ACT CTA GAG GAT C-3′) containing either A or G (X = A or G) opposite uracil. An AP-site was then generated by treatment with the purified recombinant catalytic domain of human UNG. Labelled duplex oligonucleotide (0.1 pmol) and increasing concentrations of unlabelled duplex oligonucleotide containing AP:A or AP:G were then incubated with purified APE1 under conditions similar to those in the BER assay (40 mM HEPES-KOH pH 7.8, 5 mM MgCl$_2$, 70 mM KCl, 1 mM DTT, 0.1 μg/μl BSA) at 30 °C for 5 min. Reactions were stopped by addition of formamide loading buffer containing 10 mM EDTA, heated at 90 °C for 10 min and oligonucleotides separated by 12% PAGE.

2.6. *Statistical analysis*

Linear regression analysis was employed to determine best-fit curves and corresponding coefficient of determination values (R^2). P-values were calculated to determine whether the slopes of the linear regression curves were significantly different from zero, which would be the expected result if one assumes no correlation between the content of the relevant protein and repair capacity. Finally, t-tests were performed to determine the statistical significance of the apparent preference for U, AP and nAP opposite G in the extracts. All statistical analyses were done using Excel and GraphPad Prism.

3. Results

3.1. *Preparation of nuclear extracts*

To be able to directly compare results from *in vitro* repair assays using nuclear extracts from different cell lines, we carried out a series of experiments to establish conditions for extract preparation that gave reproducible results for all cell lines used. The criteria were reproducibility in terms of BER activity, UDG-activity and yield of protein per 10^6 cells. In our hands, isolation of nuclei after Dounce homogenization of hypotonically swollen cells, followed by centrifugation at 180 × *g* and extraction in hypertonic buffer containing detergent and 200 mM KCl gave reproducible results for all cell lines. Using lower salt concentration (100 mM KCl) for extraction under otherwise identical conditions resulted in several-fold lower protein yield, as well as lower BER activity. Higher salt (500 mM KCl) followed by dialysis increased the yield of protein, but

Cell line	Type of cell	UDG-activity ± S.D.
CCD 1064	Human foreskin fibroblast	0.19 ± 0.01
AGS	Gastric adenocarcinoma	0.34 ± 0.01
SW480	Colon adenocarcinoma	0.80 ± 0.02
ZR-75-1	Breast carcinoma	0.83 ± 0.01
MDA-MB-231	Breast adenocarcinoma	0.95 ± 0.02
HCT-8	Ileoceal adenocarcinoma	0.99 ± 0.01
CX-1	Colon adenocarcinoma	1.60 ± 0.03
T-47D	Breast carcinoma	2.40 ± 0.11

One unit is defined as nmol uracil released per mg protein per minute, and standard deviations are calculated based on three independent experiments.

BER activity was markedly reduced. Extraction with 500 mM KCl in the absence of detergent also resulted in lower BER activity, and in addition resulted in more variation between different experiments (data not shown). Specific UDG-activity in nuclear extracts, measured using a substrate with uracil in a U:A context, was found to vary 12.6-fold (Table 1). The data presented for each cell line (UDG-activity) are from three independent measurements on the same preparation of nuclear extract (S.D. < 5%). However, we have also carried out similar measurements after preparation of three separate nuclear extracts from the same batch of cells (S.D. = 5%), as well as three separate nuclear extracts from different cell batches (S.D. = 16%). These results are in good agreement with the report that formed the basis for selection of cell lines [22]. However, we found higher specific activities, since we have used nuclear extracts rather than whole cell extracts of sonicated cells.

3.2.   Western blot analysis of BER proteins

The content of UNG2, TDG, APE1, Pol β and δ, XRCC1, PCNA and DNA ligase I and III in the extracts was examined by Western blot analysis, as displayed in Fig. 1A and Table 2. The results displayed are from one experiment. However, we have repeated Western blots using three independently prepared nuclear extracts for UNG2, APE1, XRCC1 and Lig III. Standard deviations were in the range 4.8–20%, demonstrating that variation between extract preparations is much smaller than between extracts from different cell lines. The mitochondrial UNG1 protein was not detected in any of the nuclear extracts, indicating that they were largely free of contaminants from mitochondria. We detected two bands for TDG—one representing an unmodified protein at an apparent molecular weight of ∼60 kDa, and another that conforms to a SUMOylated form at ∼84 kDa [31]. Quantitative Western blot analysis was limited to the 60 kDa form. We examined the possibility that expression of some of the proteins correlated with each other. However, the only significant correlation observed was that between the replication-associated proteins DNA polymerase δ and DNA ligase I ($R^2 = 0.80$ and $P = 0.0027$). Regrettably, we were unable to visualise SMUG1 in the extracts. This protein was only detectable following immuno-precipitation (data not shown), so our failure to detect SMUG1 was presumably due to low SMUG1 levels in the human nuclear extracts.

The relative protein content of UNG2 in the extracts was found to correlate strongly with UDG-activity ($R^2 = 0.92$ and $P = 0.0002$, Fig. 1B). This suggests that UNG2 was mainly responsible for the variation in UDG-activity measured under these assay conditions. Importantly, it also indicates that our quantitative Western analysis was suitable to gauge the content of BER proteins in different extracts. We found that the content of proteins frequently used as "loading control", e.g. lamin A/C, varied much between cell lines, making them useless as a general loading control in experiments involving several cell lines. Instead, we relied on protein measurements and loaded 50 μg of total protein from each nuclear extract.

3.3.   DNA–uracil and BER intermediates are repaired at different rates and the repair capacities vary among the different extracts

Nuclear extracts were used to study repair of cccDNA containing uracil, an AP-site or a nicked AP-site in a defined position (Fig. 1C). Each type of lesion was placed opposite A in the complementary strand to mimic the substrate resulting from incorporation of dUMP during replication, or opposite G to mimic the substrate resulting from cytosine deamination. Following incubation with nuclear extracts, repair was assessed by measuring the radioactivity incorporated into the fragment between the XbaI and HincII restriction sites. This is a good quantitative measure for BER, as exactly one radiolabelled nucleotide is incorporated into this fragment per BER event regardless of whether repair takes place via SP or LP subpathways. Technically, this does not provide information about the final ligation step of BER, as the product would not be different after incomplete BER in the form of unligated nick in the final intermediate. However, we found that ligation took place at high and largely similar efficiency in the extracts investigated. This control was carried out by digesting repair products with BamHI and PstI, which results in a 22-mer if the substrate is completely repaired and ligated, and a 14-mer representing an unligated, nicked repair product after one-nucleotide incorporation (Fig. 1D). We did not observe a significant accumulation of repair intermediates of size between 14 and 22 nucleotides, indicating negligible accumulation of repair intermediates other than the unligated 1 nucleotide extension product. However, with the methodology used, we cannot exclude the possibility that some intermediates containing an AP-site accumulated when starting BER with substrates containing uracil in a U:A or U:G context. Therefore, the intensity of the XbaI-HincII fragment was a useful approximation to complete BER in this system. Furthermore, the BER reaction was linear beyond the incubation time of 30 min used in subsequent experiments. The conditions used did not consume too much of the substrate for quantitative analysis, and the BER activity of the extract did not decay significantly during the incubation (Fig. 1E).

All extracts were capable of repairing the DNA substrates, although with different efficiencies (Fig. 2A and B). As expected, the substrates representing later stages in the BER pathway were generally repaired more efficiently than those representing earlier stages (U < AP < nAP), but the capacity of the extracts to repair each lesion varied several-fold. Some extracts repaired uracil and AP-sites with similar efficiencies
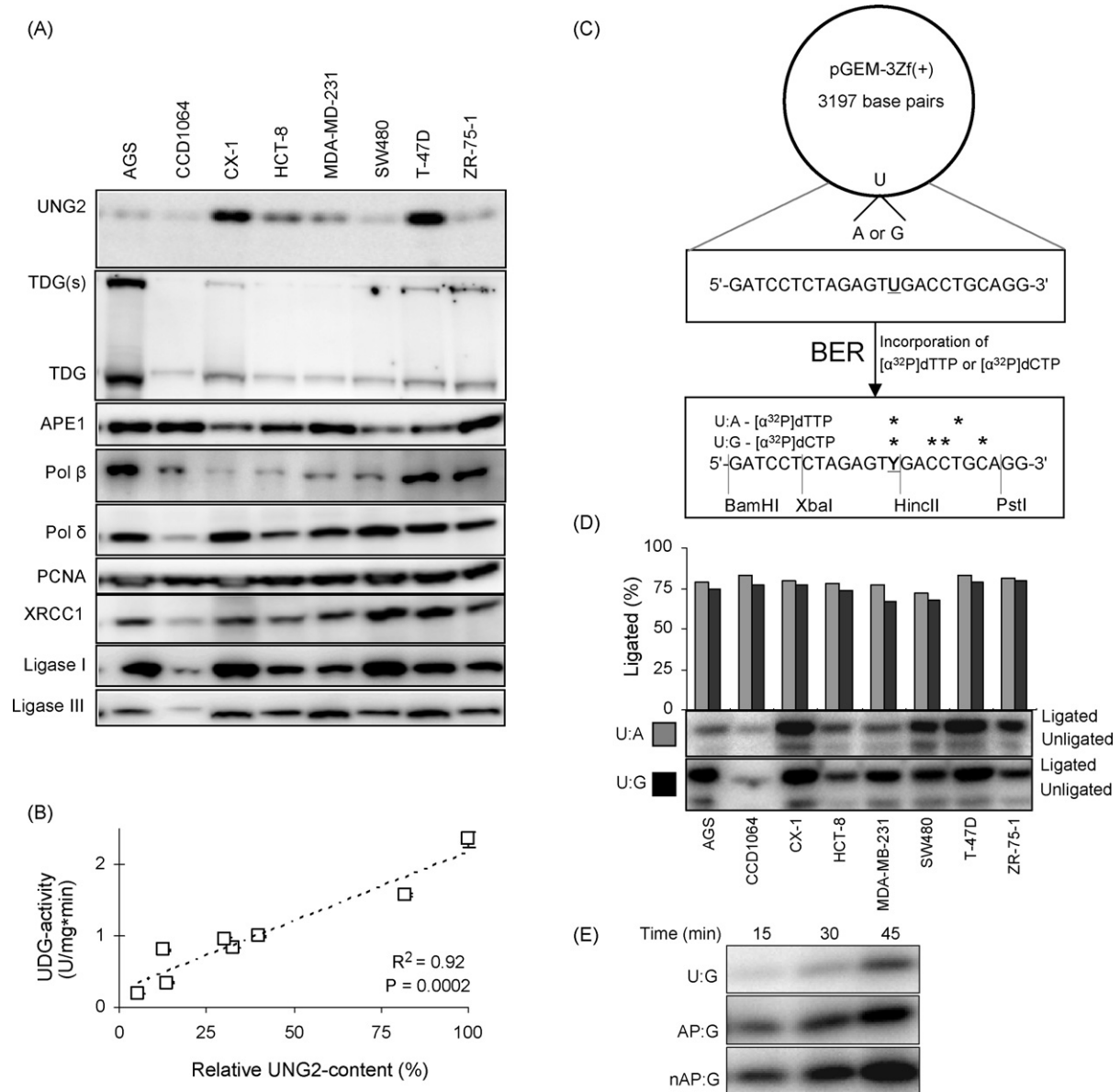
Fig. 1 – BER proteins in nuclear extracts and measurement of BER activities. (A) Western blot analysis of BER proteins in nuclear extracts. From left to right: (1) AGS, (2) CCD 1064, (3) CX-1, (4) HCT- 8, (5) MDA-MB-231, (6) SW480, (7) T-47D, (8) ZR-75-1. TDG appears as two bands at ∼60 kDa and ∼84 kDa, the latter conforming to a SUMOylated form of TDG [31]. (B) Correlation between relative UNG2 content and UDG-activity in nuclear extracts. UNG2 content was set relative to that in the T-47D extract (100%), where UNG2 was most abundant. UDG-activity was measured by the standard UDG-assay (values taken from Table 1). (C) Overview of strategy for analysing the BER process. A plasmid containing uracil or, alternatively, an AP-site or a nAP-site (not shown in figure) in a defined position is incubated with nuclear extracts. BER is then quantified in restriction fragments (routinely XbaI and HincII) after incorporation of [α^{32}P]dTTP or [α^{32}P]dCTP in the position of the original lesion. For ligation analysis we digested the substrate with BamHI and PstI. Potential incorporation sites for radiolabelled [α^{32}P]dTTP and [α^{32}P]dCTP are indicated with asterisks. Y represents incorporation of dCMP or dTMP following BER. For analysis of BER of uracil in a U:G context, the complementary strand contained G instead of A (not shown in figure). (D) Fraction of ligated product (complete repair) after in vitro BER in nuclear extracts, measured as radioactivity in BamHI-PstI fragments using U:A or U:G substrates. The lower 14-mer fragment represents an unligated single nucleotide insertion intermediate while the upper 22-mer represents completely ligated product, resembling completed short- and long patch repair products, alternatively unligated long patch repair with a patch size of eight nucleotides or above. Ligation during U:A repair appeared to be slightly more efficient than for U:G repair, with 72–83% versus 67–79%, respectively, of the intensity in the upper fragment. (E) BER as function of time. Repair of U:G, AP:G and nAP:G substrates by SW480 nuclear extract after 15 min, 30 min and 45 min incubation, as monitored after digestion with XbaI and HincII.

Table 2 – Variation in the content of BER proteins among extracts

	UNG2	TDG	APE1	Pol β	Pol δ	PCNA	XRCC1	Ligase I	Ligase III
AGS	14	**100**	91	64	61	76	52	72	58
CCD1064	5	18	**100**	33	15	83	24	15	17
CX-1	82	42	49	17	91	78	32	84	89
HCT	40	15	64	48	36	87	37	42	74
MDA-MD-231	30	12	90	44	66	92	54	37	**100**
Sw480	13	20	44	39	**100**	87	**100**	**100**	69
T-47D	**100**	32	50	91	73	100	81	60	96
ZR-75-1	33	33	92	**100**	52	81	38	38	69

Protein content is given in percent relative to the extract in which the protein in question was found to be most abundant (100%). Quantitative Western analysis was limited to the non-SUMOylated form of TDG.

(T-47D and SW480), whereas others displayed up to 3–4-fold more efficient AP-site repair compared to uracil-repair (HCT-8 and CX-1). The most efficiently repaired substrate was generally the one containing nAP, which was repaired 1.4–5.6-fold faster than AP-sites, and 1.5–12-fold faster than uracil in DNA.

3.4. BER of uracil in DNA correlates with UDG-activity and UNG2 content

Generally, the extracts with the highest UDG-activity displayed the most efficient repair of both U:A and U:G (Fig. 2C

and D, see also Fig. 4A). In particular, the repair of U:A correlated well with UDG-activity ($R^2 = 0.84$ and $P = 0.0013$) as well as with the relative content of UNG2 ($R^2 = 0.84$ and $P = 0.0007$). As shown in Fig. 1A, there is no co-variation between the DNA glycosylases or between these and other BER proteins. Thus, this highly significant linear relationship between BER and UNG2 suggests that increased expression of UNG2 results in more efficient repair of U:A, and that the glycosylase step is the rate-limiting step in BER of U:A. The correlation between UDG-activity and U:G repair was also significant, but weaker ($R^2 = 0.67$ and $P = 0.013$) and the correlation between U:G repair
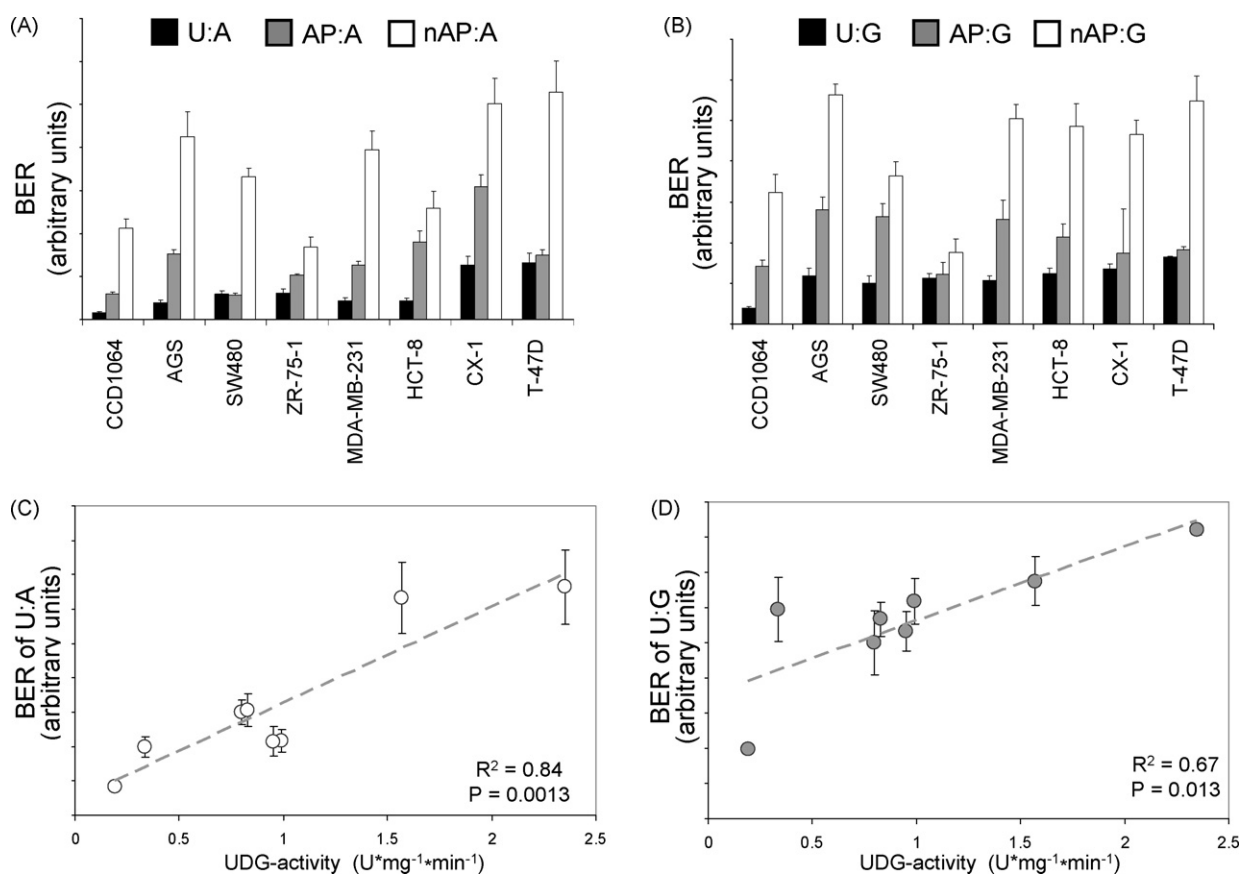
Fig. 2 – BER of circular DNA containing uracil, AP-site or nAP-site by nuclear extracts. Note that in panels A–D nuclear extracts along the X-axis are ordered according to ascending UDG-activity, as measured in the standard UDG-assay. All UDG-activity values are taken from Table 1. (A) Repair of uracil (black bars), AP-sites (grey bars) and nAP-sites (white bars) opposite A by nuclear extracts. (B) As in panel A, but lesion opposite G. (C) Correlation between BER of U:A substrate and UDG-activity. (D) Correlation between BER of U:G substrate and UDG-activity. Each experiment was conducted three times, and the error bars represent standard deviation of the mean. The units for BER (along the Y-axis) are arbitrary.
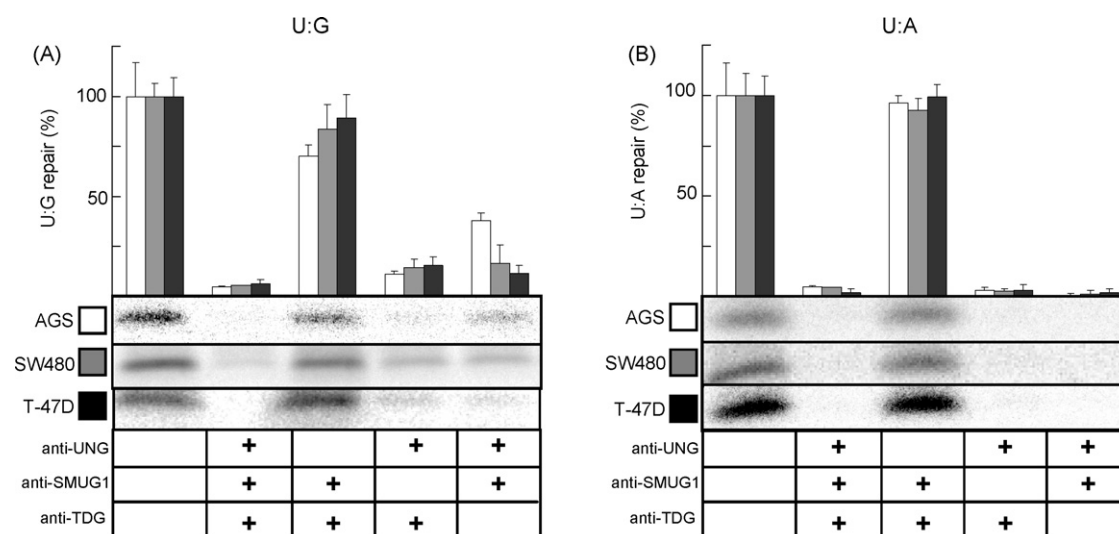
Fig. 3 – Contribution of UNG2, SMUG1 and TDG to initiation of BER of uracil in DNA. Effect of neutralising antibodies on BER of (A) U:G, and (B) U:A in nuclear extract from AGS, SW480 and T-47D cell lines. The effects of neutralising antibodies towards UNG2, SMUG1 and TDG were examined in otherwise standard BER assay. Detail on antibody dilutions are given in Section 2.4. Each experiment was conducted three times. Error bars represent the standard deviation of the mean and units are arbitrary. A representative image of the bands of one experiment is shown.

and the relative content of UNG2 ($R^2 = 0.64$ and $P = 0.017$) was weaker as well. The weaker correlation with U:G substrate was essentially caused by one outlier in Fig. 2D, the extract from the AGS cell line. This extract displayed a significantly higher U:G repair than expected from its measured UDG-activity, and interestingly also contained the highest content of TDG of all the extracts. Because the UDG-activity assay measures excision of uracil from U:A base pairs, the contribution of other glycosylases with a relative preference for U:G mispairs (i.e. SMUG1, TDG and MBD4, reviewed in [10]) is most likely underestimated using this assay. We therefore investigated whether U:G repair in the AGS extract could be initiated by other U:G glycosylases to any significant extent. By adding neutralising antibodies against UNG2, SMUG1 and TDG to the reaction mixtures, we were able to inhibit BER of U:G in the AGS extract by ~95% (Fig. 3A), indicating that these glycosylases are the main enzymes initiating repair of U:G under these experimental conditions. The residual ~5% activity could either be due to incomplete inhibition by the three antibodies or result from activity of MBD4, which we did not have the means to selectively inhibit. By omitting one of the three neutralising antibodies from the reaction mixture, we were able to estimate the individual contribution to U:G repair from each glycosylase. Our results indicate that in extract of AGS cells, TDG initiated ~30% of the U:G repair, but even here UNG2 appeared to be the major uracil-excising activity, initiating ~60% of the repair events. SMUG1 appeared to initiate only ~5% of total repair. However, in extracts from cell lines SW480 and T-47D, which contained more UNG2 and less TDG than the AGS extract, UNG2 initiated almost 90% of U:G repair, whereas TDG and SMUG1 contributed roughly equally (~5% each) to the rest of the BER initiations (Fig. 3A). To our knowledge, this is the first demonstration of a significant contribution of TDG to initiation of BER of uracil in a system mimicking a more com-

plex cellular system. In agreement with previous studies using other methods [2,27,32], UNG2 was apparently the sole activity initiating repair of the U:A substrate in all three extracts (Fig. 3B).

Next we analysed the relationship between the content of individual BER proteins in the extract and the repair capacity of uracil, AP-site and nAP substrates (Fig. 4) and examined possible correlation to the rate of BER by linear regression analysis. Resulting coefficients of determination (R^2) are displayed in Table 3. We quantified each protein relative to the extract in which the protein in question was most abundant, e.g. for UNG2, the reference extract was T-47D. Except for UNG2, the most significant correlation appeared to be that between the content of DNA ligase III and U:G repair ($R^2 = 0.68$ and $P = 0.012$), thus suggesting that it was important for the efficiency of U:G repair. However, we observed no general correlation between the content of DNA ligase III (or any other protein) and the ligation efficiency of U:A or U:G (Fig. 1D), and the correlation between DNA ligase III and the repair of AP- and nAP-substrates was also low. We did not observe any clear relationships between the content of any of the other proteins and repair of the other substrates, except for APE1 which displayed a weak negative correlation with U:A repair ($R^2 = 0.52$ and $P = 0.045$).

3.5. Uracil and AP-sites are repaired more rapidly opposite G than opposite A

In six out of eight extracts U:G was repaired 1.7–3-fold more efficiently than U:A ($P < 0.05$), and AP-sites opposite G were repaired 1.2–4.7-fold more efficient than AP-sites opposite A in five ($P < 0.05$) (Fig. 5A and B). For repair of nAP-sites, the preference for G opposite the lesion was less obvious (Fig. 5C), with only four extracts displaying a significantly
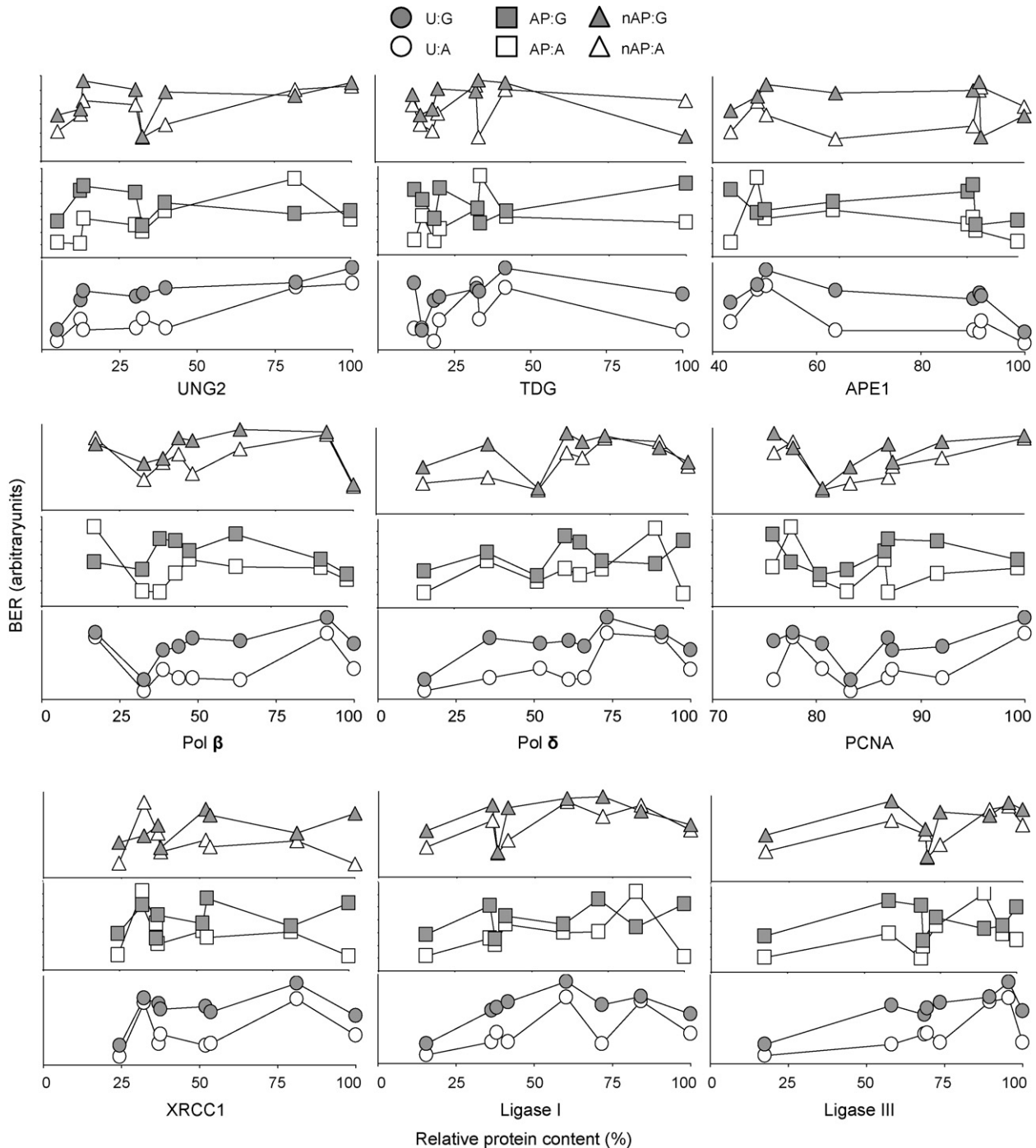
Fig. 4 – Relationship between content of different BER proteins in nuclear extracts and BER. Data on BER protein content (X-axis) is from Table 2 while data on repair (Y-axis) of U:A (open circles), AP:A (open squares), nAP:A (open triangles), U:G (closed circles), AP:G (closed squares) or nAP:G (closed triangles) is from Fig. 2A and B. Protein levels for each BER protein are given relative to the extract in which each specific protein was most abundant (100%), e.g. for UNG2 the 100% reference extract was T-47D (see Table 2).

more efficient nAP:G repair (1.3–1.8-fold, $P < 0.05$). As the differences between substrates containing A or G opposite the lesion were more pronounced for uracil and AP-sites this could suggest that the base opposite the AP-site may affect binding and/or the activity of APE1. However, this effect cannot be explained from the known properties of

AP-endonuclease, the major one being APE1 in mammalian cells [8,33]. Moreover, we found that the purified APE1 incised AP-sites with equal efficiency in both contexts (Fig. 5D). Equal incision opposite A and G has also been observed by others using the synthetic AP-site analog tetrahydrofuran [34].

Table 3 – Correlations between relative protein content and base excision repair capacities of nuclear extracts						
	U:A	AP:A	nAP:A	U:G	AP:G	nAP:G
UNG2	0.87**(0.85)*	0.44	0.45	0.64*(0.85)*	0.08	0.14
TDG	0.00	0.07	0.12	0.06	0.10	0.11
APE1	0.52*	0.15	0.28	0.35	0.03	0.08
DNA Polymerase β	0.02	0.08	0.02	0.17	0.06	0.04
DNA Polymerase δ	0.40	0.09	0.41	0.31	0.17	0.06
PCNA	0.09	0.06	0.07	0.10	0.00	0.08
XRCC1	0.08	0.11	0.15	0.13	0.28	0.05
DNA Ligase I	0.26	0.10	0.32	0.22	0.24	0.08
DNA Ligase III	0.43	0.25	0.38	0.68*	0.07	0.18

Correlation is displayed as coefficient of determination (R^2)-values of best-fit linear regression curves. **$P < 0.001$ and *$0.001 < P < 0.05$. Values within parentheses represent R^2 from a data set in which the AGS extract is excluded on the basis of its high TDG content (outlier).
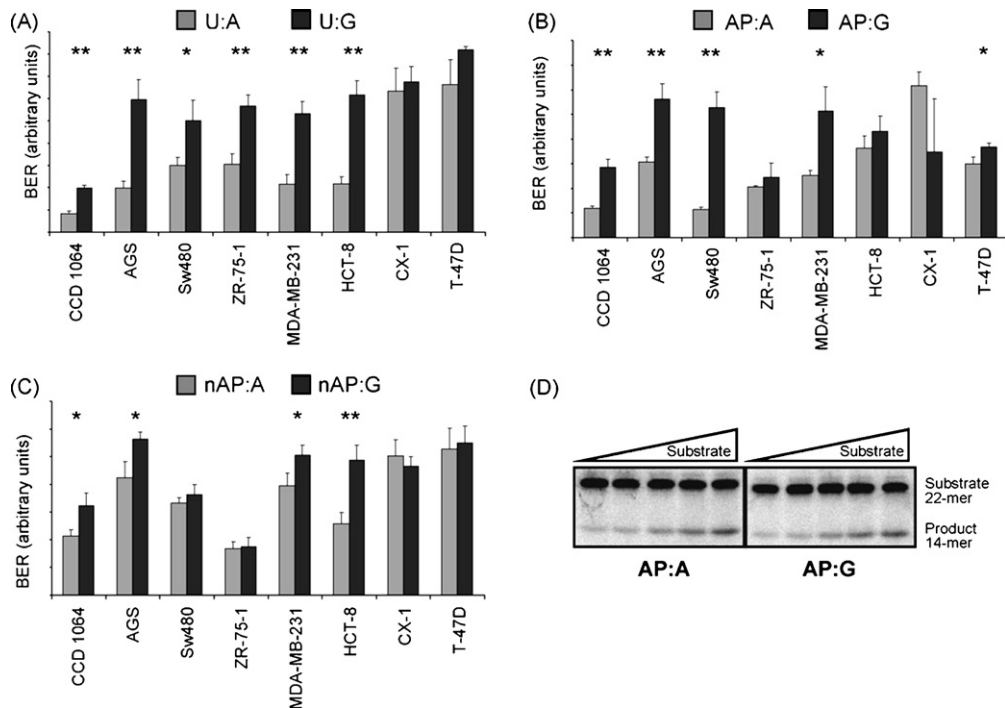
Fig. 5 – Effect of opposite base in BER of circular DNA containing U, AP-site or nAP-site opposite A (grey bars) or G (black bars). From left to right in panels A–C—1: CCD1064 (0.19), 2: AGS (0.34), 3: SW480 (0.80), 4: ZR-75-1 (0.83), 5: MDA-MB-231 (0.95), 6: HCT-8 (0.99), 7: CX-1 (1.6), 8: T-47D (2.4). (A) Repair of U positioned opposite A or G. (B) Repair of AP-site opposite A or G. (C) Repair of nAP-site opposite A or G. The units for repair are arbitrary. All experiments were performed in triplicates, and error bars represent standard deviations of the mean.*$0.01 < P < 0.05$ and **$P < 0.01$. (D) A fixed amount of purified APE1 was incubated with 0.1 pmol labelled 22-mer oligonucleotide containing an AP-site opposite A or G as well as increasing concentrations of unlabelled 22-mer duplex oligonucleotide. Incision of the 22-mer at the AP-site results in the 14-mer product.

4. Discussion

We aimed at identifying rate-limiting factor(s) in BER of uracil and the roles of uracil-excising glycosylases UNG2, SMUG1 and TDG in this process. For this purpose, we used nuclear extracts from eight human cell lines and a BER system that carries out all the steps in the repair process. Uracil in DNA is found in two very different contexts, U:A pairs as a result of a replication errors and U:G mismatches as a consequence of cytosine deamination. There is no *a priori* reason to assume

that the processing of these structurally and biologically quite different lesions should involve the same proteins or protein complexes. However, we found that UNG2 is the major glycosylase for initiation of BER of both lesions. Furthermore, the concentration of DNA–uracil substrate (1 lesion per plasmid) in our studies is only approximately 3 nM, while the K_M for UNG2 is approximately 1000-fold higher [9]. The substrate concentration is therefore far below saturating conditions, but in spite of this UNG2 is a major rate-limiting factor in overall BER of uracil in DNA. Our results also indicate that, at least for proliferating human cells, measurements of UDG-activity

on DNA nick-translated in the presence of ^{3}H-labelled dUTP, in which U is found in a U:A context, is a good biomarker for the capacity of a cell to repair uracil in DNA. As mentioned above, other studies have proposed removal of 5′dRP by Pol β [25] or ligation [26] as the rate-limiting step in mammalian BER. However, in the first study [25], BER was reconstituted using equimolar amounts of recombinant BER proteins, which may not resemble physiological conditions. In the second study the initial steps were not examined [26]. A third study using extracts from male germ cells demonstrated that uracil–DNA glycosylase was rate-limiting for BER in extracts from young mice, but not from old. In contrast, APE1 was apparently rate-limiting in old mice, but not in young [35]. Thus, the rate-limiting step in BER may change with age, at least in male murine germ cells. In addition, the rate-limiting step may vary depending on the type of lesion involved. The very strong correlation between uracil repair and the content/activity of UNG2 observed here indicates that in human cancer cells, the rate of uracil BER is controlled by the initiating glycosylase. In line with this, another study observed virtually no detectable repair intermediates during repair of uracil, 8-oxoguanine and hypoxanthine when examining complete repair [36]. This suggests that repair intermediates are very rapidly processed, and supports our data demonstrating that the rate-limiting step for repair of uracil resides in the first step of the BER pathway. This would seem to make sense, since constriction of repair at a later step could cause accumulation of repair intermediates that are more cytotoxic than the original lesion. This is thought to be the mechanism behind the cytotoxicity observed when glycosylases are highly overexpressed in the presence of agents which damage DNA, as demonstrated for N-methylpurine DNA glycosylase, 8-oxoguanine-DNA glycosylase 1 and human homologue of endonuclease III [37–39].

Generally, UNG2 initiates all U:A repair and the largest fraction of U:G repair. However, in the gastric carcinoma cell line AGS that contained very high TDG levels this glycosylase contributed to initiation of ∼30% of U:G repair, as demonstrated using neutralising antibodies. Even here, UNG2 was the major contributor to BER initiation (∼60%), while SMUG1 contributed far less (∼5%) to U:G repair. In other cell lines (SW480 with medium level of UNG2, medium level of TDG, and T-47D with high UNG2, medium TDG) the contribution of TDG and SMUG1 to initiation of U:G BER was roughly equal (5–10%), but small compared with UNG2 (∼90%). TDG has previously been thought to have a specialised or quantitatively minor role in U:G repair, due to the very low turnover number of the purified enzyme compared to UNG2 and SMUG1 [10,40], but here we find that it is quantitatively at least as important as SMUG1 in human cells. The catalytic turnover of TDG is strongly inhibited by binding to the product AP-site, but factors that displace TDG from the AP-site stimulate its turnover. Such factors include the cell cycle checkpoint complex Rad9-Rad1-Hus1 [41], the XPC-RAD23B protein complex [42], APE1 [43] and SUMOylation [31]. These factors may well contribute to increase the catalytic efficiency of TDG in the BER system employed here, and in intact cells. To our knowledge the present work is the first study to demonstrate a considerable contribution from TDG to BER of uracil in DNA, although U:G repair activity independent of UNG and SMUG1 has been reported [9,16]. In conclusion, UNG2 appears as the sole glyco-sylase initiating BER in a U:A context, and the major initiator of U:G repair, but if UNG2 is poorly expressed TDG and SMUG1 may significantly complement UNG2.

UNG2 has the highest level of expression in the S-phase [18], and TDG peaks in the G1-phase of the cell cycle [44]. However, since all cells were harvested during exponential growth (50–70% confluency) and there was no inverse correlation between the content of UNG2 and that of TDG among the cell lines, the low UNG2 and high TDG content in the AGS extract likely reflect an intrinsic property of the cell line. Furthermore, the low UDG-activity (measured on a U:A substrate) in AGS cells was also reported in a previous study [45] and reproduced in independent experiments here. It was reported that TDG is 12–300-fold more active on U:G than on T:G mispairs, depending on surrounding sequence context [46]. Considering this result and data on cell cycle expression [44], TDG may well represent a major activity for repair of U:G mispairs outside the S-phase in human cells, a task that is likely to be shared by SMUG1, which is equally expressed in all phases of the cell cycle (unpublished data). UNG2 is present in BER complexes in replication foci during S-phase [2,14] and also probably in pre-assembled BER complexes [16]. Rapid post-replicative removal of incorporated dUMP depends on functional UNG2-activity [2,32]. In addition, the rate of removal of uracil in U:A pairs by purified UNG2 [9] (and also from U:G and U in single-stranded DNA) is orders of magnitude higher than that of TDG [30] and SMUG1 [9]. UNG2 is therefore an ideal enzyme for removal of incorporated dUMP-residues at a speed keeping up with the movement of the replication fork. It is conceivable that complexes containing UNG2 may be evolutionary optimised to process uracil in one of the two main lesion contexts, while all three glycosylases contribute to U:G repair. Generation of U:G mispairs from deamination is largely independent of the cell cycle, and probably infrequent compared with dUMP incorporation [1]. U:G mispairs present in the S-phase may need to be repaired relatively rapidly to avoid mutations and UNG2 may be a good candidate for this task. In agreement with this, MEFs from *Ung* knockout mice display ∼5.2-fold increase in mutation rates, but SMUG1-deficient cells also show ∼2.4-fold increase. For both cell types G:C to A:T transition mutations were the most common changes observed. This strongly indicates that both UNG2 and SMUG1 contribute to U:G repair and that they are not redundant. One possible explanation for this could be that they act mainly in different cell cycle phases, which also fits with the additive, rather than synergistic, effect of deficiency in both glycosylases in MEFs [47]. Little is known about the repair phenotype of *Tdg* knockout mice, as they lose viability midway through the gestation period [48].

We also found that the content of DNA ligase III correlates significantly with U:G repair ($R^2 = 0.68$ and $P = 0.012$) and must therefore be considered as a possible rate-limiting factor in U:G repair as well. This idea has considerable biological merit, as several studies have implicated the XRCC1–DNA ligase III complex in BER [11,49,50]. However, if the rate-limiting step for U:G repair resided at the ligation step, one might have expected that this would be the case for AP:G and nAP:G repair as well, but it is not. In addition, if data from the AGS extract are excluded on the basis of its high TDG content, we find that the correlation between UDG-activity/UNG2 content and U:G repair is as strong as that observed for U:A ($R^2 = 0.85$ and

$P = 0.003$ for both, Table 3). Moreover, we did not observe accumulation of unligated fragments during U:G repair, nor did we find a strong correlation between the content of DNA ligase III and the proportion of ligated to unligated fragment ($R^2 = 0.16$, Fig. 1D). We did not observe correlation between the content of any other BER protein and repair, except in the case of APE1, which has a weak inverse correlation with U:A repair ($R^2 = 0.52$ and $P = 0.045$). Our failure to find correlation between the content of BER proteins and AP-site or nAP-site repair may have several explanations. The rate-limiting step of AP and nAP repair may be controlled by some factor not considered here, and/or the Western blot analysis may not reflect the content of functional protein in the extracts. For example 30% of cancers characterised to date, somewhat surprisingly, express variant forms of DNA polymerase β, some of which contain altered polymerase and dRPase activity [51,52]. Furthermore, Bcl-2 was recently shown to modulate the activity of APE1 [53], indicating that AP-site processing is a rather complex issue. In addition, numerous protein–protein interactions and post-translational modifications are known to occur in the BER pathway and may be important in AP-site processing [2,14–18]. One of the unexpected features observed here was that AP-sites (and uracil) were generally more efficiently repaired when opposite G compared to opposite A. This was apparently not due to the properties of APE1 since incision at AP:A and AP:G by purified APE1 (Fig. 5D) was equally efficient in both contexts. This has also been observed by others [34], albeit with the AP-site analogue tetrahydrofuran. It thus appears likely that other factors may contribute to more efficient repair of AP-sites opposite G in most extracts.

Cancer cells are reported to be genetically unstable [54], and our results from a relatively modest number of cell lines demonstrate a wide variation in DNA repair protein expression and DNA repair capacity. Because of this variation results from one cell line may not be extrapolated to cancer cells in general. It is likely that both too low expression, too high expression and unbalanced expression may contribute to genetic instability. Unbalanced expression may contribute to variable responses to cytostatic drugs, including resistance. Identification of factor(s) governing the rate of DNA repair in cancer cells may therefore improve diagnosis and treatment of cancer.

Conflicts of interest

None.

Acknowledgements

references

[1] S. Andersen, T. Heine, R. Sneve, I. Konig, H.E. Krokan, B. Epe, H. Nilsen, Incorporation of dUMP into DNA is a major source of spontaneous DNA damage, while excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts, Carcinogenesis 26 (2005) 547–555.

[2] M. Otterlei, E. Warbrick, T.A. Nagelhus, T. Haug, G. Slupphaug, M. Akbari, P.A. Aas, K. Steinsbekk, O. Bakke, H.E. Krokan, Post-replicative base excision repair in replication foci, EMBO J. 18 (1999) 3834–3844.

[3] B.C. Blount, M.M. Mack, C.M. Wehr, J.T. MacGregor, R.A. Hiatt, G. Wang, S.N. Wickramasinghe, R.B. Everson, B.N. Ames, Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 3290–3295.

[4] D.K. Rogstad, P. Liu, A. Burdzy, S.S. Lin, L.C. Sowers, Endogenous DNA lesions can inhibit the binding of the AP-1 (c-Jun) transcription factor, Biochemistry 41 (2002) 8093–8102.

[5] B. Kavli, M. Otterlei, G. Slupphaug, H.E. Krokan, Uracil in DNA—general mutagen, but normal intermediate in acquired immunity, DNA Repair (Amst) 6 (2007) 505–516.

[6] T. Lindahl, Instability and decay of the primary structure of DNA, Nature 362 (1993) 709–715.

[7] H. Nilsen, K.A. Haushalter, P. Robins, D.E. Barnes, G.L. Verdine, T. Lindahl, Excision of deaminated cytosine from the vertebrate genome: role of the SMUG1 uracil–DNA glycosylase, EMBO J. 20 (2001) 4278–4286.

[8] M. Akbari, M. Otterlei, J. Pena-Diaz, H.E. Krokan, Different organization of base excision repair of uracil in DNA in nuclei and mitochondria and selective upregulation of mitochondrial uracil–DNA glycosylase after oxidative stress, Neuroscience 145 (2007) 1201–1212.

[9] B. Kavli, O. Sundheim, M. Akbari, M. Otterlei, H. Nilsen, F. Skorpen, P.A. Aas, L. Hagen, H.E. Krokan, G. Slupphaug, 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, J. Biol. Chem. 277 (2002) 39926–39936.

[10] H.E. Krokan, F. Drablos, G. Slupphaug, Uracil in DNA—occurrence, consequences and repair, Oncogene 21 (2002) 8935–8948.

[11] Y. Kubota, R.A. Nash, A. Klungland, P. Schär, D.E. Barnes, T. Lindahl, Reconstitution of DNA base excision-repair with purified human proteins: interaction between DNA polymerase beta and the XRCC1 protein, EMBO J. 15 (1996) 6662–6670.

[12] Y. Matsumoto, K. Kim, J. Hurwitz, R. Gary, D.S. Levin, A.E. Tomkinson, M.S. Park, Reconstitution of proliferating cell nuclear antigen-dependent repair of apurinic/apyrimidinic sites with purified human proteins, J. Biol. Chem. 274 (1999) 33703–33708.

[13] B. Pascucci, M. Stucki, Z.O. Jonsson, E. Dogliotti, U. Hubscher, Long patch base excision repair with purified human proteins. DNA ligase I as patch size mediator for DNA polymerases delta and epsilon, J. Biol. Chem. 274 (1999) 33696–33702.

[14] E. Parlanti, G. Locatelli, G. Maga, E. Dogliotti, Human base excision repair complex is physically associated to DNA replication and cell cycle regulatory proteins, Nucleic Acids Res. 35 (2007) 1569–1577.

[15] K.H. Almeida, R.W. Sobol, A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification, DNA Repair (Amst) 6 (2007) 695–711.

[16] M. Akbari, M. Otterlei, J. Pena-Diaz, P.A. Aas, B. Kavli, N.B. Liabakk, L. Hagen, K. Imai, A. Durandy, G. Slupphaug, H.E. Krokan, Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells, Nucleic Acids Res. 32 (2004) 5486–5498.

[17] J. Fan, D.M. Wilson 3rd, Protein–protein interactions and posttranslational modifications in mammalian base excision repair, Free Radic. Biol. Med. 38 (2005) 1121–1138.

[18] L. Hagen, B. Kavli, M.M. Sousa, K. Torseth, N.B. Liabakk, O. Sundheim, J. Pena-Diaz, M. Otterlei, O. Hørning, O.N. Jensen, H.E. Krokan, G. Slupphaug, Cell cycle-specific UNG2 phosphorylations regulate protein turnover, activity and association with RPA, EMBO J. 27 (2008) 51–61.

[19] S.L. Allinson, I.I. Dianova, G.L. Dianov, Poly(ADP-ribose) polymerase in base excision repair: always engaged, but not essential for DNA damage processing, Acta Biochim. Pol. 50 (2003) 169–179.

[20] J. Zhou, J. Ahn, S.H. Wilson, C. Prives, A role for p53 in base excision repair, EMBO J. 20 (2001) 914–923.

[21] A. Das, I. Boldogh, J.W. Lee, J.A. Harrigan, M.L. Hegde, J. Piotrowski, N. de Souza Pinto, W. Ramos, M.M. Greenberg, T.K. Hazra, S. Mitra, V.A. Bohr, The human Werner syndrome protein stimulates repair of oxidative DNA base damage by the DNA glycosylase NEIL1, J. Biol. Chem. 282 (2007) 26591–26602.

[22] K. Kvaløy, H. Nilsen, K.S. Steinsbekk, A. Nedal, B. Monterotti, M. Akbari, H.E. Krokan, Sequence variation in the human uracil–DNA glycosylase (UNG) gene, Mutat. Res. 461 (2001) 325–338.

[23] H. Krokan, A. Haugen, B. Myrnes, P.H. Guddal, Repair of premutagenic DNA lesions in human fetal tissues: evidence for low levels of O6-methylguanine–DNA methyltransferase and uracil–DNA glycosylase activity in some tissues, Carcinogenesis 4 (1983) 1559–1564.

[24] B. Myrnes, K.E. Giercksky, H. Krokan, Interindividual variation in the activity of O6-methyl guanine–DNA methyltransferase and uracil–DNA glycosylase in human organs, Carcinogenesis 4 (1983) 1565–1568.

[25] D.K. Srivastava, B.J. Berg, R. Prasad, J.T. Molina, W.A. Beard, A.E. Tomkinson, S.H. Wilson, Mammalian abasic site base excision repair.Identification of the reaction sequence and rate-determining steps, J. Biol. Chem. 273 (1998) 21203–21209.

[26] S.L. Oei, M. Ziegler, ATP for the DNA ligation step in base excision repair is generated from poly-(ADP-ribose), J. Biol. Chem. 275 (2000) 23234–23239.

[27] G. Slupphaug, I. Eftedal, B. Kavli, S. Bharati, N.M. Helle, T. Haug, D.W. Levine, H.E. Krokan, Properties of a recombinant human uracil–DNA glycosylase from the UNG gene and evidence that UNG encodes the major uracil–DNA glycosylase, Biochemistry 34 (1995) 128–138.

[28] G. Frosina, E. Cappelli, P. Fortini, E. Dogliotti, In vitro base excision repair assay using mammalian cell extracts, Methods Mol. Biol. 113 (1999) 301–315.

[29] H. Nilsen, S.P. Yazdankhah, I. Eftedal, H.E. Krokan, Sequence specificity for removal of uracil from U:A pairs and U:G mismatches by uracil–DNA glycosylase from Escherichia coli, and correlation with mutational hotspots, FEBS Lett. 362 (1995) 205–209.

[30] P. Neddermann, P. Gallinari, T. Lettieri, D. Schmid, O. Truong, J.J. Hsuan, K. Wiebauer, J. Jiricny, Cloning and expression of human G/T mismatch-specific thymine–DNA glycosylase, J. Biol. Chem. 271 (1996) 12767–12774.

[31] U. Hardeland, R. Steinacher, J. Jiricny, P. Schär, Modification of the human thymine–DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover, EMBO J. 21 (2002) 1456–1464.

[32] H. Nilsen, I. Rosewell, P. Robins, C.F. Skjelbred, S. Andersen, G. Slupphaug, G. Daly, H.E. Krokan, T. Lindahl, D.E. Barnes, Uracil–DNA glycosylase (UNG)-deficient mice reveal a primary role of the enzyme during DNA replication, Mol. Cells 5 (2000) 1059–1065.

[33] M.Z. Hadi, K. Ginalski, L.H. Nguyen, D.M. Wilson 3rd, Determinants in nuclease specificity of Ape1 and Ape2, human homologues of Escherichia coli exonuclease III, J. Mol. Biol. 316 (2002) 853–866.

[34] D.M. Wilson 3rd, M. Takeshita, A.P. Grollman, B. Demple, Incision activity of human apurinic endonuclease (Ape) at abasic site analogs in DNA, J. Biol. Chem. 270 (1995) 16002–16007.

[35] G.W. Intano, C.A. McMahan, J.R. McCarrey, R.B. Walter, A.E. McKenna, Y. Matsumoto, M.A. MacInnes, D.J. Chen, C.A. Walter, Base excision repair is limited by different proteins in male germ cell nuclear extracts prepared from young and old mice, Mol. Cell. Biol. 22 (2002) 2410–2418.

[36] S.L. Allinson, K.M. Sleeth, G.E. Matthewman, G.L. Dianov, Orchestration of base excision repair by controlling the rates of enzymatic activities, DNA Repair (Amst) 3 (2004) 23–31.

[37] M. Rinne, D. Caldwell, M.R. Kelley, Transient adenoviral N-methylpurine DNA glycosylase overexpression imparts chemotherapeutic sensitivity to human breast cancer cells, Mol. Cancer Ther. 3 (2004) 955–967.

[38] M.L. Rinne, Y. He, B.F. Pachkowski, J. Nakamura, M.R. Kelley, N-Methylpurine DNA glycosylase overexpression increases alkylation sensitivity by rapidly removing non-toxic 7-methylguanine adducts, Nucleic Acids Res. 33 (2005) 2859–2867.

[39] N. Yang, H. Galick, S.S. Wallace, Attempted base excision repair of ionizing radiation damage in human lymphoblastoid cells produces lethal and mutagenic double strand breaks, DNA Repair (Amst) 3 (2004) 1323–1334.

[40] U. Hardeland, M. Bentele, T. Lettieri, R. Steinacher, J. Jiricny, P. Schär, Thymine DNA glycosylase, Prog. Nucleic Acid Res. Mol. Biol. 68 (2001) 235–253.

[41] X. Guan, A. Madabushi, D.Y. Chang, M.E. Fitzgerald, G. Shi, A.C. Drohat, A.L. Lu, The human checkpoint sensor Rad9–Rad1–Hus1 interacts with and stimulates DNA repair enzyme TDG glycosylase, Nucleic Acids Res. 35 (2007) 6207–6218.

[42] Y. Shimizu, S. Iwai, F. Hanaoka, K. Sugasawa, Xeroderma pigmentosum group C protein interacts physically and functionally with thymine DNA glycosylase, EMBO J. 22 (2003) 164–173.

[43] T.R. Waters, P. Gallinari, J. Jiricny, P.F. Swann, Human thymine DNA glycosylase binds to apurinic sites in DNA but is displaced by human apurinic endonuclease 1, J. Biol. Chem. 274 (1999) 67–74.

[44] U. Hardeland, C. Kunz, F. Focke, M. Szadkowski, P. Schär, Cell cycle regulation as a mechanism for functional separation of the apparently redundant uracil DNA glycosylases TDG and UNG2, Nucleic Acids Res. 35 (2007) 3859–3867.

[45] K. Kvaloy, H. Nilsen, K.S. Steinsbekk, A. Nedal, B. Monterotti, M. Akbari, H.E. Krokan, Sequence variation in the human uracil–DNA glycosylase (UNG) gene, Mutat. Res. 461 (2001) 325–338.

[46] M.T. Morgan, M.T. Bennett, A.C. Drohat, Excision of 5-halogenated uracils by human thymine DNA glycosylase. Robust activity for DNA contexts other than CpG, J. Biol. Chem. 282 (2007) 27578–27586.

[47] Q. An, P. Robins, T. Lindahl, D.E. Barnes, C→T mutagenesis and gamma-radiation sensitivity due to deficiency in the Smug1 and Ung DNA glycosylases, EMBO J. 24 (2005) 2205–2213.

[48] D. Cortazar, C. Kunz, Y. Saito, R. Steinacher, P. Schär, The enigmatic thymine DNA glycosylase, DNA Repair (Amst) 6 (2007) 489–504.

[49] G.L. Dianov, K.M. Sleeth, I.I. Dianova, S.L. Allinson, Repair of abasic sites in DNA, Mutat. Res. 531 (2003) 157–163.

[50] E. Cappelli, R. Taylor, M. Cevasco, A. Abbondandolo, K. Caldecott, G. Frosina, Involvement of XRCC1 and DNA ligase III gene products in DNA base excision repair, J. Biol. Chem. 272 (1997) 23970–23975.

[51] S. Dalal, A. Chikova, J. Jaeger, J.B. Sweasy, The Leu22Pro tumor-associated variant of DNA polymerase beta is dRP lyase deficient, Nucleic Acids Res. 36 (2008) 411–422.

[52] D. Starcevic, S. Dalal, J.B. Sweasy, Is there a link between DNA polymerase beta and cancer? Cell Cycle 3 (2004) 998–1001.

[53] J. Zhao, F. Gao, Y. Zhang, K. Wei, Y. Liu, X. Deng, Bcl2 Inhibits Abasic Site Repair by Down-regulating APE1 Endonuclease Activity, J. Biol. Chem. 283 (2008) 9925–9932.

[54] J.H. Bielas, K.R. Loeb, B.P. Rubin, L.D. True, L.A. Loeb, Human cancers express a mutator phenotype, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 18238–18242.

Paper III

Cytotoxicity of 5-fluoropyrimidines is mainly through RNA incorporation and thymidylate synthase inhibition rather than DNA fragmentation from DNA excision repair

Henrik Sahlin Pettersen*, Torkild Visnes*, Cathrine Broberg Vågbø, Berit Doseth, Bodil M. Kavli and Hans E. Krokan

* Authors contributed equally

Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, N-7489 Trondheim, Norway

Corresponding author:

Hans E. Krokan

Department of Cancer Research and Molecular Medicine, NTNU, Erling Skjalgssons gate 1, N-7489 Trondheim, Norway

Fax: +47 2576400

Phone: +47 2573074

E-mail address: hans.krokan@ntnu.no

ABSTRACT

The cytotoxicity of 5-fluorouracil (5-FU) is thought to be mediated via thymidylate synthase inhibition by 5-FdUMP, and by incorporation of 5-FdUTP and 5-FUTP into DNA and RNA, respectively. Recently, cytotoxicity due to repair of 5-FU-containing DNA and subsequent DNA fragmentation has received considerable attention. This may involve mismatch repair (MMR) and base excision repair (BER) initiated by uracil-DNA glycosylases UNG2, SMUG1, TDG and MBD4, but their relative significance has not been examined. In extracts from human cancer cells (HeLa, SW480), we find that only BER repairs 5-FU:A, while BER and MMR both repair 5-FU:G. The major mechanism *in vitro* is BER initiated by UNG2. However, cytotoxicity was neither affected by siRNA-knock-down of either glycosylase, nor by inhibition of the common steps in BER. Furthermore, accumulation of 5-FU was ~3000-fold higher in RNA than in DNA in 5-FU treated cells. Although the mechanisms contributing to cytotoxicity were different for 5-FU, 5-F(rU) and 5-F(dU), reversal experiments by dT, dU and rU indicated that cytotoxic effects of fluoropyrimidines are mainly attributed to RNA effects and thymidylate synthase inhibition. BER apparently has a minor role in cytotoxicity and DNA repair by MMR is limited to a 5-FU:G context in human cancer cells.

INTRODUCTION

5-fluorouracil (5-FU) is one of the most widely used drugs in the treatment of cancer, including breast cancer, colorectal cancer and other gastrointestinal malignancies. It has been used as an anti-cancer drug for five decades, and presently some 2 million patients are treated each year. However, for approximately one-half of the patients given 5-FU based combination therapies the treatment has no positive effect, highlighting the need for more knowledge of its complex mechanism of action. 5-FU is a prodrug that is converted to several active metabolites that are thought to mediate cytotoxicity directly and indirectly by interfering with RNA and DNA functions. (Fig. 1) (1). Incorporation of 5-fluorouridine triphosphate (5-FUTP) into RNA causes disruption of rRNAs (2,3), tRNAs (4) and snRNA processing (5) and inhibits the conversion of uridine to pseudouridine in RNA (6). DNA metabolism is perturbed by 5-fluorodeoxyuridine monophosphate (5-FdUMP), which inhibits thymidylate synthase (TS) and thereby *de novo* synthesis of dTMP, resulting in dTTP deficiency, imbalanced nucleotide pools, and an increased incorporation of dUTP and 5-FdUTP into DNA (7).

Genomic uracil and 5-fluorouracil are repaired by base excision repair (BER) initiated by DNA-glycosylases. Purified recombinant forms of all four known human uracil-DNA glycosylases are able to excise 5-FU from DNA *in vitro*. Uracil-DNA glycosylase (UNG2), Single-strand selective monofunctional uracil-DNA glycosylase 1 (SMUG1) and thymine-DNA glycosylase (TDG) may all excise 5-FU in 5-FU:A- and 5-FU:G contexts, while Methyl-binding domain 4 protein (MBD4) is active only on 5-FU:G (8-11). In addition, mismatch repair (MMR) can process 5-FU:G in a nicked plasmid *in vitro*, and has been suggested to be able to repair 5-FU:A base pairs as well (12). However, the quantitative contribution from MMR and BER, as well as the possible role of individual DNA glycosylases, remain obscure.

Deficiency in DNA repair is associated with tolerance to 5-FU in several cell systems, indeed suggesting a role of DNA-repair in cytotoxicity. Accumulated repair intermediates in BER, such as abasic sites and cleaved DNA strands, are more cytotoxic than the original base lesion, and may therefore contribute to cell killing (13). Furthermore, synthesis of long repair tracts during MMR may be cytotoxic and mutagenic in cells having imbalanced and dTTP-depleted nucleotide pools (7,14). MMR may also act as a DNA damage sensor, inducing a rapid G2 arrest following 5-F(dU) treatment (15). A 5-FU-tolerant phenotype has been reported for both human and murine cells deficient in MMR (15,16). The evidence linking BER to fluoropyrimidine cytotoxicity is more ambiguous. Mouse embryonic fibroblasts (MEFs) derived from gene-targeted knockouts of the genes encoding TDG, MBD4 and DNA polymerase β did show an increased tolerance to fluoropyrimidines (17-20). In contrast, siRNA knock-down of *Smug1* in MEFs reduced the tolerance to fluoropyrimidines while *Ung*$^{-/-}$ MEFs displayed essentially identical sensitivity to fluoropyrimidines as wild type (11,21). This was also the case for human cells expressing the UNG-specific inhibitor Ugi, and down-regulation of human DNA polymerase β had no effect on 5-FU cytotoxicity (11,22). One open question is whether MEF knockout cells, yeast mutants, and indeed individual human cancer cell lines in culture, are good models to study the mechanism of 5-FU in human cancer, since they may convert this prodrug to different levels of active metabolites.

In this paper we analyse the relative contribution of the BER- and MMR pathways to 5-FU DNA-repair in extracts of human cancer cells and intact cells. Furthermore, we determine the relative efficiency of each DNA glycosylase in initiating BER of 5-FU in DNA. In addition, we investigate the effect on cytotoxicity of BER inhibitors and down-regulation of individual DNA glycosylases by siRNA. Finally, we examine the ability of deoxy- and ribonucleosides

to reverse the effect of 5-fluoropyrimidines, and the incorporation of 5-FU into DNA and RNA. We find that BER is not likely to be a major factor mediating toxicity, and a role for DNA fragmentation to MMR would be initiated through a 5-FU:A context. Our results also suggest that cytotoxic mechanisms involving perturbation of RNA functions and TS inhibition hold up as major contributors to toxicity of 5-FU and its metabolites in human cancer cells.

MATERIALS AND METHODS

Cell lines, chemicals and enzymes

The human cancer cell lines HeLa S3 (cervix adenocarcinoma), SW480 (colon adenocarcinoma) and CX-1 (colon adenocarcinoma) were purchased from ATCC and cultured in DMEM (4500 mg/l glucose) with 10% FCS, 0.03% L-glutamine, 0.1 mg/ml gentamicin and 2.3 µg/ml fungizone at 37°C and 5% CO_2. MEFs were cultured as described (21). 5-FU, 5-F(dU), 5-F(rU), 5-hm(dU), methoxyamine, 4-amino-1,8-naphthalimide, nucleosides and oligodeoxynucleotides were from Sigma-Aldrich. siRNA targeting *UNG* (Assay ID: 36376), *SMUG1* (AM16708A, ID: 21193, 140141, 21109) and *TDG* (Assay ID: 12923) were purchased from Ambion. Radionucleotides were from Perkin-Elmer. Restriction endonucleases were from New England Biolabs. Recombinant human His-tagged APE1, UNG2, SMUG1 and TDG were purified as described (9,23).

Preparation of nuclear extracts

Cultured cells were harvested at 50-70% confluency by trypsination, followed by washing in ice-cold PBS. Nuclear extracts were prepared as described (24). Protein concentrations were measured using the Bradford method (BioRad).

Combined MMR and BER assay

To generate a substrate for both BER and MMR a unique Nt.BbvCI site was introduced into the substrate plasmid (pGEM-3Zf+) at position 388 using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) protocol. This allowed the introduction of a nick that serves as a strand distinguishing signal for MMR. Substrates for BER and MMR containing 5-FU opposite A or G in otherwise identical sequence contexts were then prepared essentially as described (24).

Substrate (300 ng cccDNA pretreated with 5 U Nt.BbvCI when indicated) were incubated with 40 µg nuclear extract (TDG depleted and preincubated with Ugi and neutralizing SMUG1 antibodies when indicated) in BER buffer (40 mM HEPES-KOH pH 7.8, 70 mM (or 110mM) KCl, 5 mM MgCl$_2$, 0.5 mM DTT, 250 µM NAD$^+$, 2 mM ATP, 50 µM of each dNTP, 4.4 mM phosphocreatine, 2.5 µg creatine phosphokinase) at 37°C for the times indicated. The reactions were stopped by addition of 25 mM EDTA , 0.5% SDS, 150 µg/µl proteinase K and incubation at 55°C for 30 min. DNA was purified by phenol-chloroform extraction and ethanol precipitation with 10 µg glycogen as carrier. DNA was then treated with purified recombinant UNG (0.1 µg/µl)(25) (U- and 5-FU-substrates) or purified human TDG (0.5 µg/µl) (T:G substrates), as well as 50 mM methoxyamine (MX) and 0.2 µg/µl RNaseA (NEB buffer 2 +0.1 µg/µl BSA) for one hour at 37°C, followed by treatment with restriction endonucleases XmnI and HincII (5U each) for one hour. Restriction fragments were analysed on 2% agarose gels, stained with ethidium bromide and band intensities were quantified using ImageJ software (http://rsb.info.nlh.gov/ij/).

DNA-glycosylase activity assays

5-FU- and 5-hmU-DNA excision activities were measured using a 22-mer oligonucleotide containing a centrally positioned modified base (5'GATCCTCTAGAGT-X-GACCTGCA-3', where X = 5-FU, 5-hmU or U). The oligonucleotides were labelled on the 5' end with FAM or ^{33}P. Double-stranded substrates were prepared by annealing the labelled strand to a complementary strand containing either A or G opposite the modified base. Base excision activity was measured in a an assay mixture containing 5 µg nuclear extract (or 10 µg total extract or various amount of recombinant human His-tagged UNG2, SMUG1, TDG), 0.1 pmol oligonucleotide substrate, 20mM Tris-HCl pH 7.5, 50 mM NaCl, 1mM EDTA, 1mM DDT, 0.5 mg/ml BSA (UDG buffer) and 0.1 pmol recombinant human APE1 at 37°C for 30

min. The extracts were pre-incubated on ice with 0.1 µg UGI, 0.1 µg neutralizing SMUG1 antibody (PSM1) (8) and 1 µl anti-TDG antiserum (diluted 1:3) (26) when indicated. The reactions were stopped and analyzed as previously described (8).

UDG activity assays using [^{3}H]-labelled calf thymus DNA substrates (U:A substrate) were performed in UDG buffer with 3 µg whole cell extract at 30°C for 10 min essentially as described (8)

BER incorporation assay

The assay were performed as described (24). Briefly, 300 ng cccDNA substrate and 10 µg nuclear extract were incubated in BER buffer, supplemented with 50 nM dCTP and 3 µCi [α^{33}P]dCTP for substrates containing 5-FU and 5-hmU opposite G, and 50 nM dTTP and 3µCi [α^{33}P]dTTP for substrates containing 5-FU opposite A. Ugi (0.1 µg), neutralizing SMUG1 antibody (0.1 µg) and 1 µl anti-TDG antiserum (diluted 1:3) where added when indicated. For PARP-1 inhibition increasing concentrations of 4-amino-1,8-naphtalimide (4-AN) were included for the indicated times. Reactions were stopped by addition of EDTA (25 mM), SDS (0.5%) and proteinase K (150 µg/µl). DNA was purified and treated with restriction endonucleases XbaI and HincII to release radiolabelled fragments.

AP-site incision assay

An AP-site substrate was generated by incubating 0.2 µM (20 pmol) 5'FAM-labelled 19mer double-stranded uracil containing oligonucleotide (U141A) (8) with 5 ng/µl (0.5 µg) purified recombinant UNG (25) in 100 µl UDG buffer at 30ºC for 20 minutes Subsequently, 30 ng/µl (0.3 µg) Ugi was added to inactivate the glycosylase. Methoxyamine-modified AP-sites were generated by incubating AP-site substrate (0.2 pmol) in 0.5, 5 and 50 mM MX pH 7.2 (adjusted with NaOH) or corresponding concentrations of NaCl for 20 minutes at 30ºC. AP-

site cleavage assays was performed using 100 fmol purified human AP endonuclease 1 (27) for 10 minutes at 30ºC in 10 µl UDG buffer, supplemented with 7.5 mM $MgCl_2$. Reactions were stopped by adding 15 µl 95 % formamide.

Transfection with siRNA

160000 cells per well were plated in a 6 well dish in 1600 µl antibiotic free DMEM (4500 mg/l glucose) with 10% FCS and 0.03% L-glutamine, and cultured over night. siRNA targeting SMUG1 (a mix of three, final concentration 30 nM each), UNG (60 nM final), and TDG (60 nM final) was dissolved in OptiMEM (Invitrogen) and incubated with 4 µl/well Dharmafect transfection agent (Dharmacon) for 20 min, before adding the mixture to the culture, according to the manufacturers protocol. After 24 h cells were treated with trypsin, counted and replated in medium with antibiotics.

Preparation of whole cell extracts from siRNA transfected cells

Cells from 6 well dishes were harvested by trypsination 48 h post transfection. Cell pellets were dissolved in 100 µl buffer containing 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1xComplete protease inhibitor (Roche), and 0,5 % NP-40 and sonicated for 3 x 30 seconds at 4ºC. Cell debris was removed by centrifugation at 13000 x *g* for 15 min. Supernatants were snap-frozen in liquid nitrogen and stored at -80°C.

Western analysis

50-100 µg whole cell extract were treated with DNase and RNase for 10 min at room temperature, denatured at 70°C in LPS loading buffer, separated on the NuPage electrophoresis system (Invitrogen) and electro-blotted onto Immobilon PVDF membranes (Millipore). UNG was detected using the polyclonal UNG antibody PU059 (25), SMUG1 by

the polyclonal SMUG1 antibody PSM1 (8), TDG by hTDG-antiserum (26), and β-actin was detected using mouse monoclonal ab8226 (AbCam). The membranes were analyzed using HRP swine anti-rabbit (1:5000; DakoCytomation) and HRP rabbit anti-mouse (1:5000, DakoCytomation) secondary antibodies and Super Signal West Femto substrate (Pierce) on a KODAK Image Station 2000R.

Cytotoxicity assays

2000-4000 cells/well were plated on a 96 well plate in complete DMEM (10 % FBS, L-glutamine, gentamicin, and amphotericin B). Cells were exposed to the cytostatic drugs and nucleosides 24 hours after plating and cultured for further 96 hours. Living cells were then quantified using the MTT-assay. Growth medium was replaced with 100 µl fresh medium containing 0.5 mg/ml MTT, and the plates were further incubated at 37°C for 4 h. 50 µl of medium was subsequently removed, and 100 µl 2-propanol with HCl (0.1 M) were added. Plates were transferred to a mechanical shaker until the MTT-formazan was dissolved. The optical density of each well was read on a Titertek Multiscan Plus Reader at wavelength 588 nm.

FACS analysis of cell cycle

Cells were plated in a 6-well dish at approximately 25 % confluence and grown for 24 h followed by exposure to cytostatic drugs for 48 hours. Cells were harvested by trypsination, fixed by 70 % methanol and washed twice with PBS. Then cells were treated with 50 µl RNaseA (100 µg/ml in PBS) at 37 ºC for 30 min before DNA staining using 200 µl propidium iodide (50 µg/ml in PBS) at 37ºC for 30 min. Cell cycle analyses were performed using a FACS Canto flow cytometer (BD-Life Science).

Thymidylate synthase assay

TS activity was measured as previously described (28) with minor modifications. Cells were seeded in 24-well plates at a density of 70.000 cells per well. Following overnight incubation, each well was treated with fluoropyrimidines, and/or varying concentrations of rU diluted in growth medium for one hour, followed by the addition of 1 µCi [5-^{3}H]deoxyuridine (specific activity 20 Ci/mmol, Moravek Biochemicals Inc.) and incubated for 90 min, in a final volume of 500 µl. The reaction was stopped by transferring 400 µl of the growth medium to an equal volume 150 mg/ml activated charcoal suspension in 5% trichloroacetic acid. The samples were vortexed and centrifuged at 16.000 x g at 4°C for 15 min or more. Radioactivity in a 400 µl aliquot of the supernatant was counted using a liquid scintillation counter, and each value was corrected for background counts.

Quantitation of 5-FU in DNA and RNA by LC/MS/MS

Nucleic acids were isolated from fluoropyrimidine-treated cells by the Blood and cell culture mini DNA isolation kit (Qiagen) and by the mirVana RNA-isolation kit (Ambion). The DNA or RNA samples were enzymatically hydrolyzed to nucleosides using nuclease P1, phosphodiesterase I from Crotalus adamanteus venom, and alkaline phosphatase (all from Sigma-Aldrich) as described (29), followed by addition of 3 vol of methanol and centrifugation (16000 × g, 30 min). The supernatants were dried under vacuum and the resulting residues dissolved in 50 µl 5% methanol in water (v/v) for analysis of 5-F(dU) and 5-F(rU) by LC/MS/MS. A portion of each sample was diluted for the quantitation of the unmodified nucleosides (dA, dC, dG, dT, rA, rC, rG, and rU). Chromatographic separation of nucleosides was performed on a Shimadzu Prominence HPLC system with a Zorbax SB-C18 2.1x150 mm i.d. (3.5 µm) reverse phase column equipped with an Eclipse XDB-C8 2.1x12.5 mm i.d. (5 µm) guard column (all from Agilent Technologies), at ambient temperature and a

flow rate of 0.2 ml/min. For 5-F(dU) and 5-F(rU) separation the mobile phase consisted of water and methanol, starting with a 3.5-min linear gradient of 5-70% methanol, followed by 1 min with 70% methanol and 6.5 min re-equilibration with the initial mobile phase conditions. Chromatography of unmodified nucleosides was performed under isocratic conditions with water/methanol/formic acid in ratio 85/15/0.1% for deoxyribonucleosides, or 92/8/0.1% for ribonucleosides. Online mass spectrometry detection was performed using an Applied Biosystems/MDS Sciex 5000 triple quadrupole mass spectrometer (Applied Biosystems) with TurboIonSpray probe operating in negative electrospray ionization mode for 5-F(dU) and 5-F(rU), or positive electrospray ionization mode for unmodified nucleosides. LC/MS/MS chromatograms showing 5-F(dU) in DNA and 5-F(rU) in RNA hydrolysates are shown in the supplementary figure 1. The nucleosides were monitored by multiple reaction monitoring using the mass transitions 245.2→129.1 (5-F(dU)), 261.2→129.1 (5-F(rU), 252.2→136.1 (dA), 228.2→112.1 (dC), 268.2→152.1 (dG), 243.2→127.1 (dT), 268.2→136.1 (rA), 244.2→112.1 (rC), 284.2→152.2 (rG), and 245.2→113.1 (rU). Quantitation was accomplished by comparison with pure nucleoside standards run intermediate the samples.

RESULTS

BER is the dominant pathway for repair of genomic 5-FU in human cancer cell lines *in*

***vitro*, and contribution by MMR is limited to a 5-FU:G context**

In vitro studies suggest that both BER and MMR contribute to repair 5-FU in DNA. However, their relative contribution has not been established (12). We examined the contribution of BER and MMR to repair of 5-FU:A and 5-FU:G in DNA in nuclear extracts from human cancer cell lines. For this purpose we employed an *in vitro* assay using a circular covalently closed DNA substrate (cccDNA) containing a single 5-FU at a defined position, as well as a unique recognition sequence for the nicking endonuclease Nt.BbvCI positioned at 298 bp 3' to the lesion (Fig. 2A). Any mismatch in the plasmid can be repaired by the MMR system when the plasmid is nicked (12). Distinction between substrate and product in *in vitro* MMR assays has traditionally been obtained using restriction endonucleases unable to cut mismatched recognition sequences. To study MMR-mediated 5-FU:A repair by this approach is not straight-forward, because restriction endonucleases that discriminate between 5-FU:A in the substrate from T:A in the product are not available (12). Thus, we exploited the fact that restriction endonuclease HincII (GTY^RAC, Y=C/T, R= A/G) is unable to digest a recognition sequence containing a centrally positioned abasic site (AP-site). By treating the incubated substrate DNA with a DNA glycosylase that excises the damaged base prior to HincII digestion, all unrepaired substrate is converted to non-cleavable substrate (due to the AP-site in the HincII recognition site). If the lesion is repaired, however, it will not be recognized by the glycosylase, thus the product (T:A or C:G in the HincII recognition site) will be cleaved by HincII (Fig. 2B). To validate the assay we used nuclear extract from SW480 and verified repair of a T:G mismatch in a nick-dependent manner, consistent with an active MMR system. The same extract under identical assay conditions also carried out repair of cccDNA-U:A substrate, but this process was completely inhibited by the UNG inhibitor

Ugi, consistent with BER initiated by UNG (Fig. 2C). Thus, these substrates can be used to measure both BER and MMR. One or both pathways can be specifically inactivated; BER by directly inhibiting the initiating glycosylase, and MMR by removing the strand-break that repair is critically dependent on. We then measured repair in nuclear extracts from SW480 and HeLa on a cccDNA-5-FU:A substrate. Repair of 5-FU:A was completely inhibited by the presence of the UNG2 inhibitor Ugi and anti-SMUG1 antibodies in extract from both cell lines. This was also the case for the nicked substrate, with no detectable repair after 60 min in the presence of Ugi and neutralizing SMUG1 antibodies, both at 70 mM KCl (Fig. 2D) and 110 mM KCl (data not shown). This indicates that BER is the main, possibly sole, pathway for repair of 5-FU:A. Surprisingly, we could not detect any contribution from TDG on 5-FU:A repair in either extract. The repair of 5-FU:G was also mainly performed by BER, as most of the cccDNA 5-FU:G substrate was repaired after 15 min whether it was nicked or not (Fig. 2E). Inhibition of BER by the addition of Ugi and anti-SMUG1 antibody to a TDG-depleted extract (12) inhibited all detectable repair of 5-FU:G (un-nicked) substrate, while a marked reduction was observed when using nicked substrate. These results indicate a dominant role for BER in repair of 5-FU in DNA, with a smaller contribution of MMR to 5-FU:G repair.

DNA repair of 5-FU in human cancer cells is predominantly initiated by UNG2, while SMUG1 and TDG are more important in mouse embryonic fibroblasts

Purified recombinant UNG2, SMUG1, TDG and MBD4 have all been reported to excise 5-FU from DNA *in vitro* (8-10,12). However, their relative importance in 5-FU-DNA repair in different cells has so far not been investigated. We analysed the contribution of UNG2, SMUG1, and TDG to the excision of 5-FU from DNA in nuclear extracts from three human cancer cell lines (SW480, HeLa and CX-1) as well as in MEFs. Nuclear extracts were

incubated with duplex oligonucleotides with a central 5-FU paired with adenine (5-FU:A), guanine (5-FU:G), or as single-stranded DNA (5-FU). SMUG1 and TDG activities in the extracts were inhibited using neutralising antibodies as described (8,26), while UNG2 activity was inhibited with Ugi. Note that MBD4 did not appear to be significantly involved quantitatively, as inhibition of UNG2, SMUG1 and TDG was sufficient to abolish essentially all measurable 5-FU excision in the extracts (Fig. 3A, line 5). UNG2 was the dominant activity with all the 5-FU substrates in extracts from human cancer cell lines (SW480, HeLa and CX1), while SMUG1 and TDG activities were measurable only on the 5-FU:G substrate (Fig 3A). In contrast, 5-FU excision by UNG2 was hardly detectable in the MEF extract, where it was dependent mainly on SMUG1 and TDG. Moreover, excision activity in a 5-FU:A context and in 5-FU in single stranded substrate was very low (Fig. 3A). In accordance with this, measurements of UDG activity in the extracts using a ^{3}H-labelled calf thymus U:A-DNA substrate (which detects mainly UNG-activity) revealed very low UDG activity in the MEF extract compared to extracts of human cancer cells (Fig. 3B). This difference is interesting because results from 5-FU treatment of MEF cell systems are often assumed to be valid for human cells as well. We have, in fact, found that these differences apply to murine tumour cell lines as well (Doseth et al., unpublished data). To determine substrate preference and specific activity, experiments with purified recombinant human UNG2, SMUG1 and TDG were performed under identical conditions. The results confirmed that 5-FU is substrate for all three UDGs, with UNG2 as the most efficient enzyme on 5-FU:A and especially on 5-FU in a single-stranded context, while SMUG1 was the most efficient enzyme on 5-FU:G. As expected, TDG excised 5-FU efficiently from a 5-FU:G context (Fig. 3C), in accordance with the analysis of 5-FU-excision in nuclear extracts. A dominant role for UNG2 in BER of 5-FU:A was also substantiated by assays measuring complete BER incorporation assays in SW480 and HeLa extracts, since all detectable 5-FU:A repair activity was abolished when

Ugi was added to the nuclear extracts (Fig. 2D). On the other hand, UNG2, SMUG1 and TDG were all able to initiate 5-FU:G repair, although with varying efficiency.

Cellular sensitivity to fluoropyrimidines is not affected by siRNA knock-down of *UNG*, *SMUG1* or *TDG* and 5-FU accumulates abundantly in RNA compared with DNA

Our *in vitro* data from nuclear extracts suggested that 5-FU in DNA was predominantly repaired by BER, initiated by UNG2, SMUG1 or TDG. We therefore used specific siRNAs to examine the *in vivo* effects of these UDGs in mediating fluoropyrimidine cytotoxicity in SW480 and HeLa cells. To avoid selection bias and phenotypic drift, we employed transient siRNA-mediated silencing. UNG, SMUG1 and TDG activities were reduced at least 70-90% 48 h after transfection (Fig. 4A), and western blots verified knock-down at the protein level (Fig. 4B). Generally, the knock-down effect was stronges at 48 hrs after transfection and then gradually faded out towards 50 to 75 % residual activity after six days (data not shown). Thus, we exposed SW480 and HeLa cells to 5-FU and its metabolites 5-F(dU) and 5-F(rU) 48 h post transfection.

The DNA-directed effects of fluoropyrimidines would be analogous to the effect of 5-hydroxymethyl-2'-deoxyuridine (5-hm(dU)), which is incorporated into DNA, which in turn may lead to DNA strand breaks and apoptosis through excessive 5-hm(dU) excision by SMUG1 (8,30-32). Thus, since 5-hmdUMP does not inhibit thymidylate synthase (33), has no known RNA-directed effects, and is removed by a distinct DNA glycosylase, it constitutes an ideal positive control for the concept of DNA repair-directed cytotoxicity of fluoropyrimidines. We tested hmU-excision in nuclear extracts from SW480, HeLa and CX-1 and verified that SMUG1 was the only glycosylase having detectable activity on this substrate (Figure 4C). In accordance with this, SW480 *SMUG1*-knock down cells were more tolerant to

5-hm(dU) than control cells, while *UNG* and *TDG* knock downs had no effect on relative cytotoxicity after exposure to 5-hm(dU) (Fig. 5). These results demonstrate that BER can in principle enhance cytotoxicity of pyrimidine antimetabolites incorporated into DNA. HeLa tolerated very high doses of 5-hm(dU), but the *SMUG1* knock-down effect was still detected at high concentrations also in this cell line.

To differentiate between the different proposed cytotoxic mechanisms of 5-FU, we also exposed the knock-down cells to varying concentrations of 5-F(dU) and 5-F(rU) to predominantly induce DNA- and RNA-mediated cytotoxicity, respectively (Fig. 1). However, we were not able to observe any significant change in sensitivity to 5-FU, 5-F(dU) or 5-F(rU) in *UNG*, *SMUG1* or *TDG* knock down cells (Fig. 5).

Furthermore, while 5-hm(dU)-exposed cells were shifted from G1/S arrest towards G2/M arrest in *SMUG1* knock-down cells, the cell cycle profiles after 5-FU and 5-F(dU) treatment were unaffected by knock-down of the glycosylases (Fig. 6A). The G1/S cell cycle arrest induced by both 5-FU, 5-F(dU), and 5-F(rU) was completely reversed by thymidine, both in SW480 and HeLa cells (Fig. 6B), indicating that the G1/S cell cycle arrest is due to thymidine starvation (presumably induced via TS inhibition). Importantly, both 5-FU- and 5-F(dU)-treated cells contained measurable quantities of 5-FU in DNA. However, 5-FU was more abundant in RNA compared to DNA, both after 5-FU treatment (~2000-3000-fold) and surprisingly also after 5-F(dU) treatment (~6 fold) (Table 1).

5-FU, 5-F(dU) and 5-F(rU) inhibit TS with similar efficiency in HeLa and SW480 cells
To further elucidate how the different fluoropyrimidines mediates cytotoxicity, we measured the activity of TS in HeLa and SW480 in the presence of varying concentrations of 5-FU, 5-

F(dU) and 5-F(rU) (Fig. 7). 5-F(dU) was by far the most potent inhibitor, inhibiting 50% of the TS activity (IC_{50}) at 2.0 and 2.7 nM for HeLa and SW480, respectively. The IC_{50}-values for 5-F(rU) were 0.45 and 0.63 μM for HeLa and SW480, respectively, while the corresponding values for 5-FU were 98 and 32 μM. Thus, as 5-FU, 5-F(dU) and 5-F(rU) inhibit TS with a rather similar efficiency in HeLa cells and SW480 cells, it seems that TS inhibition is insufficient to explain the large variations in cytotoxicity observed in Fig. 5.

Inhibition of PARP-1 and AP-site processing does not affect 5-F(dU) cytotoxicity

Lack of detectable effects on fluoropyrimidine cytotoxicity after knock-down of the individual 5-FU-DNA glycosylases may be due to redundancy, in which the different DNA glycosylases may substitute for each other in the repair process. Thus, to further explore whether BER mediates 5-F(dU) cytotoxicity we employed inhibitors that target the common steps of BER. Methoxyamine (MX) reacts with and inhibits processing of AP-sites by APE1 (34) and 4-AN is a potent inhibitor of PARP-1 polyribosylation (35). These inhibitors have previously been shown to affect cytotoxicity mediated by BER (36,37). The effect of MX on AP-site cleavage was tested *in vitro* by hAPE1 activity measurements. We employed an AP-site-containing duplex oligonucleotide pre-treated for 20 min with pH adjusted MX (~1:1 molarity of MX-HCl and NaOH to pH 7), and used corresponding NaCl concentrations as controls. MX treatment of AP-sites clearly inhibited cleavage by APE1 in a concentration dependent manner (Fig. 8A). From the results in Fig. 8A we decided to use 20 mM MX in the following cell culture experiments. To verify the effect of 4-AN on BER *in vitro*, we utilized a BER incorporation assay with HmU opposite G in the cccDNA substrate. More than 50 % inhibition of BER was achieved in presence of 10 μM 4-AN (Fig. 8B).

We further tested MX and 4-AN *in vivo* by concurrent 5-hm(dU) and 5-F(dU) exposure of SW480 and HeLa cells. The cytotoxicity of MX and 4-AN treatment in itself had an approximately 10–20 % growth inhibitory effect on the cells, for which the data were normalized. The presence of 20 mM MX clearly enhanced the cytotoxicity of 5-hm(dU) in both SW480 and HeLa cells (Fig. 8C). Similarly, treatment with 10 μM 4-AN resulted in an enhanced 5-hm(dU) cytotoxicity at low concentrations. The effect of 4-AN was most pronounced in HeLa cells. At higher concentrations of 5-hm(dU) , 4-AN had a protective, rather than aggravating effect on cytotoxicity. Importantly, however, both MX and 4-AN failed to affect the cytotoxicity of 5-F(dU)-treated cells in any discernible way (Fig. 8D). These results indicate that BER mediates the cytotoxicity of 5-hm(dU) cytotoxicity, but not that of 5-F(dU).

Cytotoxicity of 5-FU, 5-F(dU) and 5-F(rU) is differentially reversed by dT, dU and rU, indicating quantitatively different mechanisms of action

Since BER did not affect fluoropyrimidine sensitivity significantly, we wanted to explore the mechanisms further by attempting to reverse cytotoxicity by ribonucleosides (rU) and deoxynucleosides (dT, dU). For these reversal experiments, we exposed HeLa- and SW480 cells to fixed concentrations of 5-F(dU), 5-F(rU) and 5-FU and varying concentrations of thymidine (dT), deoxyuridine (dU), and uridine (rU) for four days. The cytotoxic effect of 5-F(dU) was partially reversed by thymidine (dT), and to a lesser extent deoxyuridine (dU), but not uridine (rU). The effects were, however, significantly different in the two cell lines (Fig. 9A). The cytotoxicity of 5-F(rU) was reversed by rU in both cell lines, but not by dU or dT (Fig. 9B). However, 5-FU toxicity was only marginally reversed by rU, and not by dU or dT (Fig. 9C). This was somewhat unexpected since dT reversed the G1/S cell cycle arrest of cells treated with either 5-FU, 5-F(dU) or 5-F(rU) (Fig. 5B). Consistent with the above results,

addition of rU strongly reduced the content of 5-FU in RNA for cells exposed to 5-F(rU), but not after exposure to 5-FU (Table 2). Since ribonucleosides may be converted to deoxyribonucleotides, we also examined the possible effect of rU on 5-F(rU)-mediated TS inhibition. Here, rU had two apparent effects. First, addition of rU alone resulted in a small to moderate decrease in apparent TS activity, which might be explained by pool effects. Secondly, and more importantly, rU alleviated TS-inhibition by 5-F(rU) at much lower concentrations than those required to reverse cytotoxicity in both cell lines (Fig. 9D). Therefore, it appears that 5-F(rU) more likely mediates cytotoxicity through interfering with RNA-functions than by inhibiting TS, in agreement with in inability of dT to reverse 5-F(rU) cytotoxicity (Fig. 9B).

In summary, cytotoxicity of 5´-subsituted pyrimidines from BER processes is a plausible mechanism, as demonstrated here in the case of 5-hm(dU). However, our results also strongly indicate that BER is not a major contributor to cytotoxicity of 5´-fluoropyrimidines in human cancer cells (HeLa and SW480), and a possible role of MMR would most likely be limited to a 5-FU:G context. Cytotoxicity of these agents appears to be mainly be attributed to TS-inhibition and dTTP-deficiency for 5-F(dU) and incorporation into RNA for 5-F(rU) and 5-FU, rather than a consequence of excessive DNA repair.

DISCUSSION

5-FU has been in clinical use for half a century and although several cytotoxic mechanisms have been proposed for the drug, their relative significance in killing cancer cells is not well understood. Several papers report that cells deficient in BER or MMR are more resistant to 5-FU or 5-F(dU) than the wild type. However, the relative quantitative contributions of these repair pathways have not been investigated. In this paper we report that BER, rather than MMR, was the sole repair activity of 5-FU in a 5-FU:A context *in vitro*, and also the dominant activity on nicked 5-FU:G substrate in nuclear extracts from human cancer cell lines (Fig. 2D&E). This may appear to be in contrast to a previous study, which found that MMR-proficient extracts incorporated more radio-labelled deoxynucleotides into nicked 5-FU:A substrates than MMR-deficient (12). However, it does not necessarily follow from this that MMR is involved in 5-FU:A repair, especially as an undamaged control substrate was not included in the study. MMR did, however, repair 5-FU:G, although MMR, at least *in vitro*, proceeded at a far lower rate than BER, which repaired ~70% of the substrate after only 15 min. It should be kept in mind that the presence of a nick in DNA is crucial for strand-discrimination, and hence for the activity of MMR. Since multiple ligases may seal the nick directly, the amount of substrate available for MMR may decline rapidly during incubation. Consequently, the relative contribution from MMR may be underestimated using this assay. In addition, it should be underlined that we have not examined the contribution of MMR to 5-FU:G repair in intact cells. While several studies have demonstrated that MMR-deficient cells are more tolerant to fluoropyrimidines than MMR-proficient, the identity of the DNA lesion that provokes MMR is not known. Our results and the inability of MMR proteins to recognise 5-FU:A in gel shift assays (12,15), indicate that MMR may act on mismatches involving 5-FU:G or alternatively mismatches introduced by DNA polymerases due to dNTP pool imbalances during TS inhibition. It is important to note that even if we only observe a

relatively modest contribution from MMR to repair of 5-FU *in vitro*, this does not exclude that MMR mediates cytotoxicity of 5-FU *in vivo* or in the clinic. Some 10-15% of colon cancers are MMR-deficient due to inactivating mutations or epigenetic silencing (38). Clinically, these patients generally respond well to 5-FU treatment (39,40), although resistance to 5-FU has been reported in cancer cell lines deficient in the MMR damage recognition proteins MSH2 (16) and MLH1 (20,41). Thus, while we observe a quantitative modest role of MMR *in vitro*, our results do not exclude a significant involvement of MMR in the 5-FU response *in vivo*.

Our results clearly demonstrate that UNG2 is the dominant glycosylase activity initiating 5-FU:A repair, at least *in vitro*. Given that UNG2 is downregulated in G1 (and vice versa for TDG) (42,43), 5-FU:A that escapes repair in S-phase may not be efficiently processed in G1. As a consequence, aberrant bases may well be present in the template strand during the subsequent S-phase. 5-FU:G, on the other hand, may be repaired by UNG2 or SMUG1 in S-phase and TDG or SMUG1 in G1, alternatively by MMR. We were not able to measure any contribution from MBD4. Our *in vitro* experiments with 5-hm(dU) demonstrate that the DNA fragmentation-hypothesis involving excision repair is a feasible idea. Furthermore, given our *in vitro* results, and that 5-FU is mainly incorporated opposite A, fragmentation of DNA by excessive glycosylase activity would be a more likely candidate than MMR. We therefore tested this hypothesis using intact human cancer cell lines, in which the different DNA glycosylases had been knocked down using siRNA, using 5-hm(dU) as a positive control for BER-mediated cytotoxicity. Knock-down of *SMUG1* in cells exposed to 5-hm(dU) lead to a shift from G1/S to G2/M arrest and also reduced cytotoxicity. Furthermore, the cytotoxicity was modulated by BER inhibitors and reversed by the addition of thymidine to the medium. While this is consistent with a cytotoxic mechanism involving BER, the 5-hm(dU) results

were in stark contrast to those obtained with fluoropyrimidines. Here, the knock-down of individual glycosylases did not affect cytotoxicity or cell cycle arrest, and the addition of BER inhibitors had no apparent effect on cytotoxicity. Collectively, these experiments indicate that BER processes are neither substantially enhancing cytotoxicity due to DNA fragmentation, nor reducing cytotoxicity due to removal of 5-FU from DNA. Taken together, our results indicate that incorporation of a 5'-substituted thymine-analogue followed by excessive excision by a DNA glycosylase most likely explains the cytotoxicity of 5-hm(dU), but not that of fluoropyrimidines. The nucleoside reversal experiments suggest that TS-inhibition and RNA-incorporation are the most likely the dominant modes of cytotoxicity for fluoropyrimidines (Fig. 9).

These results may appear to be in conflict with results from MEFs demonstrating that resistance to 5-FU depends upon SMUG1, since SMUG1 knock-down increased sensitivity (11). However, our studies indicate that the divergent results may be explained by species differences, since we find that SMUG1 has a much more prominent role in removal of 5-FU in mouse cells, compared with human cells (Fig. 3A). Another apparent species and/or cell type difference was the observed ~3000:1 preferential incorporation of 5-FU into RNA in stead of DNA in HeLa (Table 1), compared to ~11:1 reported for wild type MEFs (11). Furthermore, $Tdg^{-/-}$ mouse embryonic stem cells and fibroblasts have an increased tolerance to 5-FU, while knock-down of endogenous levels of TDG in HeLa has a much smaller effect (19). Similarly, MEFs carrying gene-targeted disruptions in the gene encoding DNA polymerase β are more resistant to 5-FU compared to wild type (18), while knock-down of the human orthologue in two human cancer cell lines did not alter the sensitivity to 5-FU (22). It is likely that DNA repair may well mediate fluoropyrimidine cytotoxicity in MEFs, but these results may not be easily extended to human cancer cell lines. In contrast, silencing of *UNG* in

a human cervix cancer cell line (HeLa) was reported to increase resistance to 5-F(dU) treatment (17). Apart from technical differences in cell culture, incubation times and assay strategies, we find it hard to reconcile this result with ours. Nevertheless, the inclusion of a positive control in the form of 5-hm(dU) in our study lends strength to the argument that the contribution of BER-mediated cytotoxicity is rather modest in human cancer cells. The fact that the prodrug 5-FU is converted to several metabolites that may affect DNA and RNA transactions is most likely the basic reason for the apparently diverse mechanisms of action of the drug. There is little doubt that several of the proposed mechanisms may play a role under different conditions, but the problem is rather to identify the practical significance of each of them for therapy. Thus, the cytotoxic mechanism of 5-FU may vary between species, cell types and even individuals. It is certainly also different for various 5-fluoropyrimidines, as clearly demonstrated here. Consequently, the research literature is filled with conflicting reports, and the quantitative contribution of different RNA and DNA-related mechanisms of cytotoxicity remains elusive.

In summary, our results and other recent results allow the conclusion that the main mechanisms that mediate 5-FU cytotoxicity in human cells are TS-inhibition and RNA incorporation rather than BER of 5-FU. Our results do not exclude a role of MMR in toxicity of 5-FU, particularly since we have only carried out *in vitro* studies. Repair patches in MMR are long, frequently several thousand nucleotides, and may therefore be more sensitive to dNTP pool imbalances decreasing repair efficiency and increasing risks of polymerase errors introducing mismatches. The importance of TS-inhibition is further corroborated by the clinical success of combining 5-FU with leucovorin that stabilizes the TS:5-FdUMP complex (44). 5-FU, 5-F(dU) and 5-F(rU) inhibited TS with similar efficiencies in HeLa and SW480 (Fig. 7), but the these cell lines displayed very different sensitivities, especially to 5-F(dU),

(Fig. 5), indicating that additional factors modulate the cytotoxicity mediated by TS-inhibition. Interestingly, in a comprehensive drug activity gene expression study, 5-FU clustered with RNA synthesis inhibitors, suggesting that a major mechanism of action is RNA-directed (45). Finally, microarray profiling of 5-FU resistant cell lines tend not to find BER genes to be differentially regulated, as one might expect if BER were an important mediator of cytotoxicity (46-51).

ACKNOWLEDGEMENTS

FUNDING

REFERENCES

1. van Laar, J.A., Rustum, Y.M., Ackland, S.P., van Groeningen, C.J. and Peters, G.J. (1998) Comparison of 5-fluoro-2'-deoxyuridine with 5-fluorouracil and their role in the treatment of colorectal cancer. *Eur J Cancer*, **34**, 296-306.
2. Ghoshal, K. and Jacob, S.T. (1994) Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil. *Cancer Res*, **54**, 632-636.
3. Sun, X.X., Dai, M.S. and Lu, H. (2007) 5-fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction. *J Biol Chem*, **282**, 8052-8059.
4. Gustavsson, M. and Ronne, H. (2008) Evidence that tRNA modifying enzymes are important in vivo targets for 5-fluorouracil in yeast. *RNA*, **14**, 666-674.
5. Patton, J.R. (1993) Ribonucleoprotein particle assembly and modification of U2 small nuclear RNA containing 5-fluorouridine. *Biochemistry*, **32**, 8939-8944.
6. Samuelsson, T. (1991) Interactions of transfer RNA pseudouridine synthases with RNAs substituted with fluorouracil. *Nucleic Acids Res*, **19**, 6139-6144.
7. Longley, D.B., Harkin, D.P. and Johnston, P.G. (2003) 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer*, **3**, 330-338.
8. Kavli, B., Sundheim, O., Akbari, M., Otterlei, M., Nilsen, H., Skorpen, F., Aas, P.A., Hagen, L., Krokan, H.E. and Slupphaug, G. (2002) 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. *J Biol Chem*, **277**, 39926-39936.
9. Hardeland, U., Bentele, M., Jiricny, J. and Schar, P. (2000) Separating substrate recognition from base hydrolysis in human thymine DNA glycosylase by mutational analysis. *J Biol Chem*, **275**, 33449-33456.
10. Turner, D.P., Cortellino, S., Schupp, J.E., Caretti, E., Loh, T., Kinsella, T.J. and Bellacosa, A. (2006) The DNA N-glycosylase MED1 exhibits preference for halogenated pyrimidines and is involved in the cytotoxicity of 5-iododeoxyuridine. *Cancer Res*, **66**, 7686-7693.
11. An, Q., Robins, P., Lindahl, T. and Barnes, D.E. (2007) 5-Fluorouracil incorporated into DNA is excised by the Smug1 DNA glycosylase to reduce drug cytotoxicity. *Cancer Res*, **67**, 940-945.
12. Fischer, F., Baerenfaller, K. and Jiricny, J. (2007) 5-Fluorouracil is efficiently removed from DNA by the base excision and mismatch repair systems. *Gastroenterology*, **133**, 1858-1868.
13. Seiple, L., Jaruga, P., Dizdaroglu, M. and Stivers, J.T. (2006) Linking uracil base excision repair and 5-fluorouracil toxicity in yeast. *Nucleic Acids Res*, **34**, 140-151.
14. Wyatt, M.D. and Wilson, D.M., 3rd. (2009) Participation of DNA repair in the response to 5-fluorouracil. *Cell Mol Life Sci*, **66**, 788-799.
15. Liu, A., Yoshioka, K., Salerno, V. and Hsieh, P. (2008) The mismatch repair-mediated cell cycle checkpoint response to fluorodeoxyuridine. *J Cell Biochem*, **105**, 245-254.
16. Meyers, M., Wagner, M.W., Mazurek, A., Schmutte, C., Fishel, R. and Boothman, D.A. (2005) DNA mismatch repair-dependent response to fluoropyrimidine-generated damage. *J Biol Chem*, **280**, 5516-5526.
17. Fischer, J.A., Muller-Weeks, S. and Caradonna, S.J. (2006) Fluorodeoxyuridine modulates cellular expression of the DNA base excision repair enzyme uracil-DNA glycosylase. *Cancer Res*, **66**, 8829-8837.
18. Li, L., Berger, S.H. and Wyatt, M.D. (2004) Involvement of base excision repair in response to therapy targeted at thymidylate synthase. *Mol Cancer Ther*, **3**, 747-753.

19.   Kunz, C., Focke, F., Saito, Y., Schuermann, D., Lettieri, T., Selfridge, J. and Schar, P. (2009) Base excision by thymine DNA glycosylase mediates DNA-directed cytotoxicity of 5-fluorouracil. *PLoS Biol*, **7**, e91.

20.   Sansom, O.J., Zabkiewicz, J., Bishop, S.M., Guy, J., Bird, A. and Clarke, A.R. (2003) MBD4 deficiency reduces the apoptotic response to DNA-damaging agents in the murine small intestine. *Oncogene*, **22**, 7130-7136.

21.   Andersen, S., Heine, T., Sneve, R., Konig, I., Krokan, H.E., Epe, B. and Nilsen, H. (2005) Incorporation of dUMP into DNA is a major source of spontaneous DNA damage, while excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts. *Carcinogenesis*, **26**, 547-555.

22.   Albertella, M.R., Lau, A. and O'Connor, M.J. (2005) The overexpression of specialized DNA polymerases in cancer. *DNA Repair (Amst)*, **4**, 583-593.

23.   Kavli, B., Otterlei, M., Slupphaug, G. and Krokan, H.E. (2007) Uracil in DNA--general mutagen, but normal intermediate in acquired immunity. *DNA Repair (Amst)*, **6**, 505-516.

24.   Visnes, T., Akbari, M., Hagen, L., Slupphaug, G. and Krokan, H.E. (2008) The rate of base excision repair of uracil is controlled by the initiating glycosylase. *DNA Repair (Amst)*, **7**, 1869-1881.

25.   Slupphaug, G., Eftedal, I., Kavli, B., Bharati, S., Helle, N.M., Haug, T., Levine, D.W. and Krokan, H.E. (1995) Properties of a recombinant human uracil-DNA glycosylase from the UNG gene and evidence that UNG encodes the major uracil-DNA glycosylase. *Biochemistry*, **34**, 128-138.

26.   Neddermann, P., Gallinari, P., Lettieri, T., Schmid, D., Truong, O., Hsuan, J.J., Wiebauer, K. and Jiricny, J. (1996) Cloning and expression of human G/T mismatch-specific thymine-DNA glycosylase. *J Biol Chem*, **271**, 12767-12774.

27.   Kavli, B., Andersen, S., Otterlei, M., Liabakk, N.B., Imai, K., Fischer, A., Durandy, A., Krokan, H.E. and Slupphaug, G. (2005) B cells from hyper-IgM patients carrying UNG mutations lack ability to remove uracil from ssDNA and have elevated genomic uracil. *J Exp Med*, **201**, 2011-2021.

28.   Yalowich, J.C. and Kalman, T.I. (1985) Rapid determination of thymidylate synthase activity and its inhibition in intact L1210 leukemia cells in vitro. *Biochem Pharmacol*, **34**, 2319-2324.

29.   Crain, P.F. (1990) Preparation and enzymatic hydrolysis of DNA and RNA for mass spectrometry. *Methods Enzymol*, **193**, 782-790.

30.   Mi, L.J., Chaung, W., Horowitz, R., Teebor, G.W. and Boorstein, R.J. (2001) Excessive base excision repair of 5-hydroxymethyluracil from DNA induces apoptosis in Chinese hamster V79 cells containing mutant p53. *Carcinogenesis*, **22**, 179-186.

31.   Boorstein, R.J., Chiu, L.N. and Teebor, G.W. (1992) A mammalian cell line deficient in activity of the DNA repair enzyme 5-hydroxymethyluracil-DNA glycosylase is resistant to the toxic effects of the thymidine analog 5-hydroxymethyl-2'-deoxyuridine. *Mol Cell Biol*, **12**, 5536-5540.

32.   Boorstein, R.J., Cummings, A., Jr., Marenstein, D.R., Chan, M.K., Ma, Y., Neubert, T.A., Brown, S.M. and Teebor, G.W. (2001) Definitive identification of mammalian 5-hydroxymethyluracil DNA N-glycosylase activity as SMUG1. *J Biol Chem*, **276**, 41991-41997.

33.   Wataya, Y., Santi, D.V. and Hansch, C. (1977) Inhibition of Lactobacillus casei thymidylate synthetase by 5-substituted 2'-deoxyuridylates. Preliminary quantitative structure-activity relationship. *J Med Chem*, **20**, 1469-1473.

34.   Liuzzi, M. and Talpaert-Borle, M. (1985) A new approach to the study of the base-excision repair pathway using methoxyamine. *J Biol Chem*, **260**, 5252-5258.

35. Schlicker, A., Peschke, P., Burkle, A., Hahn, E.W. and Kim, J.H. (1999) 4-Amino-1,8-naphthalimide: a novel inhibitor of poly(ADP-ribose) polymerase and radiation sensitizer. *Int J Radiat Biol*, **75**, 91-100.

36. Boorstein, R.J., Haldar, J., Poirier, G. and Putnam, D. (1995) DNA base excision repair of 5-hydroxymethyl-2'-deoxyuridine stimulates poly(ADP-ribose) synthesis in Chinese hamster cells. *Carcinogenesis*, **16**, 1173-1179.

37. Horton, J.K. and Wilson, S.H. (2007) Hypersensitivity phenotypes associated with genetic and synthetic inhibitor-induced base excision repair deficiency. *DNA Repair (Amst)*, **6**, 530-543.

38. Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P. *et al.* (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat Med*, **2**, 169-174.

39. Elsaleh, H., Powell, B., McCaul, K., Grieu, F., Grant, R., Joseph, D. and Iacopetta, B. (2001) P53 alteration and microsatellite instability have predictive value for survival benefit from chemotherapy in stage III colorectal carcinoma. *Clin Cancer Res*, **7**, 1343-1349.

40. Storojeva, I., Boulay, J.L., Heinimann, K., Ballabeni, P., Terracciano, L., Laffer, U., Mild, G., Herrmann, R. and Rochlitz, C. (2005) Prognostic and predictive relevance of microsatellite instability in colorectal cancer. *Oncol Rep*, **14**, 241-249.

41. Meyers, M., Wagner, M.W., Hwang, H.S., Kinsella, T.J. and Boothman, D.A. (2001) Role of the hMLH1 DNA mismatch repair protein in fluoropyrimidine-mediated cell death and cell cycle responses. *Cancer Res*, **61**, 5193-5201.

42. Hagen, L., Kavli, B., Sousa, M.M., Torseth, K., Liabakk, N.B., Sundheim, O., Pena-Diaz, J., Otterlei, M., Horning, O., Jensen, O.N. *et al.* (2008) Cell cycle-specific UNG2 phosphorylations regulate protein turnover, activity and association with RPA. *EMBO J*, **27**, 51-61.

43. Hardeland, U., Kunz, C., Focke, F., Szadkowski, M. and Schar, P. (2007) Cell cycle regulation as a mechanism for functional separation of the apparently redundant uracil DNA glycosylases TDG and UNG2. *Nucleic Acids Res*, **35**, 3859-3867.

44. Wright, J.E., Dreyfuss, A., el-Magharbel, I., Trites, D., Jones, S.M., Holden, S.A., Rosowsky, A. and Frei, E., 3rd. (1989) Selective expansion of 5,10-methylenetetrahydrofolate pools and modulation of 5-fluorouracil antitumor activity by leucovorin in vivo. *Cancer Res*, **49**, 2592-2596.

45. Scherf, U., Ross, D.T., Waltham, M., Smith, L.H., Lee, J.K., Tanabe, L., Kohn, K.W., Reinhold, W.C., Myers, T.G., Andrews, D.T. *et al.* (2000) A gene expression database for the molecular pharmacology of cancer. *Nat Genet*, **24**, 236-244.

46. Schmidt, W.M., Kalipciyan, M., Dornstauder, E., Rizovski, B., Steger, G.G., Sedivy, R., Mueller, M.W. and Mader, R.M. (2004) Dissecting progressive stages of 5-fluorouracil resistance in vitro using RNA expression profiling. *Int J Cancer*, **112**, 200-212.

47. Park, J.S., Young Yoon, S., Kim, J.M., Yeom, Y.I., Kim, Y.S. and Kim, N.S. (2004) Identification of novel genes associated with the response to 5-FU treatment in gastric cancer cell lines using a cDNA microarray. *Cancer Lett*, **214**, 19-33.

48. Kang, H.C., Kim, I.J., Park, J.H., Shin, Y., Ku, J.L., Jung, M.S., Yoo, B.C., Kim, H.K. and Park, J.G. (2004) Identification of genes with differential expression in acquired drug-resistant gastric cancer cells using high-density oligonucleotide microarrays. *Clin Cancer Res*, **10**, 272-284.

49. de Angelis, P.M., Fjell, B., Kravik, K.L., Haug, T., Tunheim, S.H., Reichelt, W., Beigi, M., Clausen, O.P., Galteland, E. and Stokke, T. (2004) Molecular

characterizations of derivatives of HCT116 colorectal cancer cells that are resistant to the chemotherapeutic agent 5-fluorouracil. *Int J Oncol*, **24**, 1279-1288.

50. Mariadason, J.M., Arango, D., Shi, Q., Wilson, A.J., Corner, G.A., Nicholas, C., Aranes, M.J., Lesser, M., Schwartz, E.L. and Augenlicht, L.H. (2003) Gene expression profiling-based prediction of response of colon carcinoma cells to 5-fluorouracil and camptothecin. *Cancer Res*, **63**, 8791-8812.

51. Zembutsu, H., Ohnishi, Y., Tsunoda, T., Furukawa, Y., Katagiri, T., Ueyama, Y., Tamaoki, N., Nomura, T., Kitahara, O., Yanagawa, R. *et al.* (2002) Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. *Cancer research*, **62**, 518-527.

Figure 1. Metabolism of 5-FU and mechanisms of cytotoxicity.

5-FU cytotoxicity is thought to be conveyed by four active metabolites (underlined in the figure); 5-FUTP (RNA incorporation), 5-FdUMP (TS-inhibition), 5-FdUTP and dUTP (DNA incorporation). The nucleotide metabolizing pathways which effectuate 5-FU cytotoxicity are indicated by full drawn arrows. Possible routes to 5-FU deactivation are indicated by dotted arrows. dUMP/dUTP (2'-deoxyuridine mono-/triphosphate), 5-F(dU) (5-fluoro-2'-deoxyuridine), 5-FU (5-fluorouracil), 5-F(rU) (5-fluorouridine), BER (base excision repair), dUTPase (deoxyuridinetriphosphatase), DHFU (dihydrofluorouracil), DHF (dihydrofolate), DPD (dihydropyrimidine dehydrogenase), MMR (mismatch repair), OPRT (orotic acid phosphoribosyl transferase), RR (ribonucleotide reductase), THF (tetrahydrofolate), TK (thymidine kinase), TP (thymidine phosphorylase), TS (thymidylate synthase), UK (uridine kinase), UP (uridine phosphorylase), 5-FUTP (5-fluorouridine triphosphate), 5-FdUMP and 5-FdUTP (5-fluoro-2'-deoxyuridine mono- and triphosphate).

Figure 2. Repair of 5-FU-DNA by BER and MMR in nuclear extracts.

A. Cartoon showing the cccDNA substrate designed to measure 5-FU:A and 5-FU:G repair by both BER and MMR. 5-FU is positioned in the HincII recognition sequence. Only the lesion containing strand is shown. The nicking endonuclease Nt.BbvCI cleaves one strand 298 bp 3' to the lesion, thus providing a strand-discrimination signal for MMR. **B.** Agarose gel showing HincII+XmnI treated cccDNA substrates containing either 5-FU:A, T:A, 5-FU:G or C:G in the HincII recognition site. Distinction between substrates (5-FU:A, 5-FU:G) and products (T:A, C:G) are performed by 5-FU excision by UNG generating AP-sites that are not cleaved

by HincII . **C.** Positive controls for MMR and BER and their inhibition. cccDNA substrate (T:G, T:G-nicked, U:A) were incubated with SW480 nuclear extract (40 µg) for 60 min, followed by treatment with recombinant TDG (T:G) or UNG (U:A) and MX before purification and HincII + XmnI digestion. Ugi was added to the U:A reactions when indicated. **D.** 5-FU:A repair by SW480 and HeLa nuclear extracts. Nuclear extracts (40 µg) were incubated with cccDNA (5-FU:A, 5-FU:A-nicked) substrates and incubated for 15, 30, 45 and 60 min. Ugi and anti-SMUG1 antibodies were added to the reactions when indicated. **E.** 5-FU:G repair by SW480 and HeLa nuclear extracts. Nuclear extracts and TDG depleted nuclear extracts were incubated with cccDNA (5-FU:G, 5-FU:G nicked) substrates for 15, 30, 45, and 60 min. Ugi and anti-SMUG1 antibodies were added to the reactions when indicated. BER + MMR is quantified from the reactions with cccDNA 5-FU:G nicked substrate, BER is quantified from the panel with cccDNA 5-FU:G substrate, MMR is quantified from the reactions with cccDNA 5-FU:G nicked substrate and TDG-depleted nuclear extract with Ugi and neutralizing SMUG1 antibody. The background (no repair) is quantified from the panel with cccDNA 5-FU:G-nicked substrate and TDG-depleted nuclear extract with Ugi and neutralizing SMUG1 antibody.

Figure 3. 5-FU and 5-hmU excision by human uracil-DNA glycosylases.

A. 5-FU excision by uracil-DNA glycosylases in nuclear extracts from human cancer cell lines (SW480, HeLa, CX1) and MEFs. Nuclear extracts (5 µg) were pre-incubated with Ugi, neutralizing SMUG1 (αSMUG1), and neutralizing TDG (αTDG) antibodies as indicated and assayed with oligonucleotide substrates containing 5-FU:A, 5-FU:G, or in a single-stranded context (5-FU). Excision of 5-FU allows piperidine cleavage of the 22-mer oligonucleotide substrate, resulting in a 13-mer product fragment. U, S and T indicate the individual activities of UNG2, SMUG1 and TDG, respectively. **B.** UDG activity in nuclear extracts nuclear

extracts from human cancer cell lines (SW480, HeLa, CX1) and MEFs. UDG activity was measured by the release of [^{3}H]uracil from labelled calf thymus DNA (U:A substrate). **C.** Varying amounts (0-1000 fmol) of purified recombinant hUNG2, hSMUG1 and hTDG assayed with oligonucleotide substrates containing 5-FU in different contexts (5-FU:A, 5-FU:G, 5-FU). **D.** BER incorporation assay using a cccDNA substrate containing 5-FU opposite A (5-FU:A) or G (5-FU:G). Nuclear extracts (10 μg) from SW480 and HeLa were pre-incubated with Ugi, neutralizing SMUG1 (αSMUG1), and neutralizing TDG (αTDG) antibodies as indicated, and BER were detected by measuring incorporation of radio-labelled nucleotides.

Figure 4. Verification of siRNA down regulation of UNG, SMUG1 and TDG in SW480 and HeLa cell lines.

A. Measurement of siRNA down regulation by specific enzyme activity assays. Whole cell extracts of SW480 and HeLa UNG, SMUG1 and TDG knock-downs (48 h post-transfection) were assayed for UNG, SMUG1 and TDG activity, respectively and compared to control cells. UNG activity was measured by the release of [^{3}H]uracil from labelled calf thymus DNA (U:A substrate). SMUG1 activity in the extract (10 μg) was measured with a uracil containing oligonucleotide annealed to a complementary strand containing G opposite U (U:G substrate) in the presence of Ugi and neutralizing TDG antibodies (37°C, 1 hour). TDG activity was measured in 10 μg extract on the same substrate, but in the presence of Ugi and neutralizing SMUG1 antibodies, and the samples were incubated at 37°C for 20 hours. **B.** Efficiency of siRNA down regulation verified by Western analysis. Western analysis of UNG, SMUG and TDG knock-down compared to control. Total extracts were prepared 48 hour post transfection. β-actin was included as loading control. **C.** hmU-excision activity of SW480, HeLa, and CX-1 nuclear extracts. Extracts (5 μg) were incubated with an oligonucleotide

containing a centrally positioned 5-hmU opposite A (5-hmU:A), opposite G (5-hmU:G) or in a single-stranded context (5-hmU) in presence or absence of neutralizing SMUG1 anti bodies.

Figure 5. Relative cytotoxicity of UNG, SMUG1, and TDG knock-down cells after continuous treatment with 5-hm(dU), 5-FU, 5-F(dU), and 5-F(rU). HeLa and SW480 transfected with SMUG1↓ (green), UNG↓ (blue), TDG↓ (red) and control siRNA (black) were treated for four days with varying concentrations of 5-hm(dU), 5-FU, 5-F(dU), or 5-F(rU). Cytotoxicity was measured by the MTT assay. The curves represent relative cytotoxicity compared to untreated cells. The data points represent the mean ± SD of at least two parallel experiments.

Figure 6. FACS analysis of cell cycle distribution.

A. FACS analysis (cell cycle profiles) of SW480 control and knock-down cells (UNG↓, SMUG1↓, TDG↓) after treatment with 100μM 5-hm(dU), 25 μM 5-FU, and 25μM 5-F(dU) for 48 hours. **B.** FACS analysis (cell cycle profiles) of SW480 and HeLa cells treated with of 5-hm(dU), 5-FU, 5-F(dU) and 5-F(rU) for 48 hours in the absence or presence of 100 μM dT. PI (Propidium Iodide).

Figure 7. Inhibition of thymidylate synthase by 5-F(dU), 5-F(rU) and 5-FU.
HeLa and SW480 was treated with varying concentrations of fluoropyrimidines. Thymidylate synthase activity was measured by counting $[^3H]H_2O$ released into the growth medium, and is plotted relative to the activity of untreated samples. The values represent the mean ± SD of at least three parallel experiments.

Figure 8. Effect of BER inhibitors on 5-hm(dU) and 5-F(dU) treated cells.

A. APE1 cleavage of MX-modified AP-sites. A double-stranded oligonucleotide containing an AP-site was pre-treated for 20 min with various concentrations of MX, and then incubated with purified hAPE1. The upper bands observed after denaturing PAGE represent uncleaved 19-mer substrate, whereas the lower bands represent the APE1 cleaved products. **B.** BER of 5-hmU:G in the presence of increasing concentrations of the PARP-1 inhibitor 4-AN. Nuclear extract from the SW480 cell line was pre-incubated with various concentration of 4-AN and assayed with the BER incorporation assay using cccDNA plasmid with a single HmU opposite G. BER was measured by monitoring the incorporation of $[\alpha^{33}P]dCTP$ at the position of hmU. **C.** Effect of BER inhibitors on the cytotoxicity of 5-hm(dU). HeLa and SW480 cells were treated for four days with varying concentrations of 5-hm(dU) in the presence of either 20 mM MX, 10 µM 4-AN or normal medium (control). The data represent the mean ± SD of at least two parallel experiments. **D.** Effect of BER inhibitors on the cytotoxicity of 5-F(dU). HeLa and SW480 cells were treated for four days with varying concentrations of 5-F(dU) in the presence of either 20 mM MX, 10 µM 4-AN or normal medium (control). The data represent the mean ± SD of at least two parallel experiments.

Figure 9. Cytotoxicity reversal experiments.

A. Relative cytotoxicity of SW480 and HeLa cells treated for four days with fixed doses of 5-F(dU) and varying concentrations of dT, dU, and rU. **B.** Relative cytotoxicity of SW480 and HeLa treated with fixed doses of 5-F(rU) and varying amounts of nucleosides. **C.** Relative cytotoxicity of SW480 and HeLa treated with fixed doses of 5-FU and varying amounts of nucleosides. The data represent the mean ± SD of at least three parallel measurements. **D.** Thymidylate synthase activity in HeLa and SW480 cells treated with varying amounts of rU

in the absence or presence of 2.5 µM 5-F(rU). The data represent the mean ± SD of three parallel measurements.

Supplementary figure 1: LC/MS/MS chromatograms showing 5-F(dU) in DNA and 5-F(rU) in RNA hydrolysates from 5-FU- or 5-F(dU)-treated HeLa cells. 5-FU:A cccDNA was employed as a positive control for 5-FU in DNA. For each mass transition the signal intensities are normalized according to the most abundant peak in the present samples. The arrows indicate the expected elution positions of the respective nucleosides.

Table 1: Incorporation of 5-FU in DNA and RNA from HeLa cells exposed to different concentrations of 5-FU or 5-F(dU) for 24 hours.

	Conc. (μM)	DNA (5-F(dU)/10^6 nt)	RNA (5-F(rU)/10^6 nt)	Ratio 5-FU (RNA/DNA)
5-FU	10	2.4 ±1.0	5419 ±90	2260
	20	2.4 ±0.22	8262 ±420	3451
	40	5.1 ±0.11	15046 ±1083	2967
5-F(dU)	1	27.5 ±5.6	169 ±8.8	6.2
	2	46.0 ±3.6	303 ±0.7	6.2
	4	72.0 ±3.4	457 ±51	6.3

Table 2: Incorporation of 5-FU in RNA from HeLa cells exposed to 5-FU or 5-F(rU) combined with increasing concentration of uridine

Uridine (μM)	5-FU in RNA (5-F(rU)/10^6 nt)	
	5-FU (10 μM)	5-F(rU) (2μM)
0	3501 ±119	35166 ±2920
10	4339 ±263	33486 ±534
100	3651 ±110	7001 ±130
1000	3417 ±22	1222 ±7

Figure 1

230 x 178 mm

Figure 2

230 x 178 mm

A

HincII
5-FU
298 bp

Nt.BbvC
(nick)

cccDNA
substrate
(3198 bp)

1301 bp

XmnI

1599 bp

B

cccDNA (HincII + XmnI)

λ 5-FU:A T:A 5-FU:G C:G

bp

3198 Substrate
1897 Product
1301 Product

UNG   UNG

C

cccDNA (HincII + XmnI)

λ   T:G   U:A

bp

3198 Substrate
1897 Product
1301 Product

nicked   Ugi

TDG   UNG

D

SW480   Ugi-αSMUG1

5-FU:A

5-FU:A
nicked

0 15 30 45 60   0 15 30 45 60
Time (min)   Time (min)

HeLa   Ugi-αSMUG1

5-FU:A

5-FU:A
nicked

0 15 30 45 60   0 15 30 45 60
Time (min)   Time (min)

E

SW480   Ugi-αSMUG1-
TDGdepl

5-FU:G

5-FU:G
nicked

0 15 30 45 60   0 15 30 45 60
Time (min)   Time (min)

HeLa   Ugi-αSMUG1-
TDGdepl

5-FU:G

5-FU:G
nicked

0 15 30 45 60   0 15 30 45 60
Time (min)   Time (min)

SW480

HeLa

■ BER+MMR
□ BER
● MMR
○ No repair

Figure 3

230 x 178 mm

Figure 4

230 x 178 mm

A

SW480   HeLa

B

SW480   HeLa

C

Figure 5

Figure 6

230 x 178 mm

Figure 7

Figure 8

230 x 178 mm

A

AP-site cleavage (%)

Sub
Pro

MX (mM): 0, 0.5, 5, 50

B

5-hmU:G repair (%)

4-AN (µM): 0, 2, 10, 50

C

SW480

Relative survival

Control
MX (20 mM)
4-AN (10 µM)

5-hm(dU) (µM)

HeLa

Relative survival

5-hm(rU) (µM)

D

SW480

Relative survival

Control
MX (20 mM)
4-AN (10 µM)

5-F(dU) (µM)

HeLa

Relative survival

5-F(dU) (µM)

Figure 9

230 x 178 mm

A SW480 + 25 µM 5-F(dU)

A HeLa + 0.25 µM 5-F(dU)

B SW480 + 2.5 µM 5-F(rU)

B HeLa + 2.5 µM 5-F(rU)

C SW480 + 15 µM 5-FU

C HeLa + 50 µM 5-FU

D

HeLa
HeLa + 2.5 µM 5-F(rU)
SW480
SW480+ 2.5 µM 5-F(rU)

Dissertations at the Faculty of Medicine, NTNU

1977
1. Knut Joachim Berg: EFFECT OF ACETYLSALICYLIC ACID ON RENAL FUNCTION
2. Karl Erik Viken and Arne Ødegaard: STUDIES ON HUMAN MONOCYTES CULTURED *IN VITRO*

1978
3. Karel Bjørn Cyvin: CONGENITAL DISLOCATION OF THE HIP JOINT.
4. Alf O. Brubakk: METHODS FOR STUDYING FLOW DYNAMICS IN THE LEFT VENTRICLE AND THE AORTA IN MAN.

1979
5. Geirmund Unsgaard: CYTOSTATIC AND IMMUNOREGULATORY ABILITIES OF HUMAN   BLOOD MONOCYTES CULTURED IN VITRO

1980
6. Størker Jørstad: URAEMIC TOXINS
7. Arne Olav Jenssen: SOME RHEOLOGICAL, CHEMICAL AND STRUCTURAL PROPERTIES   OF MUCOID SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

1981
8. Jens Hammerstrøm: CYTOSTATIC AND CYTOLYTIC ACTIVITY OF HUMAN MONOCYTES AND EFFUSION MACROPHAGES AGAINST TUMOR CELLS *IN VITRO*

1983
9. Tore Syversen: EFFECTS OF METHYLMERCURY ON RAT BRAIN PROTEIN.
10. Torbjørn Iversen: SQUAMOUS CELL CARCINOMA OF THE VULVA.

1984
11. Tor-Erik Widerøe: ASPECTS OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS.
12. Anton Hole: ALTERATIONS OF MONOCYTE AND LYMPHOCYTE FUNCTIONS IN REALTION TO SURGERY UNDER EPIDURAL OR GENERAL ANAESTHESIA.
13. Terje Terjesen: FRACTURE HEALING AND STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
17. Trond Haug: FACTORS REGULATING BEHAVIORAL EFFECTS OG DRUGS.

1985
18. Sven Erik Gisvold: RESUSCITATION AFTER COMPLETE GLOBAL BRAIN ISCHEMIA.
19. Terje Espevik: THE CYTOSKELETON OF HUMAN MONOCYTES.
20. Lars Bevanger: STUDIES OF THE Ibc (c) PROTEIN ANTIGENS OF GROUP B STREPTOCOCCI.
21. Ole-Jan Iversen: RETROVIRUS-LIKE PARTICLES IN THE PATHOGENESIS OF PSORIASIS.
22. Lasse Eriksen: EVALUATION AND TREATMENT OF ALCOHOL DEPENDENT BEHAVIOUR.
23. Per I. Lundmo: ANDROGEN METABOLISM IN THE PROSTATE.

1986
24. Dagfinn Berntzen: ANALYSIS AND MANAGEMENT OF EXPERIMENTAL AND CLINICAL PAIN.
25. Odd Arnold Kildahl-Andersen: PRODUCTION AND CHARACTERIZATION OF MONOCYTE-DERIVED CYTOTOXIN AND ITS ROLE IN MONOCYTE-MEDIATED CYTOTOXICITY.
26. Ola Dale: VOLATILE ANAESTHETICS.

1987
27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988

30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.
33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.
1989
43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.
1990
52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.
1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.
68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
76. Harold C Sexton: STUDIES RELATING TO THE TREATMENT OF SYMPTOMATIC NON-PSYCHOTIC PATIENTS.
77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
78. Terje Johannessen: CONTROLLED TRIALS IN SINGLE SUBJECTS.
79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
90. Lars Jacob Stovner: THE CHIARI TYPE I MALFORMATION.
91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
97. Bjørn Backe: STUDIES IN ANTENATAL CARE.
98. Gerd Inger Ringdal: QUALITY OF LIFE IN CANCER PATIENTS.
99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
100. Hans E. Fjøsne: HORMONAL REGULATION OF PROSTATIC METABOLISM.
101. Eylert Brodtkorb: CLINICAL ASPECTS OF EPILEPSY IN THE MENTALLY RETARDED.
102. Roar Juul: PEPTIDERGIC MECHANISMS IN HUMAN SUBARACHNOID HEMORRHAGE.
103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
106. Kirsten Rasmussen: VIOLENCE IN THE MENTALLY DISORDERED.
107. Finn Egil Skjeldestad: INDUCED ABORTION: Timetrends and Determinants.
108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.
109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANCER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
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.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

1997

124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
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.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

1998

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.

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

2002
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
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

2003
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
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES
223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
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
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

2004

235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
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

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
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
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
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
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 OF 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
290. Jakob Nakling: RESULTS AND CONSEQUENCES OF ROUTINE ULTRASOUND SCREENING IN PREGNANCY – A GEOGRAPHIC BASED POPULATION STUDY
291. Anne Engum: DEPRESSION AND ANXIETY – THEIR RELATIONS TO THYROID DYSFUNCTION AND DIABETES IN A LARGE EPIDEMIOLOGICAL STUDY
292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
295. Gunilla Klensmeden Fosse: MENTAL HEALTH OF PSYCHIATRIC OUTPATIENTS BULLIED IN CHILDHOOD
296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

2007

2009

381. Tore Grüner Bjåstad: HIGH FRAME RATE ULTRASOUND IMAGING USING PARALLEL BEAMFORMING
382. Erik Søndenaa: INTELLECTUAL DISABILITIES IN THE CRIMINAL JUSTICE SYSTEM
383. Berit Rostad: SOCIAL INEQUALITIES IN WOMEN'S HEALTH, HUNT 1984-86 AND 1995-97, THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
384. Jonas Crosby: ULTRASOUND-BASED QUANTIFICATION OF MYOCARDIAL DEFORMATION AND ROTATION
385. Erling Tronvik: MIGRAINE, BLOOD PRESSURE AND THE RENIN-ANGIOTENSIN SYSTEM
386. Tom Christensen: BRINGING THE GP TO THE FOREFRONT OF EPR DEVELOPMENT
387. Håkon Bergseng: ASPECTS OF GROUP B STREPTOCOCCUS (GBS) DISEASE IN THE NEWBORN. EPIDEMIOLOGY, CHARACTERISATION OF INVASIVE STRAINS AND EVALUATION OF INTRAPARTUM SCREENING
388. Ronny Myhre: GENETIC STUDIES OF CANDIDATE TENE3S IN PARKINSON'S DISEASE
389. Torbjørn Moe Eggebø: ULTRASOUND AND LABOUR
390. Eivind Wang: TRAINING IS MEDICINE FOR PATIENTS WITH PERIPHERAL ARTERIAL DISEASE
391. Thea Kristin Våtsveen: GENETIC ABERRATIONS IN MYELOMA CELLS
392. Thomas Jozefiak: QUALITY OF LIFE AND MENTAL HEALTH IN CHILDREN AND ADOLESCENTS: CHILD AND PARENT PERSPECTIVES
393. Jens Erik Slagsvold: N-3 POLYUNSATURATED FATTY ACIDS IN HEALTH AND DISEASE – CLINICAL AND MOLECULAR ASPECTS
394. Kristine Misund: A STUDY OF THE TRANSCRIPTIONAL REPRESSOR ICER. REGULATORY NETWORKS IN GASTRIN-INDUCED GENE EXPRESSION
395. Franco M. Impellizzeri: HIGH-INTENSITY TRAINING IN FOOTBALL PLAYERS. EFFECTS ON PHYSICAL AND TECHNICAL PERFORMANCE
396. Kari Hanne Gjeilo: HEALTH-RELATED QUALITY OF LIFE AND CHRONIC PAIN IN PATIENTS UNDERGOING CARDIAC SURGERY
397. Øyvind Hauso: NEUROENDOCRINE ASPECTS OF PHYSIOLOGY AND DISEASE
398. Ingvild Bjellmo Johnsen: INTRACELLULAR SIGNALING MECHANISMS IN THE INNATE IMMUNE RESPONSE TO VIRAL INFECTIONS
399. Linda Tømmerdal Roten: GENETIC PREDISPOSITION FOR DEVELOPMENT OF PREEMCLAMPSIA – CANDIDATE GENE STUDIES IN THE HUNT (NORD-TRØNDELAG HEALTH STUDY) POPULATION
400. Trude Teoline Nausthaug Rakvåg: PHARMACOGENETICS OF MORPHINE IN CANCER PAIN
401. Hanne Lehn: MEMORY FUNCTIONS OF THE HUMAN MEDIAL TEMPORAL LOBE STUDIED WITH fMRI
402. Randi Utne Holt: ADHESION AND MIGRATION OF MYELOMA CELLS – IN VITRO STUDIES –
403. Trygve Solstad: NEURAL REPRESENTATIONS OF EUCLIDEAN SPACE
404. Unn-Merete Fagerli: MULTIPLE MYELOMA CELLS AND CYTOKINES FROM THE BONE MARROW ENVIRONMENT; ASPECTS OF GROWTH REGULATION AND MIGRATION
405. Sigrid Bjørnelv: EATING– AND WEIGHT PROBLEMS IN ADOLESCENTS, THE YOUNG HUNT-STUDY
406. Mari Hoff: CORTICAL HAND BONE LOSS IN RHEUMATOID ARTHRITIS. EVALUATING DIGITAL X-RAY RADIOGRAMMETRY AS OUTCOME MEASURE OF DISEASE ACTIVITY, RESPONSE VARIABLE TO TREATMENT AND PREDICTOR OF BONE DAMAGE
407. Siri Bjørgen: AEROBIC HIGH INTENSITY INTERVAL TRAINING IS AN EFFECTIVE TREATMENT FOR PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE
408. Susanne Lindqvist: VISION AND BRAIN IN ADOLESCENTS WITH LOW BIRTH WEIGHT
409. Torbjørn Hergum: 3D ULTRASOUND FOR QUANTITATIVE ECHOCARDIOGRAPHY
410. Jørgen Urnes: PATIENT EDUCATION IN GASTRO-OESOPHAGEAL REFLUX DISEASE. VALIDATION OF A DIGESTIVE SYMPTOMS AND IMPACT QUESTIONNAIRE AND A RANDOMISED CONTROLLED TRIAL OF PATIENT EDUCATION