Excision repair deficiencies in man and mice; lessons from *CSA* and *Fen1* mutants.

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Oslo, November 2011
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CONTENTS

LIST OF PAPERS

This thesis is based upon the following papers, which will be referred to by their Roman numbers:

I **Kleppa L**, Kanavin ØJ, Klungland A, Strømme P. (2007) A novel splice site mutation in the Cockayne syndrome group A gene in two siblings with Cockayne syndrome. Neuroscience, 145:1397-406.

II Larsen E*, **Kleppa L***, Meza TJ, Meza-Zepeda LA, Rada C, Castellanos CG, Lien GF, Nesse GJ, Neuberger MS, Laerdahl JK, William Doughty R, Klungland A. (2008) Early-onset lymphoma and extensive embryonic apoptosis in two domain-specific *Fen1* mice mutants. Cancer Res., 68:4571-9. *Joint first authors

III **Kleppa L**, Mari PO, Larsen E, Flor Lien G, Godon C, Theil AF, Nesse GJ, Wiksen H, Vermeulen W, Giglia-Mari G and Klungland A. *In vivo* kinetics and PARP1 dependence of FEN1 in base excision repair. Manuscript.

ABBREVIATIONS

(6–4)PP	6–4 photoproduct (induced by UV light)
8-oxoA	7,8-dihydro-8-oxoadenine
8-oxoG	7,8-dihydro-8-oxoguanine
A	Adenine
AD	Alzheimer's disease
AGS	Aicardi-Goutières syndrome
ALKBH	AlkB homolog
ALS	Amyotrophic lateral sclerosis
APEX1	APEX nuclease 1
ATLD	AT (ataxia telangiectasia)-like disorder
BER	Base excision repair
BFP	Blue fluorescent protein
BLM	Bloom syndrome protein
BS	Blooms syndrome
C	Cytosine
CDKM1A	Cyclin dependent kinase inhibitor 1A (alias p21)
cis-Pt	Cisplatin (DNA-crosslinking agent)
COFS	cerebro-oculo-facio-skeletal (COFS) syndrome
CPDs	Cyclobutane pyrimidine dimers (induced by UV light)
CS	Cockayne Syndrome
CSA	Cockayne syndrome group A
CSB	Cockayne syndrome group B
CSF	Cerebrospinal fluid
CSR	Class switch recombination
DBSs	Double strand breaks
DDB1	DNA damage binding protein 1, involved in NER
DDB2	DNA damage binding protein 2, involved in NER
DNA	Deoxyribonucleic acid
DNA2	DNA replication helicase 2 homolog (yeast)
dsDNA	Double stranded DNA
EJ	End joining
EP300	E1A binding protein (alias p300)

ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1 (5' incision in NER)
ERCC5	Excision repair cross-complementing rodent repair deficiency protein 5 (alias XPG)
FEN1	Flap structure-specific endonuclease 1
FRAP	Fluorescence recovery after photobleaching
G	Guanine
G1-phase	Gap 1/growth phase 1, cell cycle stage for growth and normal function
G2-phase	Gap 2/growth phase 2, the cell resumes growth and prepares for mitosis
GFP	Green fluorescent protein
GGR	Global genomic repair
GG-NER	Global genomic nucleotide excision repair
GH	Growth hormone
HaCaT	Human keratinocyte cells
HD	Huntington's disease
HeLa	Henrietta Lacks cervical cancer cells
HR	Homologous recombination
HR23B	UV excision repair protein RAD23 homolog B
IFN-α	Interferon-α
IGF1	Insulin growth factor 1
LIG	DNA ligase
LoVo	Colon cancer cell line
M-phase	Mitotic phase (Cell division)
MBD4	Methyl-CpG binding domain protein 4
MGMT	O-6-methylguanine methyltransferase
mKate2-zyxin	Far red fluorescent protein fused to zyxin protein (z. is associated with actin)
MMC	Mitomycin C (DNA-crosslinking agent)
MMR	Mismatch repair
MNU	Methylnitrosourea
MPG	N-methylpurine-DNA glycosylase
MRE11	Meiotic recombination 11, double strand break repair protein
MRN	MRE11/RAD50/NBS1 complex
MSH	MutS homolog
Mt	Mitochondria

MUTYH	MutY homolog (E. coli) A/G-specific adenine DNA glycosylase
NBS	Nijmegen Breakage Syndrome
NBS1	Nijmegen Breakage Syndrome 1, alias nibrin (NBN), DSB repair associated protein
NEIL	Nei endonuclease VIII-like
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NTHL1	Nth endonuclease III-like 1
OGG1	8-oxoguanine DNA glycosylase
P53	Tumor suppressor protein 53
PCNA	Proliferating cell nuclear antigen
PD	Parkinson's disease
pH2AX	gamma-phosphorylated H2AX (Histone 2A), alias γH2AX
phiYFP-mito	*Phialidium* sp. mutant yellow fluorescent protein fused to mitochondria
POL	DNA polymerase
RAD50	RAD50 homolog (S. cerevisiae), DNA (DSB) repair protein
RFC	Replication factor C
RFP	Red fluorescent protein
RNAPII	RNA polymerase II
RNASEH2	Ribonuclease H2
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPA	Replication protein A (single-stranded DNA binding protein)
RTS	Rothmund-Thomson syndrome
S-phase	Synthesis phase (DNA replication, between G1 and G2 in the cells interphase)
SCID	Severe combined immunodeficiency
SHM	Somatic hypermutation
SMUG1	Single-strand-selective monofunctional uracil-DNA glycosylase
SNPs	Single nucleotide polymorphisms
SSBs	Single-strand breaks
SSBP	Single-strand binding protein
T	Thymine (Thymine+deoxyribose= thymidine)
TagBFP-H2B	Protein tag blue fluorescent protein (BFP) fused with histone H2B

TagGFP2-actin	Protein tag green fluorescent protein (GFP) fused with actin
TagRFP-golgi	Protein tag red fluorescent protein (RFP) fused with golgi (appears orange)
TC-NER	Transcription coupled nucleotide excision repair
TCR	Transcription coupled repair
TDG	Thymine-DNA glycosylase
TFIIH	Transcription factor II H
TFIIS	RNA polymerase II transcription elongation factor
Tg	Thymine glycol
TLS	Translesion synthesis
TNR	Trinucleotide repeat
TREX1	Three prime repair exonuclease 1
TTD	Trichothiodystrophy
TTDA	Trichothiodystrophy group A
UBD	Ubiquitin-binding domain
UV	Ultraviolet
UVSS	UV-sensitive syndrome
UNG	Uracil DNA glycosylase
WRN	Werner syndrome protein
WS	Werner syndrome
XAB2	XPA-binding protein-2
XP	Xeroderma pigmentosum
XPA-G, V	Xeroderma pigmentosum group A-G, V
YFP	Yellow fluorescent protein

SUMMARY

Thousands of DNA lesions, in form of chemical modifications, base loss and single-strand breaks are estimated to occur in every cell per day. Excision repair pathways and cell cycle checkpoints have evolved as part of the cellular response to DNA damage. Base excision repair (BER) can remove subtle DNA lesions, while nucleotide excision repair (NER) can remove more bulky helix distorting DNA damage.

In paper I, we characterized two Cockayne syndrome (CS) patients deficient in a sub-pathway of NER, transcription coupled (TC) NER. We hypothesized that the underlying mutation most probably would be found in one of the known CS proteins, CSA or CSB. Molecular analysis confirmed our hypothesis, and a new splice site mutation was identified in the *CSA* gene. Moreover, we reviewed on the known human mutations in the CSA protein, at the time of publication, and their possible correlation to clinical findings. In the discussion of this thesis, an update on CS proteins, their known human mutations and clinical characteristics is further reviewed. Still, a molecular explanation of the CS pathology is lacking, and the role of the CS proteins in TC-NER and possibly oxidative damage repair needs further investigation.

Flap endonuclease 1 (FEN1) is essential in mammalian long-patch (LP) BER and in removal of RNA primers in lagging strand DNA replication. Thus, it could be hypothesized that FEN1 deficiency would have detrimental consequences for cell survival and health of mutated mice. In paper II we show that *Fen1* mutations in mice result in severe phenotypes in form of embryonic lethality and early cancer development. An update on FEN1's role and regulation in the cell, and possible mechanisms causing cancer, is given in the discussion of this thesis.

Moreover, in paper III we characterized *Fen1* knock-in mice with a yellow fluorescent protein (YFP) tag fused to FEN1, in order to study FEN1-YFP kinetics in BER and DNA replication *in vivo*. For the first time, the kinetics of the FEN1-YFP protein in LP-BER, expressed from the *Fen1-YFP* gene at an endogenous level, could be investigated in living cells, following highly localized laser irradiation. This micro-irradiation method produces a high concentration (local damage) and wide spectrum of DNA lesions, including LP-BER substrates for FEN1. We found that FEN1-YFP is rapidly recruited to DNA damaged areas and were able to follow ongoing repair through the progressive disappearance of FEN1's flap substrate. Inhibition of PARP disrupted FEN1 accumulation at DNA lesions, indicating that PARP is needed for FEN1

recruitment to DNA repair intermediates in LP-BER. Fluorescence recovery after photobleaching (FRAP) measurements following local damage allowed us to study the kinetics of FEN1 binding and unbinding its flap substrate. FRAP after global damage allowed us to measure the proportion of FEN1 binding at the moment of bleaching, and to estimate how long the FEN1 molecules stay bound to the substrate. We found that FEN1 binding after local damage is very short lived. In line with FEN1's role in DNA replication and its interaction with PCNA, we compared the (co)localization of FEN1 and PCNA in S-phase DNA replication foci.

1. INTRODUCTION

1.1 DNA DAMAGE

Unlike other macromolecules, DNA has no turnover and its stability is therefore essential to maintain cellular function throughout the lifespan of a cell and an organism. Then again, cells receive tens of thousands of DNA lesions per day (Lindahl and Barnes, 2000). Time-dependent accumulation of damage in cells and organs is associated with gradual functional decline and aging (Kirkwood, 2005). Such lesions can block genome replication and transcription, and if they are not repaired or are repaired incorrectly, they lead to mutations or wider-scale genome aberrations that threaten the cell or organisms viability. Hydrolysis of DNA is the most common damage, with depurination being the most prevalent of these spontaneous chemical reactions (Lindahl, 1993). Non-enzymatic methylation of DNA bases and oxidation are also frequent occurring endogenous lesions (Sedgwick *et al.*, 2007).

1.1.1 Endogenous and exogenous DNA damage

Cellular metabolism generates reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxidation products, endogenous alkylating agents, estrogen and cholesterol metabolites, and reactive carbonyl species, all of which damage DNA (De and Van, 2004). ROS arising as by-products from oxidative respiration, redox-cycling events involving environmental toxic agents, Fenton reactions mediated by heavy metals, and ROS and RNS produced by macrophages and neutrophils at sites of inflammation and infections can induce strand breaks, oxidized bases and AP sites (Valko *et al.*, 2006;Kawanishi *et al.*, 2006). More than 80 different aberrant bases produced by ROS have been identified (Bjelland and Seeberg, 2003). Occasionally, DNA aberrations arise via physiological processes, such as DNA mismatches introduced during DNA replication and DNA strand breaks caused by abortive topoisomerase I and topoisomerase II activity. Single-strand breaks (SSBs) that occur in close proximity, or certain other lesions that are encountered by the DNA replication apparatus, form double-strand breaks (DSBs). Although DSBs do not occur as frequently as the lesions listed above, they are more complicated to repair and extremely toxic to the cell (Khanna and Jackson, 2001).

In addition to spontaneous reactions and reactive species from cell metabolism, exogenous physical and chemical agents damage DNA. The damage from environmental agents, such as

ultraviolet (UV) radiation, ionizing radiation and genotoxic chemicals is to some extent avoidable. UV radiation gives rise to intrastrand crosslinks between adjacent pyrimidines in the DNA and creates free radicals. After a single day in strong sunlight, up to 10^5 UVA and UVB photoproducts are induced in each exposed keratinocyte (Hoeijmakers, 2009). Ionizing radiation also generates various forms of DNA damage, the most toxic of these being DSBs (Ward, 1988). Some ionizing radiation results from radioactive decay of naturally occurring radioactive compounds. Uranium decay, for example, produces radioactive radon gas that accumulates in some homes and contributes to lung-cancer incidence. Environmental and industrial chemicals create a huge diversity of DNA adducts and crosslinking of DNA. The most prevalent environmental cancer-causing chemicals today are those produced by tobacco products, which cause various cancers, most notably those of the lung, oral cavity and adjacent tissues (Doll and Peto, 1981;Wogan *et al.*, 2004). Cancer-causing DNA damaging chemicals can also contaminate foods, such as heterocyclic amines in over-cooked meats and aflatoxins in contaminated peanuts (Wogan *et al.*, 2004).

Cells that accumulate large numbers of DNA damage can no longer effectively repair injured DNA, and where mutations are induced, can go into senescence, programmed cell death/apoptosis or unregulated cell division, the latter which can lead to cancer. The outcome of the cell depends of the type of damage. Some lesions are primarily mutagenic, while others are mainly cytotoxic or cytostatic. Both types of outcomes in different ratios can result from many DNA lesions, depending on the location and number of lesions, cell type, and stage in the cell cycle and differentiation (Akbari and Krokan, 2008;Hoeijmakers, 2009). The oxidative lesion 7,8-dihydro-8-oxoguanine is a mutagenic lesion, pairing equally well with cytosine (normal pairing) and guanine (abnormal pairing) during DNA replication, causing GC→TA transversions (Akbari and Krokan, 2008). DSBs induced by ionizing radiation or that occur during the processing of interstrand crosslinks are primarily cytotoxic or cytostatic. Over time the accumulation of DNA damage contributes to a gradual decline in cellular function and manifestation of aging (Izzotti *et al.*, 1999;Mecocci *et al.*, 1999;Lu *et al.*, 2004;Siomek *et al.*, 2007).

1.2 DNA REPAIR SYSTEMS AND CONSEQUENCES OF THEIR DEFICIENCY

A complex genome maintenance apparatus controls DNA damage. It consists of multiple repair pathways, each usually involving a number of proteins for detection and repair of damaged DNA (Figure 1). Each pathway focus on a specific category of DNA lesion, various

checkpoint, signal transduction, and effector systems connected with replication, transcription, recombination, chromatin remodeling and differentiation (Harper and Elledge, 2007;Altieri *et al.*, 2008). There also exists DNA repair where only one protein is involved, direct lesion removal. Some alkylations of bases in DNA are repaired by direct removal of the alkyl adduct from the damaged base. This mechanism does not require a template for repair, and does not involve incision of the phosphodiester backbone (Friedberg *et al.*, 1995;Falnes *et al.*, 2007). The massive investment cells make in genome maintenance is illustrated by the class of repair proteins that can be used only once. For instance O-6-methylguanine methyltransferase (MGMT or AGT) repairs a single O-6 methylguanine lesion by transferring the methyl from a guanine in DNA to a cysteine in the enzyme, thereby inactivating itself (Xu-Welliver and Pegg, 2002). All organisms from bacteria to man have evolved distinct repair systems to combat the threats posed by DNA damage (Taylor and Lehmann, 1998;Hoeijmakers, 2001;Weller *et al.*, 2002).

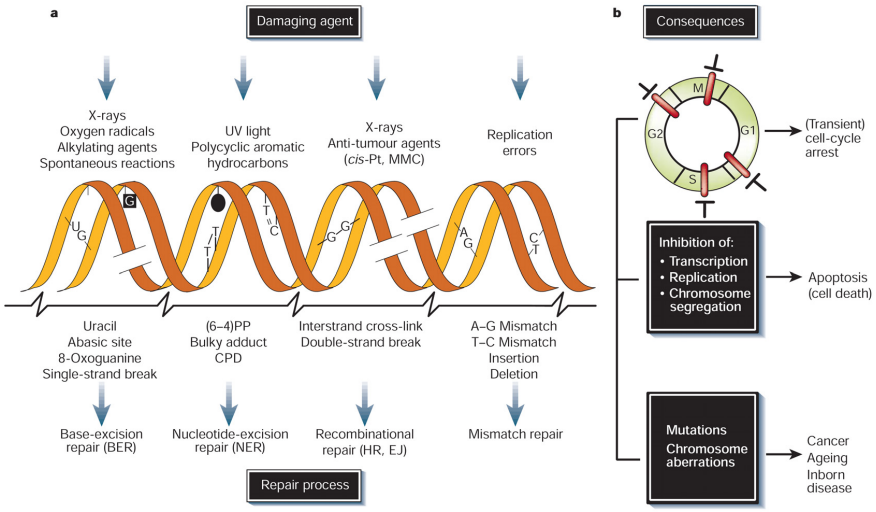

Figure 1 **DNA damage, repair mechanisms and consequences**. **a**, Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle); and most relevant DNA repair mechanism responsible for the removal of the lesions (bottom). **b**, Acute effects of DNA damage on cell-cycle progression, leading to transient arrest in the G1, S, G2 and M phases (top), and on DNA metabolism (middle). Long-term consequences of DNA injury (bottom) include permanent changes in the DNA sequence (point mutations affecting single genes or chromosome aberrations which may involve multiple genes) and their biological effects. Abbreviations: *cis*-Pt and MMC, cisplatin and mitomycin C, respectively (both DNA-crosslinking agents); (6–4)PP and CPD, 6–4 photoproduct and cyclobutane pyrimidine dimer, respectively (both induced by UV light); BER and NER, base and nucleotide-excision repair, respectively; HR, homologous recombination; EJ, end joining. (Figure and legend from (Hoeijmakers, 2001)).

In addition to direct lesion reversal, different multistep DNA repair systems exist: base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) (Figures 1 and 2). Some DNA damages escape detection by repair proteins and specialized polymerases allow replication to bypass such lesions in the template through translesion synthesis (TLS) ((Andersen *et al.*, 2008) and Figure 2).

So far, three mammalian DNA repair proteins working by direct reversal have been ascribed. These include the MGMT mentioned above, and the *E.coli* AlkB homologs 2 and 3 (ALKBH2 and ALKBH3) (Duncan *et al.*, 2002). Homozygous *Mgmt*$^{-/-}$ mice are viable with no increase in spontaneous mutagenesis, however, they are very sensitive to chemotherapeutic alkylating agents and develop liver and lung tumors (Iwakuma *et al.*, 1997). Mice lacking functional *Alkbh2* and *Alkbh3* genes are viable with no overt phenotype. However, *Alkbh2* deficient mice accumulate significant levels of 1meA in the genome, and embryonic fibroblast cells from these mice are not able to remove methyl methane sulfate (MMS)-induced 1meA lesions from genomic DNA, and display increased cytotoxicity after MMS exposure (Ringvoll *et al.*, 2006).

The mismatch repair pathway removes mispaired nucleotides and insertion/deletion loops that result from slippage of the DNA polymerase during replication ((Jiricny, 2006) and Figure 1). Identification of mutations in human MMR genes in patients with hereditary non-polyposis colorectal cancer (HNPCC) revealed the importance of the MMR pathway in human etiology (Papadopoulos *et al.*, 1994;Vasen, 2007).

The removal of helix-distorting lesions that interfere with base pairing and obstruct replication and transcription are dealt with by the NER pathway ((Cleaver *et al.*, 2009) and Figures 1 and 2). Transcription-coupled repair (TCR), one of the two sub-pathways of NER (Figures 2 and 3), targets only lesions that hinder transcription, through arresting RNA polymerase II (Fousteri and Mullenders, 2008). Global genome (GG-) NER surveys damage to DNA that occurs anywhere in the genome (Figures 2 and 3). Individuals with inherited defects in NER are characterized with hypersensitivity to the sun. This is due to defective handling of UV damage, however, other NER associated clinical features are extremely heterogenous (Cleaver *et al.*, 2009). Xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) are three rare syndromes arising from mutations in genes

coding for NER proteins (Bootsma *et al.*, 1995;Kraemer *et al.*, 2007). XP patients have an extremely high risk of developing skin cancer at young age. This is a feature not found in individuals with CS and TTD (Cleaver, 2005). Neurodegeneration and developmental disorders are major features of all three syndromes, including growth retardation, cognitive impairment and ataxia. A TTD typical feature is brittle hair and nails, whereas CS patients are characterized by microcephaly, cachetic dwarfism and developmental delay (Andressoo and Hoeijmakers, 2005).

BER is the main pathway for removal of DNA damage due to cellular metabolism and targets small chemical alterations of DNA bases ((Baute and Depicker, 2008;Zharkov, 2008) and Figures 1 and 2). Mice lacking individual BER proteins either show no particular phenotype, or a severe, mostly embryonic lethal phenotype. This can be explained by backup mechanisms for many of the BER glycosylases which initiate the BER pathway, whereas enzymes handling BER intermediates (see below) are essential and it seem that no efficient backup exist. MUTYH is a DNA glycosylase excising adenine (A) misincorporated opposite 8-oxoG during replication and in humans, MUTYH deficiencies is the underlying factor of the disorder MUTYH-associated polyposis (MAP) (Al-Tassan *et al.*, 2002;Jones *et al.*, 2002;Sieber *et al.*, 2003;Dallosso *et al.*, 2008). Biallelic germline mutations in the *MUTYH* gene cause increased GC to TA transversions in the *APC* gene, which controls the proliferation of colon cells (Fearnhead *et al.*, 2001). Uracil DNA N-glycosylase has been shown to be involved in immunoglobulin (Ig) class-switch recombination (CSR) and somatic hypermutation (SHM) generation (Rada *et al.*, 2002). Recessive mutations of the gene encoding UNG are linked to defects in CSR and the SHM pattern in patients with hyper-IgGM syndrome. UNG deficient mice were shown to have a highly elevated risk, 22 fold, of developing B-cell lymphoma (Andersen *et al.*, 2005). In mice, the single knockouts of *Ogg1* and *MutY* appear normal with no obvious cancer phenotype. However, the double knockout *Ogg1*/*Mutyh* develop lung tumors (Xie *et al.*, 2004). Polymorphisms in BER genes are associated with increased risk for certain cancers (Xu *et al.*, 2008), however, conflicting reports necessitate functional studies of these polymorphisms. Accumulating evidence also suggests that BER might play a fundamental role in the development of age-related neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD) (Yang *et al.*, 2008;de Souza-Pinto *et al.*, 2008;Xu *et al.*, 2008;Coppede and Migliore, 2010a).

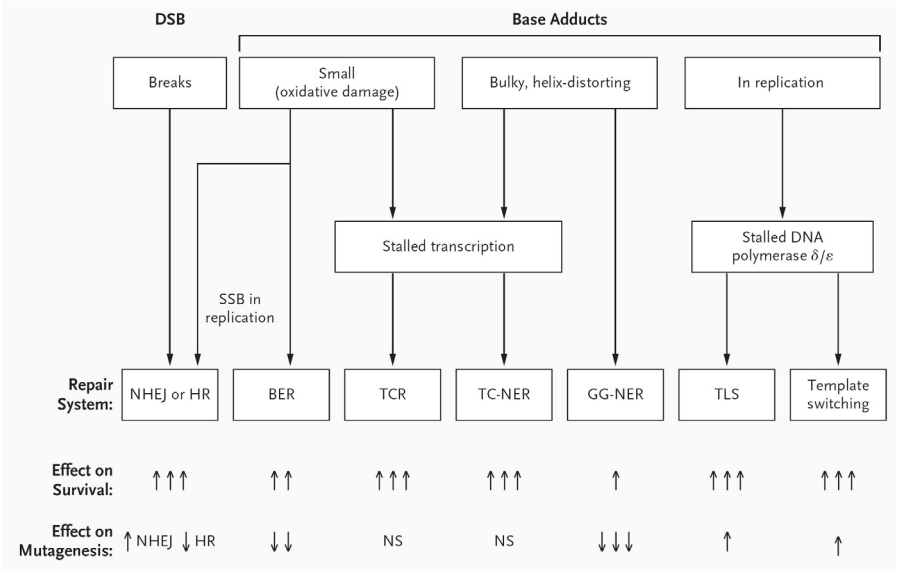

Figure 2 **DNA lesions, Corresponding DNA repair Maintenance Systems, and their Effect on Cellular Survival and Mutagenesis**. Double-strand breaks (DSBs) in DNA are highly cytotoxic and cytostatic forms of damage. They are repaired through nonhomologous end-joining (NHEJ), which simply joins the ends of DNA strands and is associated with an elevated risk of mutagenesis, or through homologous recombination (HR), which takes place after replication and uses the intact copy on the sister chromatid to properly align and seal the broken ends in an error-free manner. HR is also involved in bypassing interstrand cross-links (not shown) and in repairing single-strand breaks (SSBs) and blocking lesions encountered during replication. In mammals, NHEJ is important for the repair of somatic (differentiated) cells and proliferating cells in the G1 stage, whereas HR is important for early embryogenesis and repair of proliferating cells in the S or G2 stage. NHEJ promotes cellular survival in the presence of highly cytotoxic DSBs and may thereby enhance mutagenesis. HR also promotes cellular survival, but without inducing mutations. Base-excision repair (BER) is involved with small DNA adducts (mainly oxidative and alkylating lesions), some of which may be highly mutagenic (e.g., 7,8-dihydro-8-oxoguanine), and some cytotoxic. When these lesions block elongating RNA polymerase, transcription-coupled repair (TCR) removes the damage, allowing the vital transcription to resume. BER prevents mutagenesis and promotes cellular survival. Transcription-coupled nucleotide-excision repair (TC-NER) is specific to transcription-blocking bulky adducts, which are eliminated throughout the entire genome by the global genome nucleotide-excision repair (GG-NER) system. DNA damage that blocks the regular replication machinery involving DNA polymerase δ/ε (e.g., breaks and cross-links) can be repaired, bypassed by homologous recombination, which involves template switching and strand displacement, or bypassed by translesional synthesis (TLS), a specialized, relatively error-free (but still somewhat mutagenic) means of bypassing a specific subgroup of lesions. Arrows pointing upward indicate increases in cell survival or mutagenesis after DNA damage, and arrows pointing downward indicate decreases; the greater the number of arrows, the stronger the effect. NS denotes no significant effect. Figure and legend from (Hoeijmakers, 2009)).

Homologous recombination and non-homologous end-joining repair various types of double strand breaks ((Huertas, 2010) and Figure 1 and 2). NHEJ, which accounts for about 90% of the DSB repair, is highly efficient in ligating DNA ends. However, the repair is relatively error prone as it might involve loss or addition of bases as the joining occurs (Lieber, 2008). This

inaccurate process takes place mostly before replication, in the absence of an identical copy of DNA. NHEJ is also acting upon V(D)J and class-switch recombination intermediates (Rooney *et al.*, 2004;Chaudhuri and Alt, 2004). Inactivation of essential components of NHEJ in mice results in a severe combined immunodeficiency (SCID) phenotype (Bosma *et al.*, 1983;Blunt *et al.*, 1995;Kirchgessner *et al.*, 1995). In the S or G2 phase of the cell cycle, HR uses the identical sister chromatid to align the broken ends and accurately insert missing information through a set of pathways (Krogh and Symington, 2004). This second DSB repair mechanism is also dealing with stalled and collapsed replication forks (Aguilera and Gomez-Gonzalez, 2008). Defects in HR cause the human syndromes AT (ataxia telangiectasia)-like disorder (ATLD) and Nijmegen Breakage Syndrome (NBS), and predisposition has been linked to mutations in the MRN (MRE11/RAD50/NBS1) complex (Thompson and Schild, 2002). ATLD is characterized by progressive neurodegeneration, whereas NBS is characterized by microcephaly, growth retardation, immunodeficiency and predisposition to tumors (Taylor *et al.*, 2004;Czornak *et al.*, 2008). The RecQ helicases are required for efficient HR, and mutations in the RecQ helicase genes are associated with Werner Syndrome (WS), Rothmund-Thomson syndrome (RTS) and Blooms syndrome (BS) (Hickson, 2003). WS and RTS are characterized with a premature aging, BS patients have a strong predisposition to cancer (Bohr, 2008). Defects in DSB repair genes cause embryonic lethality, developmental disorders, sterility, immune deficiencies, and predisposition to neurodegenerative diseases and cancer (Phillips and McKinnon, 2007).

1.2.1 The nucleotide excision repair (NER) pathway

Key enzymes in NER sense the presence of a lesion through the distortion of the DNA helix structure, and there is not a collection of specific enzymes each recognizing a different lesion, as is the case for BER (de Laat *et al.*, 1999;Batty and Wood, 2000). The helix distorting lesions sensed by NER can be structurally unrelated, such as UV-induced photoproducts and numerous bulky DNA adducts induced by mutagenic chemicals from the environment or by cytotoxic drugs used in chemotherapy (Nouspikel, 2009). Two major UV photolesions repaired by NER are cyclobutane pyrimidine dimer (CPD) and the pyrimidine-pyrimidone (6-4) photoproduct (6-4PP). Benzo(a)pyrene DNA adducts induced by cigarette smoke and lesions formed by chemical carcinogens, like cisplatin, are also recognized and removed by NER (Wood, 1996;Sancar, 1996;Friedberg *et al.*, 2006). After sensing the lesion, the damaged strand is identified, and a short (24 to 32 nucleotides long) oligonucleotide spanning the lesion is excised, leaving a gap that is filled by the replicative polymerases (Figure 3). Thus,

many different lesions can be handled by a common set of enzymes, a sequential action involving over 30 proteins. Depending on whether the damage occurs in a transcriptionally inactive or active domain, repair can occur by two sub-pathways: global genomic repair (GGR) or transcription-coupled repair (TCR) (Friedberg, 1996). GGR and TCR differ in their mode of damage recognition; however, all subsequent steps are common to these two repair pathways (Figure 3). TCR ensures that the transcribed strand of active genes is repaired with higher priority than the rest of the genome, probably by using RNA polymerase II (RNAPII) as a lesion sensor (Mellon *et al.*, 1986). In GGR in human cells, the heterodimer XPC/HR23B (XPC in figure 3) appears to be the major damage recognition factor, detecting the helix distortion and stabilizing the DNA bend. XPC/HR23B recruits transcription factor TFIIH, a ten-subunit complex (including XPB, XPD and TTDA), at the site of the lesion (Figure 3). Upon ATP addition, TFIIH unwinds the DNA helix, until one of its helicase subunits (XPD) encounters a chemically modified base; the second helicase subunit (XPB) goes on unwinding the DNA to create a 20 base pair opened "bubble" structure. RPA, XPA and XPG are then recruited to assemble the "preincision" complex; ERCC1-XPF joins the complex and the dual incision (5' by ERCC1-XPF and 3' by XPG) occurs. RPA remains bound to the ssDNA and facilitates the transition to repair-synthesis by POL δ(or ε) supported by RFC and PCNA; ligase I finally seals the nick (Gillet and Scharer, 2006).

Hereditary defects in NER are associated with several human autosomal recessive genetic disorders, such as xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) (Lehmann, 2001;Bootsma *et al.*, 2001). These three syndromes are all characterized by UV sensitivity, however, they display complex and varying pathologies that involve most systemic and neural tissues and organs (Cleaver *et al.*, 2009). XP patients suffer from a high incidence of skin cancer, more than 1,000 times as high as the incidence in the general population (Benhamou and Sarasin, 2000). Mutations in 13 genes (*XPA-G*, where *DDB1* and *DDB2* encode two subunits of the XPE complex, *ERCC1*, *XPV*, *CSA*, *CSB*, *TTDA*) associated with NER cause a wide range of clinical symptoms, from mild solar sensitivity to severe skin cancers, developmental disorders and neurodegeneration (Kraemer *et al.*, 1987;Cleaver, 2005). The sites of mutations in the NER genes, protein-protein interactions, the regulation of protein expression and turnover, and external or endogenous damage are all modulators of the phenotypic features of NER diseases (Cleaver *et al.*, 2009). Mutations in *XPC* or *XPE*, which exclusively affect the GGR pathway, are associated with skin cancer, whereas mutations in the *CSA* and *CSB* in the TCR pathway contribute to more

complex developmental and neurological disorders (Cleaver, 2005). Mutations in *XPD*, which lies at the convergence of the GGR and TCR pathways, are associated with the most varied and complex combinations of clinical features (Cleaver *et al.*, 2009).

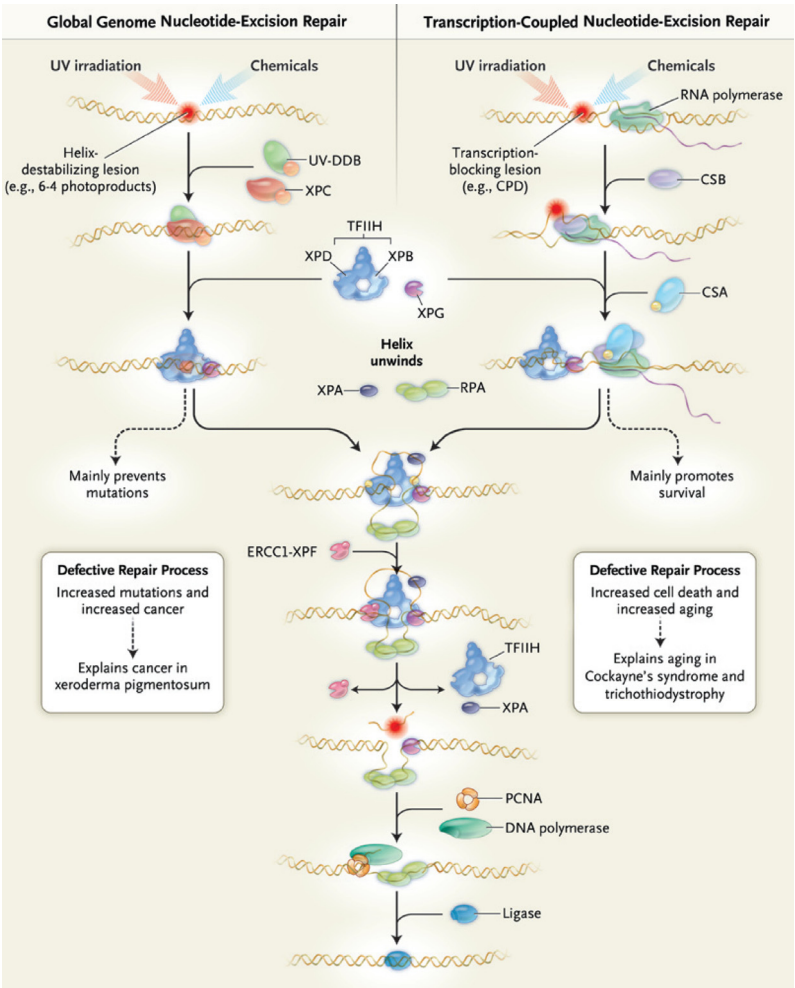

Figure 3 **Molecular mechanisms of nucleotide excision repair**. Damage to DNA that occurs anywhere in the genome (e.g., photoproducts resulting from exposure to ultraviolet [UV] radiation) is recognized by the XPC and XPE (or UV-DDB) protein complexes, which are specific components of the global genome nucleotide-excision repair (NER) system. Damage that actually blocks transcription (e.g., cyclobutane pyrimidine dimers [CPDs] resulting from exposure to UV radiation) is detected by the transcription-coupled NER system (TC-NER) system, which involves the CSB and CSA proteins. The DNA helix is opened by the XPB and XPD helicases of the repair and transcription factor IIH (TFIIH), allowing damage verification by the XPA protein. Single-strand binding protein RPA prevents reannealing, and dual incisions in the damaged strand are made by the ERCC1-XPF and XPG endonucleases, excising the damage as part of a piece of 25 to 30 bases. The single-strand gap is filled by the replication machinery, and the final nick sealed by DNA ligase. (Figure and legend from (Hoeijmakers, 2009)).

1.2.2 Transcription coupled NER, TCR

When DNA damage is located in the transcribed regions of the genome, a unique problem arises. Mistakes may be introduced at a miscoding or non-informational damage site if RNA polymerase (RNAP) continues transcribing its product past a lesion, potentially leading to transcriptional mutagenesis in a non-dividing cell (Saxowsky and Doetsch, 2006). A lesion may alter the expression level of a gene, through altering the transcription rate, some lesions could transiently arrest the elongation process, while others may arrest the RNAP complex completely (Tornaletti, 2009). The arrested RNAP complex may not be stable, resulting in release of the arrested polymerase and nascent RNA product, or if stable, represent a strong signal for accumulation of p53 and apoptosis (Yamaizumi and Sugano, 1994;Ljungman and Zhang, 1996;Derheimer *et al.*, 2007).

In TCR, lesion recognition occurs through arrest of the elongating RNAPII when it encounters the damage. Briefly, the stalled RNAPII at the damage site is recognized by CSB which in turn recruits CSA. Then the arrested RNAPII transcript, assisted by CSA and CSB, attracts core NER repair factors which remove the lesion ((Altieri *et al.*, 2008) and Figures 3 and 4). TCR operates on bulky lesions like UV-induced CPDs (Mellon *et al.*, 1987) and helix distorting DNA damage like cisplatin DNA crosslinks (Zhen *et al.*, 1993) and DNA adducts formed by benzopyrene-diolepoxide (Chen *et al.*, 1992). Oxidative lesions and non-bulky DNA adducts, like abasic sites and their oxidative derivatives may be sufficient to initiate TCR *in vivo*, however, the current model postulate that only lesions that block RNAPII will be subject to TCR (Tornaletti, 2009). The *in vitro* and *in vivo* TCR studies performed so far have shown various levels of RNAPII bypass of 8-oxoG and thymine glycol (Tg), suggesting that other factors, such as the source of RNAPII; the sequence context and/or the transcription system might play a role in the extent of lesion bypass (Larsen *et al.*, 2004;Hanawalt and Spivak, 2008;Tornaletti, 2009).

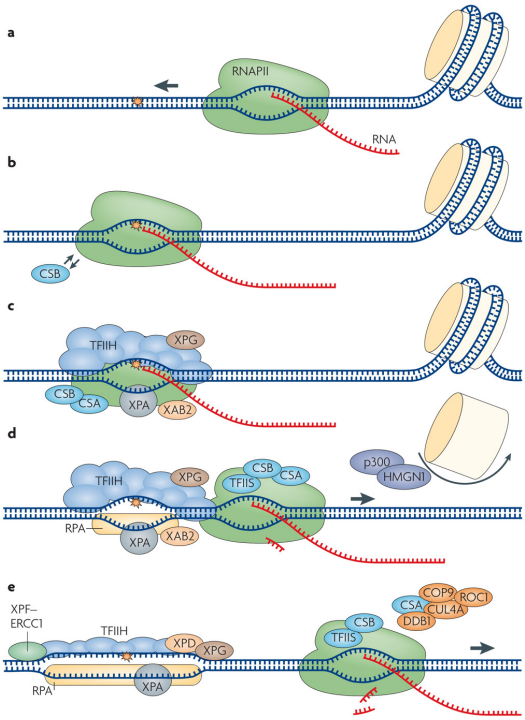

Figure 4 **A suggested scenario for initiation of repair through TCR**. **a** As RNA polymerase-II (RNAPII) translocates along the DNA, nucleosomes are dislodged in front of the polymerase and reassembled behind it. **b** Transcription is arrested when RNAPII encounters an obstacle. **c** Cockayne syndrome type B protein (CSB) becomes tightly bound to the arrested RNAPII and recruits factors that are needed to accomplish transcription-coupled repair. TFIIH localizes to the arrested elongation complex with xeroderma pigmentosum complementation group G (XPG) and XPA (which is possibly brought to the scene by XPA-binding protein-2 (XAB2)); replication protein A (RPA) arrives simultaneously or shortly thereafter. **d** The chromatin remodelling factors high-mobility group nucleosome-binding domain-containing protein-1 (HMGN1) and p300 loosen the nucleosome structure behind the polymerase; RNAPII reverses direction, backtracking from the obstacle and degrading the nascent RNA product through its cryptic 3′–5′ exonuclease activity, which is activated by TFIIS. TFIIH with associated XPG, XPA and RPA remain at the site of the obstacle, possibly maintaining the bubble of denatured DNA, but without the RNA–DNA hybrid. XPA and RPA bind the single-stranded DNA in the vicinity of the obstruction, providing lesion verification and strand specificity before the next steps. **e** Once RNAPII has backtracked, TFIIH extends the denatured region around the lesion to ~30 nucleotides, thus setting up the substrate for the subsequent DNA nicking by the structure-specific endonucleases XPG and the XPF–ERRC1 complex. CSA, as a component of a cullin-containing ubiquitylation E3 ligase complex, might facilitate resumption of transcription (once the repair process has been completed) by removing or deactivating factors, including CSB. The sizes of the respective proteins and complexes, and the expected distance of RNAPII backtracking are not drawn to scale, nor do they indicate their respective footprints on the DNA substrate. Abbreviations: CUL4A, cullin-4A; DDB1, DNA damage-binding-1. (Figure and legend from (Hanawalt and Spivak, 2008)).

1.2.3 Cockayne syndrome and the CSA protein

Cockayne syndrome (CS) is a very rare autosomal recessive neurodegenerative disorder characterized by severe postnatal growth failure, photosensitivity, microcephaly, psychomotor delay, retinal degeneration, sensorineural deafness and lipoatrophy (Nance and Berry, 1992;Pasquier *et al.*, 2006;Laugel *et al.*, 2009). CS belongs to the family of NER disorders, and CS cells are specifically defective in TCR. Mutations in *CSA* (also known as *ERCC8*) or *CSB* (also known as *ERCC6*), identified in 1995 and 1992, respectively, are responsible for most cases of CS (Troelstra *et al.*, 1992;Troelstra *et al.*, 1993;Henning *et al.*, 1995). Clusters of missense mutations in *CSA* and *CSB* can be recognized and highlight the role of particular motifs in the proteins, however, many types of mutations are scattered along the whole coding sequence of both genes (Laugel *et al.*, 2009). Rare cases of mixed XP/CS phenotypes have been linked to specific mutations in the *XPB*, *XPD* and *XPG* genes. These XP/CS patients show a specifically severe clinical photosensitivity, cancer-proneness and have a combined defect in the TCR and GGR pathways, causing mutagenesis and cancer in some tissues and accelerated cell death and premature aging in others (Andressoo and Hoeijmakers, 2005). In patients (Weidenheim *et al.*, 2009), and mouse models (Laposa *et al.*, 2007), the Purkinje cells of the cerebellum seem to be sensitive to degeneration and contains many markers of DNA damage. In contrast to CS individuals, *Csa*- and *Csb*- mutant mice are prone to UV-induced skin cancer (van der Horst *et al.*, 1997;van der Horst *et al.*, 2002). The average life span for CS patients is 12 years (Andressoo and Hoeijmakers, 2005).

The *CSA* gene, located on chromosome 5q11, encodes a 44kDa protein of 396 amino acids with seven predicted WD-40 repeats (Henning *et al.*, 1995;Zhou and Wang, 2001). Trp-Asp (WD) amino acid residues are usually found at the end of the motif and the WD repeat proteins are thought to form circularized beta propeller structures in which the repeat units may serve as a scaffold for protein-protein interactions (Li and Roberts, 2001). CSA has been shown to interact with CSB, XAB2, and the p44 subunit of TFIIH (Henning *et al.*, 1995;Nakatsu *et al.*, 2000). The E3 ubiquitin ligase complex contains CSA (Groisman *et al.*, 2003), and this complex is required for the recruitment of other ancillary NER factors to the repair site ((Fousteri *et al.*, 2006) and Figure 4) and is probably able to trigger the degradation of CSB at a late stage of the TCR process (Groisman *et al.*, 2006). After UV-irradiation, and in the presence of functional CSB and TFIIH, the CSA protein is translocated to the nuclear matrix (Saijo *et al.*, 2007) and colocalizes with the hyperphosphorylated form of RNAPII stalled at the lesion. CSA is also involved in the response to oxidative stress and

contributes to prevent the accumulation of various oxidized bases *in vivo* (Frosina, 2007;D'Errico *et al.*, 2007;Nardo *et al.*, 2009).

1.2.4 The base excision repair (BER) pathway

The multistep BER pathway is the main pathway for correcting nonbulky single-base lesions, AP sites and single-strand breaks in DNA. Such lesions are introduced by reactive oxygen species, methylation, deamination and hydroxylation (Lindahl, 1993;Seeberg *et al.*, 1995;Dalhus *et al.*, 2009). The BER pathway is responsible for removal of more than ten thousand DNA lesions daily in each human cell (Lindahl, 1993). Two sub-pathways exist, short-patch (SP) BER and long-patch (LP) BER ((Robertson *et al.*, 2009) and Figure 5). The SP-BER replaces a single nucleotide, whereas the LP-BER sub-pathway results in the incorporation of 2-13 nucleotides (Kubota *et al.*, 1996;Klungland and Lindahl, 1997). The core BER pathway requires a damage-specific DNA glycosylase, an AP endonuclease or AP DNA lyase, a DNA polymerase, and a DNA ligase. BER can be initiated in three ways, either i) by a glycosylase removing a modified or mismatched base, ii) by non-enzymatic hydrolytic depurination leading to base loss, as well as iii) by SSBs with 3' and 5' ends that require processing prior to ligation (Krwawicz *et al.*, 2007). To date, 11 different mammalian DNA glycosylases have been identified. Some are highly specific whereas other recognize apparently unrelated types of base lesions (Dalhus *et al.*, 2009;Robertson *et al.*, 2009). Some DNA glycosylases possess an additional intrinsic AP lyase activity which cleaves the DNA chain 3' to the AP site forming a 5' phosphate and a 3'-fragmented deoxyribose. The resulting cytotoxic and mutagenic AP site needs to be further processed. AP endonuclease 1 (APE1) is the major AP endonuclease in mammalian cells (Loeb, 1985). APE1 produces a nick in the backbone of the phosphodiester bond 5' to the AP site, which creates a 5'-deoxyribose phosphate (dRP) group that is cleaved by DNA polymerase β (Allinson *et al.*, 2001). In humans, a second AP endonuclease has been identified, APE2. APE2 show much lower endonuclease activity than APE1 (Hadi *et al.*, 2002). An AP endonuclease independent BER pathway has also been identified. Here, polynucleotide kinase (PNK) is processing the gap left by the mammalian DNA glycosylases NEIL1 and NEIL2 (Wiederhold *et al.*, 2004;Das *et al.*, 2006).

The major polymerase in BER is POLβ, which mainly insert single-nucleotides. However, POLβ is also able to insert the first nucleotide in LP-BER (Podlutsky *et al.*, 2001;Beard *et al.*, 2006). LP-BER DNA synthesis is mainly carried out by DNA polymerase δ (POLδ), a high fidelity replicative DNA polymerase with intrinsic exonuclease (proof-reading) activity, but

also POLε has been implicated, together with POLβ (Stucki *et al.*, 1998;Podlutsky *et al.*, 2001;Dianov *et al.*, 2003;Asagoshi *et al.*, 2010). The 5' single-strand DNA displaced by the polymerase in LP repair is removed by the flap structure specific endonuclease 1 (FEN1) (Klungland and Lindahl, 1997). Finally the newly synthesized DNA is sealed by DNA ligase III in SP and ligase I in LP-BER (Mortusewicz *et al.*, 2006;Fortini and Dogliotti, 2007).

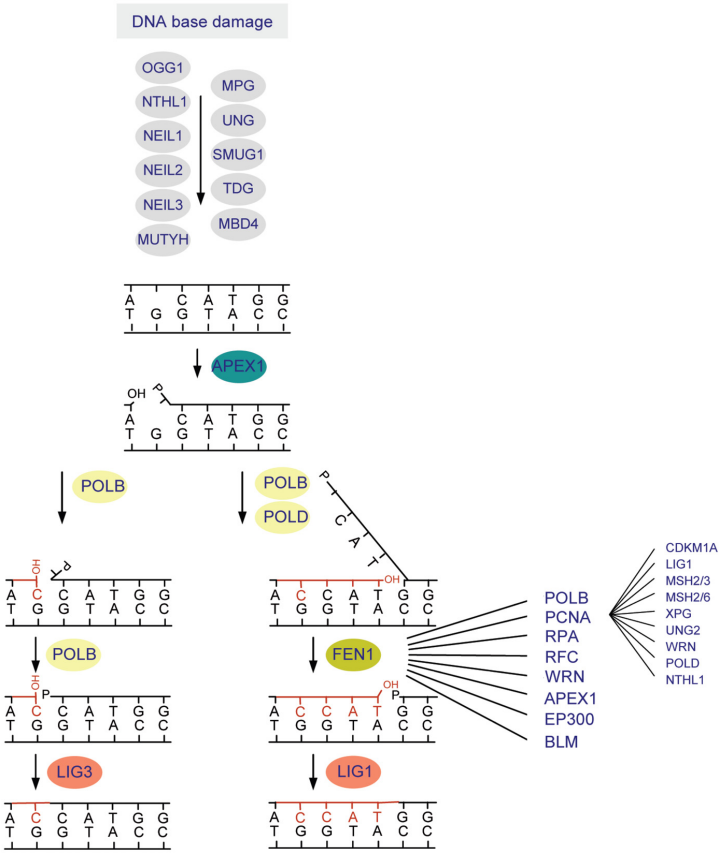

Figure 5 **Selected protein interactions in the BER pathway**. This figure shows the plethora of proteins and DNA interactions in both the short-patch (left branch) and long-patch (right branch) mammalian BER pathways. OGG1, 8-oxoguanine-DNA glycosylase; NTHL1, nth endonuclease III-like 1; NEIL, nei endonuclease VIII-like; MUTYH, muty homolog; MPG, N-methylpurine-DNA glycosylase; UNG, uracil-DNA glycosylase; SMUG1, single-strand-selective monofunctional uracil-DNA glycosylase; TDG, thymine-DNA glycosylase; MBD4, methyl-CpG binding domain protein 4; APEX1, APEX nuclease 1; POL, DNA polymerase; FEN1, flap structure-specific endonuclease 1; LIG,DNA ligase; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; RFC, replication factor C; WRN, Werner syndrome protein; EP300, E1A binding protein (alias p300); BLM, bloom syndrome protein; CDKM1A, cyclin dependent kinase inhibitor1A (alias p21); MSH,mutS homolog, ERCC5, excision repair cross-complementing rodent repair deficiency protein 5 (alias XPG). Gene names by www.genenames.org. (Figure and legend from (Robertson *et al.*, 2009)).

1.2.5 Flap structure-specific endonuclease 1 (FEN1)

Flap structure specific endonuclease 1 (FEN1) is best known for its involvement in RNA primer removal during Okazaki fragment processing in lagging strand DNA replication, and for its 5'-flap cleavage in LP-BER (Figure 5) (Harrington and Lieber, 1994;Klungland and Lindahl, 1997;Bambara *et al.*, 1997;Lieber, 1997;Liu *et al.*, 2004). FEN1 is both a structure specific 5'-flap endonuclease and a 5'-3-exonuclease, and it has also been shown to possess a gap endonuclease activity (Harrington and Lieber, 1994;Murante *et al.*, 1994;Parrish *et al.*, 2003;Zheng *et al.*, 2005). The preferred substrate for FEN1 is a double flap structure containing a 1-nucleotide 3'-tail on the upstream primer adjacent to the 5' flap (Kaiser *et al.*, 1999;Storici *et al.*, 2002;Kao *et al.*, 2002;Friedrich-Heineken *et al.*, 2003;Finger *et al.*, 2009). The multiple biochemical activities of FEN1 have been reviewed recently (Shen *et al.*, 2005;Zheng *et al.*, 2011b).

In higher organisms, FEN1 has a C-terminal conserved domain mediating interaction with proliferating cell nuclear antigen (PCNA), the "DNA sliding clamp" which stimulates FEN1 by enhancing FEN1's binding stability and cleavage efficiency *in vitro* and *in vivo* (Li *et al.*, 1995;Klungland and Lindahl, 1997;Gary *et al.*, 1999;Tom *et al.*, 2000;Gomes and Burgers, 2000).

FEN1 belongs to class II of the XPG/RAD2 family of structure-specific nucleases evolutionary conserved between Archaea and Eukarya (Lieber, 1997;Ceska and Sayers, 1998;Shen *et al.*, 1998;Ip *et al.*, 2008). The key amino acid residues involved in substrate binding and enzyme catalysis are highly conserved (Shen *et al.*, 1998).

Recently, the crystal structure of human FEN1 complexed with PCNA was reported (Sakurai *et al.*, 2005), this was the first structure of eukaryotic FEN1 (Figure 6). In the proposed model a hinge region present between the core domain and the C-terminal tail of FEN1 plays a role in switching the orientation of FEN1 from an active into an inactive orientation. In an inactive/locked-down orientation, maintained by interactions on the interfaces, rapid tracking of DNA can occur with the central hole of PCNA for sliding along DNA preserved. In the sequential processes of base excision repair and DNA replication, the PCNA trimer also stimulates DNA polymerase δ/ε and DNA ligase I, and FEN1 needs to remove its core domain from the excision point after flap cleavage, utilizing its hinge region, to provide access for incoming proteins, such as DNA ligase 1 (Sakurai *et al.*, 2005).

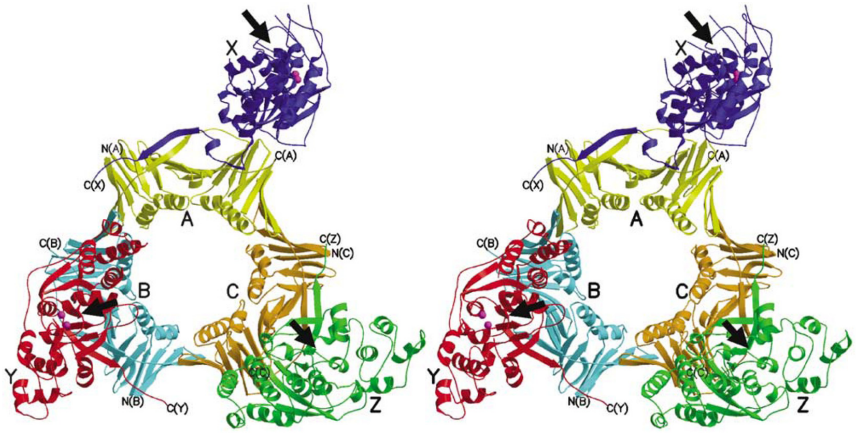

Figure 6 A **stereo view of the human FEN1–PCNA** complex. Three FEN1 molecules are colored in blue (X), red (Y) and green (Z), and the three subunits of the PCNA trimer in yellow (A), cyan (B) and orange (C). The C-termini of FEN1 and PCNA are labeled. Metal ions bound to the active sites of FEN1 (X and Y) are shown in magenta. Proposed catalytic faces of FEN1 are indicated by arrows. (Figure and legend from (Sakurai *et al.*, 2005)).

In the C-terminal of eukaryotic FEN1, the very last 26 amino acids represent the motif responsible for nuclear localization of the protein. In mammals, FEN1 is the only known nuclear flap endonuclease. A few years ago, LP-BER was discovered also in mitochondria along with 5' exo/endonuclease activity (Liu *et al.*, 2008;Szczesny *et al.*, 2008;Akbari *et al.*, 2008). Until recently, mitochondria were thought to have only SP-BER, and although the studies disagree on some details, they all detect LP-BER in mitochondria. In mitochondrial extracts from HeLa and HaCaT cells, generation and removal of 5' flaps as in LP-BER was identified, whereas FEN1 was not (Akbari *et al.*, 2008). On the contrary, mitochondrial extract from human lymphoblasts was found to contain FEN1, and FEN1 was proposed to have a role in mitochondrial LP-BER (Liu *et al.*, 2008). The third lab, which prepared mitochondrial extracts from mouse tissue and human colon cancer HCT116 cells, observed an unidentified LP-BER 5' exo/endonuclease activity. The activity could not be credited FEN1, however, FEN1 was observed in the mitochondria and could cut 5' flaps, e.g. generated during DNA synthesis (Szczesny *et al.*, 2008). The missing 5' flap endonuclease was then found by Zheng and colleagues (Zheng *et al.*, 2008), who show that the helicase/nuclease hDNA2 participates, together with FEN1, in human mitochondria LP-BER. DNA2 was originally identified in yeast as a nuclear DNA helicase with endonuclease activity, processing 5' flaps together with FEN1 (Budd and Campbell, 1997). A few years ago, DNA2 was also

identified in the nucleus of human cells, and the conserved enzymatic activities of DNA2 function in replication and double strand break repair in both mitochondria and nucleus of human and yeast cells (Duxin *et al.*, 2009;Budd *et al.*, 2011;Fortini *et al.*, 2011).

The important role of FEN1 (RAD27) in DNA metabolism is demonstrated by the severe biological effects upon loss of it (Reagan *et al.*, 1995;Kucherlapati *et al.*, 2002;Larsen *et al.*, 2003). In yeast, *RAD27* null mutants are conditionally lethal at high temperatures, with a defect in DNA replication. Mutants show sensitivity to UV radiation and alkylation agents and also deficiencies in telomere maintenance (Reagan *et al.*, 1995;Sommers *et al.*, 1995;Parenteau and Wellinger, 1999). In mice, deletion of both *Fen1* alleles results in early embryonic lethality, with death at the blastocyst stage (Larsen *et al.*, 2003). Haploinsufficiency of *Fen1* in combination with a mutation in the adenomatous polyposis coli (*Apc*) gene results in a mild tumor predisposition phenotype (Kucherlapati *et al.*, 2002).

The *RAD27* deletion strains are strong mutators with destabilized repetitive sequences, and based on these results in yeast, it was suggested that mammalian FEN1 was involved in mechanisms through which trinucleotide repeat (TNR) expansions occur (Gordenin *et al.*, 1997;Spiro *et al.*, 1999). In more than 30 human neurological, neurodegenerative and neuromuscular diseases repeat expansion has been identified as a cause of the disease. These includes Huntington disease (CAG/CTG), myotonic dystrophy type 1 (CTG/CAG), Friedreich ataxia (GAA/TTC), Fragile X syndrome (CGG/CCG), and many others (Paulson and Fischbeck, 1996;Pearson *et al.*, 2005;Lopez *et al.*, 2010). CAG repeat instability is also associated with human cancer (Andersson *et al.*, 2006;Schildkraut *et al.*, 2007). A linkage between repeat expansion in brain and oxidative DNA damage has been established, in a study where OGG1 was found to initiate age-dependent CAG repeat expansion in somatic cells (Kovtun *et al.*, 2007). Recent studies show that FEN1 can promote CAG repeat expansion, if the coordination of POLβ and FEN1 is disturbed during LP-BER (Liu *et al.*, 2009;Goula *et al.*, 2009). Tissue specific levels of these BER proteins may explain the increased CAG instability observed in striatum compared to the cerebellum in Huntington disease (HD) mice (Goula *et al.*, 2009).

FEN1 has also been implicated in non-homologous end joining (Wu *et al.*, 1999), microhomology-mediated end-joining (MHEJ) (Liang *et al.*, 2005), telomere stability (Parenteau and Wellinger, 1999;Parenteau and Wellinger, 2002;Saharia *et al.*, 2008;Vallur

and Maizels, 2010), recombination (Negritto *et al.*, 2001;Kikuchi *et al.*, 2005), HIV replication (Rumbaugh *et al.*, 1998;Brin *et al.*, 2000;Faust and Triller, 2002) and apoptosis (Parrish *et al.*, 2003).

1.3 *IN VIVO* IMAGING

The cloning of genes encoding fluorescent proteins, e.g. green fluorescent protein (GFP) from jellyfish *Aequorea victoria* (Shimomura *et al.*, 1962), and the engineered fluorescent protein derivatives have allowed detailed studies of protein expression and mobility by fluorescence microscopy (Chalfie *et al.*, 1994;Tsien, 1998). Current advances in applications of optical techniques together with expression of fluorescent proteins have allowed amazing localization and quantification studies in live cells (Waters, 2007;Day and Schaufele, 2008;Wang *et al.*, 2008). Fluorescence microscopy of GFP-tagged proteins in intact living cells allows the study of dynamic cellular processes under physiological conditions, which represents an immense advantage to fixed cells (Essers *et al.*, 2006;Giepmans *et al.*, 2006). A wide range of genetically encoded fluorescent proteins are available for live cell imaging, ranging from deep blue to deep red of the visible spectrum ((Day and Schaufele, 2008) and Figure 7). Thus, several proteins can be visualized simultaneously using fluorescent proteins from different parts of the spectrum, and protein-protein interactions can be monitored. Moreover, some of the fluorescent proteins have unusual characteristics that make them useful reporters of the dynamic behavior of proteins inside cells (Day and Schaufele, 2008).

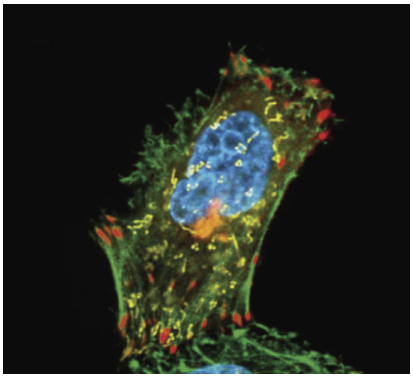

Figure 7 **Multicolor labeling, living HeLa cells**, Olympus FV1000 confocal microscope. TagBFP-H2B (blue), TagGFP2-actin (green), phiYFP-mito (yellow), TagRFP-golgi (orange), mKate2-zyxin (red). (Figure and legend from (Chudakov *et al.*, 2010).)

1.3.1 Yellow fluorescent protein (YFP) and fluorescent internal markers in living cells

Enhanced yellow fluorescent protein (EYFP) is one of many mutant forms of the *Aequorea victoria* GFP. The *EYFP* gene contains four amino acid substitutions previously published as GFP-10C (Ormo *et al.*, 1996). The fluorescence excitation maximum of EYFP is 513 nm, and the emission spectrum has a peak at 527 nm, which is in the yellow-green region. EYFP gives a bright fluorescent signal. The barrel structure made from the 11 β-sheets of EYFP protects the fluorophore, and the molecule is photostable. In addition to the chromophore mutations, *EYFP* contains >190 silent mutations that create an open reading frame comprised almost entirely of preferred human codons (Haas *et al.*, 1996;Yang *et al.*, 1996). Furthermore, upstream sequences flanking *EYFP* have been converted to a Kozak consensus translation initiation site (Kozak, 1987). These changes increase the translational efficiency of the *EYFP* mRNA and consequently increase the expression of EYFP in mammalian and plant cells (Tsien, 1998). EYFP with N-terminal fusion moieties retains the fluorescent properties of the native protein and thus can be used to localize fusion proteins *in vivo*. In 2008, the Nobel prize in chemistry was awarded professors Shimomura, Chalfie and Tsien for "the discovery and development of the green fluorescent protein, GFP". Applications for the fluorescent proteins range from fusion proteins designed to monitor intracellular dynamics and organelle-targeted markers to reporters of transcriptional regulation and *in vivo* probes for whole-body imaging and detection of cancer ((Gross and Piwnica-Worms, 2005) and Figure 8). Fluorescent proteins have facilitated the engineering of highly specific biosensors to visualize intracellular processes including protein kinase activity, apoptosis, membrane voltage, cyclic nucleotide signaling, tracing neuronal pathways, pH and metal–ion concentration (Lalonde *et al.*, 2005;Li *et al.*, 2006;Wang *et al.*, 2008;Stepanenko *et al.*, 2008). Recent achievements using fluorescent proteins include the Brainbow project, an elegant experiment where individual neurons in the brain are mapped with fluorescent proteins and fluoresce with a distinct color ((Livet *et al.*, 2007) and Figure 9) and the tracking of adult stem cells, recently reviewed (Snippert and Clevers, 2011).

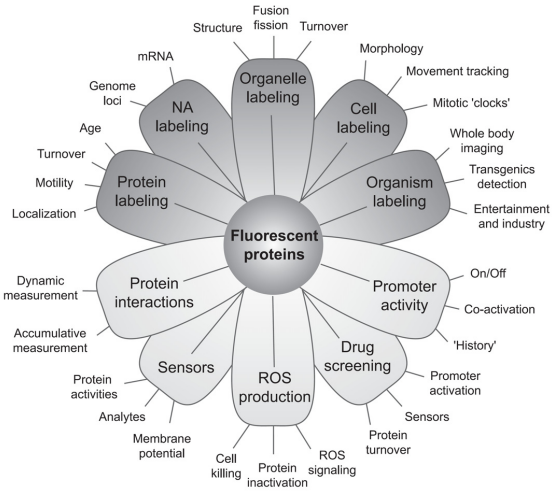

Figure 8 A **Main areas of applications of fluorescent proteins.** Dark gray and light gray petals show structural and functional studies, respectively, although boundaries between them are often quite fuzzy. (Figure and legend from (Chudakov *et al.*, 2010)).

1.3.2 Fluorescence microscopy

Fluorescent microscopes use a specific wavelength of light to excite fluorochromes (Lichtman and Conchello, 2005). In the biological field, antibodies are labelled with fluorochromes and used to tag cellular structures. Emitted fluorescence is passed through an emission filter and collected using a cooled charge-coupled device (CCD) camera ((Lichtman and Conchello, 2005) and Figure 9 *Left*). Thick samples will cause out-of-focus light to blur images making it difficult to resolve fine details. Confocal microscopy uses a pinhole to block out of focus light and therefore increase optical resolution ((Furrer and Gurny, 2010) and Figure 9 *Middle*). The light source is usually a single wavelength laser allowing tight illumination focus (Furrer and Gurny, 2010). Samples can be optically sectioned (z stacks) and the resulting images can then be reconstructed into a 3D data set (Brakenhoff *et al.*, 1985;Conchello and Lichtman, 2005). Multiphoton fluorescence microscopy allows optical sectioning of thick samples using two photons of light and leads to excitation only at the focal point ((Denk *et al.*, 1990;Hadjantonakis *et al.*, 2003) and Figure 9 *Right*). Therefore, all light collected by the system must be from the plane of focus. Because two-photon emission must inherently occur at a discrete point, pinholes are not required (Nowotschin *et al.*, 2009). Using longer wavelengths (near infra red) provides several benefits including less phototoxicity and deeper penetration, allowing imaging around 500 microns into a sample (Zipfel *et al.*, 2003;Helmchen and Denk, 2005;Makale *et al.*, 2009;Andresen *et al.*, 2009;Ntziachristos, 2010). Live cell imaging is made possible by including an incubation

chamber which tightly controls the environment of the cells and keep living cells in a healthy state during long term imaging (Baker, 2010). Investigation, e.g. by multiphoton microscopy, of three-dimensional cultures like organotypic explants can elucidate essential cellular functions like tissue-specific architecture, mechanical and biochemical signals and cell–cell communication, which is lost in monolayer "petri-dish" cell cultures (Pampaloni *et al.*, 2007).

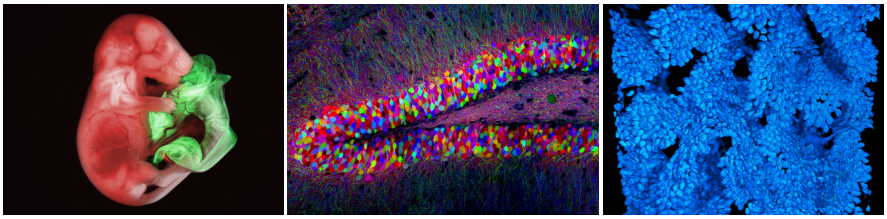

Figure 9 **Fluorescence microscopy images**. *Left* A widefield microscopy image showing a double transgenic mouse embryo, 18.5 days (17x). The image was captured using brightfield as well as green and red fluorescent filters in darkfield (Gloria Kwon, Memorial Sloan-Kettering Insititute - New York). *Middle* A confocal fluorescence microscope image of "Brainbow" transgenic mouse hippocampus (40x) (Dr. Tamily Weissman, Harvard University, Cambridge and (Livet *et al.*, 2007)). *Right* A two-photon fluorescence microscope 3D image of cell nuclei in the mouse colon (740x) (Dr Paul Appleton, University of Dundee). (All 3 images are taken from http://www.nikonsmallworld.com/.)

1.3.3 Fluorescence recovery after photobleaching (FRAP)

Fluorescence recovery after photobleaching (FRAP) is a method developed to detect motions of substances in the cell (Axelrod *et al.*, 1976). During FRAP a region is photobleached with high-power laser irradiation followed by low-power laser scanning to observe and measure the speed of fluorescence recovery in the bleached region (Houtsmuller and Vermeulen, 2001;Wang *et al.*, 2008). The characteristics of redistribution give information on diffusion, mobile fraction and duration of transient immobilization of the molecule under investigation ((Houtsmuller and Vermeulen, 2001;Lippincott-Schwartz *et al.*, 2003) and Figure 10).

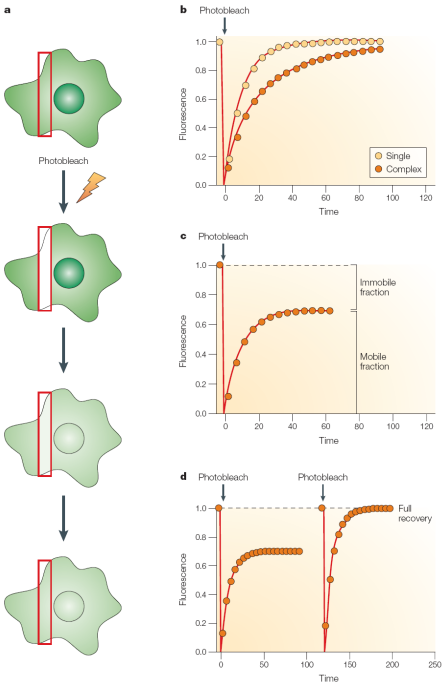

Figure 10 **Fluorescence recovery after photobleaching. a** A cell expressing fluorescent molecules is imaged with low light levels before and after photobleaching the strip outlined in red. Recovery of fluorescent molecules from the surrounding area into the photobleached region is monitored over time. Analysis usually includes compensation for the reduction in whole-cell fluorescence (depicted in the bottom cartoons). **b** Fluorescence recovery into the photobleached region can be quantified in a fluorescence recovery after photobleaching (FRAP) curve. These plots depict the recovery for a single species (simulated by a single exponential curve shown in yellow circles) or the kinetics for two equal populations recovering at two different rates (simulated by a double exponential curve shown in orange circles). Note that the kinetics for recovery of the latter takes much longer to plateau. **c** The level of fluorescence recovery in the photobleached region reveals the mobile and immobile fractions of the fluorophore in the cell. **d** A simple test for photo-induced immobile fractions is to perform a second FRAP experiment in the same region of interest. In the example here, the mobile fraction of the initial FRAP experiment is ~70%. The level of recovery can be determined by normalizing the fluorescent signal in the region and repeating the FRAP experiment. In the absence of photodamage, full recovery should be observed. (Figure and legend from (Lippincott-Schwartz *et al.*, 2003)).

2. PRESENT INVESTIGATION

2.1 AIMS OF THE STUDY

The projects presented here were initiated to increase our knowledge of DNA repair and investigate consequences of deficient DNA excision repair in human and mice. In the first part of the work, we aimed to find the disease-causing mutation in two patients with clinical features typical of Cockayne Syndrome (CS). Given that the number of studies linking clinical findings in CS patients and molecular findings of their respective CS mutations is relatively low, especially for the CSA protein where we found a novel mutation, a secondary aim was to review the CSA protein and its currently known mutations in humans.

In the last two projects described in this thesis, we wanted to further characterize the role of mouse FEN1. FEN1 is essential for DNA replication and LP-BER. Previous studies in our lab revealed early embryonic lethality of *Fen1* knock-out mice. Two *Fen1* knock-in models were generated, carrying mutations in conserved amino acids. In one model, the PCNA-binding domain of FEN1 was mutated, while the other model had a mutated FEN1 active site. Our aims were to characterize the *Fen1* mutants, study the role of FEN1 and the consequences of disrupted nuclease activity, and elucidate the importance of PCNA interaction.

Finally, the ultimate goal was to study the kinetics of mouse FEN1 in DNA repair. To obtain this, we generated and characterized knock-in mice expressing FEN1 fused to YFP, and performed multiphoton laser irradiation experiments. Our project is innovative in that the expression, localization and distribution of FEN1 will be investigated *in vivo* in live mice and cells.

Understanding the DNA metabolic pathways, their mechanisms at a fundamental level, and the consequences of deficiency or distortions of proteins involved in DNA repair can help us understand the cause, origin, and nature of disease, among them neurodegeneration and cancer.

2.2 SUMMARY OF PAPERS

PAPER I

Cockayne syndrome (CS) is mainly caused by mutations in the Cockayne syndrome group A or B (*CSA* or *CSB*) genes which are required for a sub-pathway of nucleotide excision repair entitled transcription coupled repair. Approximately 20% of the CS patients have mutations in *CSA*, which encodes a 44 kDa tryptophane (Trp, W) and aspartic acid (Asp, D) amino acids (WD) repeat protein. Up to now, nine different *CSA* mutations have been identified. We examined two Somali siblings 9 and 12 years old with clinical features typical of CS including skin photosensitivity, progressive ataxia, spasticity, hearing loss, central and peripheral demyelination and intracranial calcifications. Molecular analysis showed a novel splice acceptor site mutation, a G to A transition in the -1 position of intervening sequence 6 (g.IVS6-1G>A), in the *CSA* (excision repair cross-complementing 8 (ERCC8)) gene. IVS6-1G>A results in a new 28 amino acid C-terminus and premature termination of the CSA protein (G184DFs28X). A review of the CSA protein and the 10 known *CSA* mutations is also presented.

PAPER II

Flap endonuclease 1 (FEN1) processes Okazaki fragments in lagging strand DNA synthesis, and FEN1 is involved in several DNA repair pathways. The interaction of FEN1 with the proliferating cell nuclear antigen (PCNA) processivity factor is central to the function of FEN1 in both DNA replication and repair. Here we present two gene-targeted mice with mutations in FEN1. The first mutant mouse carries a single amino acid point mutation in the active site of the nuclease domain of FEN1 ($Fen1^{E160D/E160D}$), and the second mutant mouse contains two amino acid substitutions in the highly conserved PCNA interaction domain of FEN1 ($Fen1^{\Delta PCNA/\Delta PCNA}$). $Fen1^{E160D/E160D}$ mice develop a considerably elevated incidence of B-cell lymphomas beginning at 6 months of age, particularly in females. By 16 months of age, more than 90% of the $Fen1^{E160D/E160D}$ females have tumors, primarily lymphomas. By contrast, $Fen1^{\Delta PCNA/\Delta PCNA}$ mouse embryos show extensive apoptosis in the forebrain and vertebrae area and die around stage E9.5 to E11.5.

PAPER III

The structure specific flap endonuclease 1 (FEN1) is known to play an essential role in long-patch base excision repair (BER) and in DNA replication. Here, we have generated a novel mouse model which allows for monitoring the kinetics of FEN1 in response to DNA damage in live cells. The expression of the enhanced yellow fluorescent protein (eYFP), is here regulated by the endogenous *Fen1* promoter, and is fused to FEN1. The FEN1-YFP mouse enabled us to characterize expression levels and distribution of FEN1-YFP in cultured mouse cells and in live tissues. According to its role in processing of Okazaki fragments in lagging strand DNA synthesis, FEN1 expression is most readily found in highly proliferating tissue, however, FEN1 expression was also seen in the brain. Moreover, the FEN1-YFP fusion protein allowed us to investigate repair kinetics in cells challenged with local and global DNA damage and following poly (ADP-ribose) polymerase (PARP) inhibition. *In vivo* 2-photon fluorescence microscopy demonstrates rapid relocation of FEN1 at local DNA damage sites in the laser-irradiated nuclei, providing evidence of a highly mobile protein which accumulates fast at DNA lesion sites with high turnover rate. Inhibition of PARP disrupts FEN1 accumulation at sites of DNA damage, indicating that PARP is needed for FEN1 recruitment to DNA repair intermediates in BER.

3. DISCUSSION

3.1 A SUMMARY OF EXCISION REPAIR DEFICIENCIES AND DISEASE

When it comes to nuclear DNA repair, BER is the DNA repair pathway handling the highest number of lesions. While the lesions handled by BER are relatively small, NER is dealing with larger helix-distorting lesions generally obstructing transcription and replication (Nordstrand *et al.*, 2007). Deficiencies in BER and NER can range from a complete lack of essential protein(s) to subtle mutations and SNPs which can result in a broad spectrum of phenotypes, as illustrated in papers I and II, and studies referred to throughout the introduction and discussion of this thesis. Premature aging, cancer, neurodegeneration and developmental disorders are the main diseases resulting from deficient excision repair (Xu *et al.*, 2008;Niedernhofer, 2008;Cleaver *et al.*, 2009).

Aging is a complex phenomenon, characterized by increased susceptibility to cellular loss and functional decline, where mitochondrial DNA mutations and mitochondrial DNA damage response are thought to play important roles. Mitochondria are the major source of ROS in the cell, and are shown to utilize mitochondrial BER (mtBER) in order to remove oxidative damage and avoid mutations in the mitochondrial DNA (Larsen *et al.*, 2005;Gredilla, 2010;Boesch *et al.*, 2011). Emanating ROS from the electron transport chain can cause mtDNA damage and mutations which might result in dysfunctional mitochondrial respiration, further rising ROS generation and oxidative damage. This so-called “vicious cycle” of ROS generation and oxidative damage is one hypothesis for aging associated pathologies, and is particularly devastating in post-mitotic differentiated neuronal tissues (Jang and Remmen, 2009). Moreover, age-related decline in mtBER in skeletal muscles has been suggested to contribute to sarcopenia (loss of muscle mass) (Szczesny *et al.*, 2010). The mtBER pathway is similar to the nuclear version, however, mtBER proteins are encoded by the nuclear genome and most are splice variants, alternative translation-initiation products or post-translationally modified versions of the nuclear-encoded proteins. NER is not thought to be active in mitochondria, at least not in the classical nuclear form, and classical NER substrates like thymidine-dimers, cisplation intrastrand cross-links and complex alkyl damage are not repaired in mtDNA (Larsen *et al.*, 2005;Boesch *et al.*, 2011). However, a recent study revealed that NER proteins CSA and CSB rush into mitochondria upon oxidative damage, and interact with BER proteins (Kamenisch *et al.*, 2010). The authors suggest that

the buildup of mitochondrial gene mutations could be responsible for the subcutaneous fat loss characteristic of CS (Kamenisch *et al.*, 2010).

Initiation and progression of cancer and accelerated aging can be caused by DNA damage. The outcome depends on the amount and type of DNA damage, the location of damage and different cells also respond differently to damage. Unrepaired DNA damage may cause cell death and senescence leading to accelerated aging while protecting against cancer (Hoeijmakers, 2009). Reducing ROS and the damage load through avoidance or limited exposure to exogenous genotoxins and suppressed metabolism can delay cancer development or the aging process (Blagosklonny *et al.*, 2010). Both caloric restriction and DNA damage can elicit a protective survival response that promotes longevity and healthy aging (Garinis *et al.*, 2008;Schumacher *et al.*, 2009). This survival response includes suppression of growth hormone (GH)/insulin growth factor (IGF)-1 somatotroph axis and suppression of oxidative metabolism (Niedernhofer *et al.*, 2006;van, I *et al.*, 2007). The shift from growth and proliferation to preservation of somatic maintenance also involves upregulation of antioxidant defence and stress responses, along with a clear tendency to store glycogen and fat (Niedernhofer *et al.*, 2006;van, V *et al.*, 2006;van, I *et al.*, 2007).

BER deficiency increases susceptibility to mutagenesis and tumorigenesis, illustrated by high lymphoma incidence in *Ung*$^{-/-}$ mice and lymphoid hyperplasia and adenocarcinoma in *Polβ*$^{+/-}$ mice (Nilsen *et al.*, 2003;Cabelof *et al.*, 2006). Moreover, the combined deletion of two DNA glycosylases, OGG1 and MUTY, result in high incidence of lymphomas, lung tumors and ovarian tumors (Xie *et al.*, 2004). BER gene polymorphisms, including mouse *Fen1* and human *FEN1* variants, are associated with an increased risk for certain cancers (Zheng *et al.*, 2007b;Xu *et al.*, 2008;Yang *et al.*, 2009). Specific SNPs in *OGG1*, *POLβ* and *PARP1* genes have been associated with bladder cancer risk (Figueroa *et al.*, 2007).

Given the severe cancer-prone phenotype of XP patients one could question whether variation in NER capacity in the general population is associated with more subtle risk of sporadic cancer. One study identified an association of SNPs in the *XPC* gene with increased lung cancer risk in a Chinese population (Bai *et al.*, 2007). Common variation of other NER associated proteins, like the MMS19L protein which interacts with XPD, is linked with increased risk of pancreatic cancer (McWilliams *et al.*, 2009). Further research, in large

confirmatory studies, is needed to conclude whether SNPs in NER genes correlate with increased incidence of cancer.

NER deficiencies cause progressive neurodegeneration, and is exposed in three syndromes, XP, CS and TTD, mentioned in the introduction of this thesis, and in the case of CS described more in detail in paper I. Although some overlapping symptoms exist between XP, CS and TTD, pathogenesis differs among and within the syndromes, with the primary defect in XP being loss of neurons, whereas abnormal myelin is the major neuropathological feature in TTD and CS (Brooks *et al.*, 2008). It is speculated that the classic DNA damage accumulation model is applicable to neuronal death due to defective DNA repair, while the myelination defects and brain calcification pathology are better explained by other mechanisms (Brooks *et al.*, 2008). Interestingly, mutated versions of one of the genes defective in XP individuals, *XPD*, can result in all three NER disorders (Lehmann, 2001). XPD patients with mutations that specifically affect the NER function of XPD develop a progressive neurodegenerative disease similar to XPA patients, a "pure" XP phenotype. On the other hand, mutations in XPD that either destabilize TFIIH or affect the transcription function result in TTD or the XP-CS complex. TFIIH open DNA during NER and transcription, however, it also functions as a kinase that phosphorylates nuclear hormone receptors (Rochette-Egly *et al.*, 1997;Chymkowitch *et al.*, 2011). Moreover, TFIIH can function as a co-activator for thyroid hormone-dependent gene regulation, and the neurologic disease in human TTD patients might be explained by dysregulation of thyroid dependent gene expression in the brain, resulting in aberrant myelin gene expression (Compe *et al.*, 2007). "Pure" CS cells display normal nuclear hormone-dependent transcription, whereas reduced phosphorylation of nuclear hormone receptors levels explains the hormone-dependent transcription defect observed in XPG-CS cells (Ito *et al.*, 2007). Despite the phenotypic similarity between CS, XPG-CS, and TTD, the underlying molecular defects appear to be different (Brooks *et al.*, 2008;Hashimoto and Egly, 2009).

Abnormal myelin is, as mentioned above, the most prominent neuropathological finding in TTD and CS, and also in another rare genetic disease, Aicardi-Goutières syndrome (AGS) (Brooks *et al.*, 2008). Myelin, the "white matter", lipid-rich insulating material forming a layer around the axons of neurons, is synthesized by specific types of glial cells called oligodendrocytes (Brooks *et al.*, 2008). AGS can be caused by mutations in either *TREX1* or *RNASEH2*, and is characterized by failure to degrade endogenous DNA in the absence of

these nucleases, where upon undegraded endogenous nucleic acids activate an innate immune response, resulting in increased proinflammatory cytokine IFN-α production (Brooks *et al.*, 2008;van Heteren *et al.*, 2008;Stephenson, 2008). Secretion of IFN-α in the extracellular space of the brain, and therefore into the cerebrospinal fluid (CSF), can act on other brain cell types, including vasculature and oligodendrocytes, resulting in inflammation, dysmyelination and brain calcification (Lebon *et al.*, 2002;Brooks *et al.*, 2008). Vascular degeneration, particularly seen in microvessels, is present in CS (Weidenheim *et al.*, 2009), moreover, gene expression changes (Newman *et al.*, 2006) and evidence of inflammation has been observed in CS-cells (Weidenheim *et al.*, 2009). The defective TC-NER may contribute to the "vicious cycle" of neuroinflammation and cell death in CS brain. Overlap in neurological symptoms between CS and AGS suggests that vascular changes, and perhaps alterations in gene expression may play a role in the white matter phenotypes and brain calcifications in both diseases (Brooks *et al.*, 2008). This hypothesis of involvement of inflammation in NER disorders await additional evidence.

Brain has less nuclear and mitochondrial BER activity than other somatic tissues, yet it uses more oxygen than other organs and is vulnerable to ROS-induced damage (Intano *et al.*, 2001;Karahalil *et al.*, 2002;Hegde *et al.*, 2011). Age-associated decline of BER activity in the central nervous system has been linked to neurodegenerative disorders, implying a role for impaired BER in the pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) (Xu *et al.*, 2008;Coppede and Migliore, 2010b). Impaired BER activity has also been demonstrated in tissues from AD individuals (Coppede and Migliore, 2010b). In accordance with impaired BER, OGG1 showed decreased activity in AD brain and ALS motor neurons. It has been suggested that increased oxidative damage in AD patients could indirectly impair DNA repair proteins, e.g. by post-translational oxidative induced modifications or degradation of OGG1 activity (Shao *et al.*, 2008;Hill *et al.*, 2008). On the other hand, increased OGG1 levels were observed in the *substantia nigra* of PD patients (Fukae *et al.*, 2005), and OGG1 was linked to somatic CAG repeat expansion in HD (Kovtun *et al.*, 2007). On the contrary, increased PARP1 activity has been observed in AD, PD and ALS brain tissues (Soos *et al.*, 2004;Kim *et al.*, 2004;Kauppinen and Swanson, 2007), and increased APE1 expression was observed in AD brain regions and ALS motor neurons (Tan *et al.*, 1998;Shaikh and Martin, 2002;Davydov *et al.*, 2003;Marcon *et al.*, 2009). The understanding of the involvement of DNA repair in AD and other neurodegenerative diseases is currently at its beginning and need further research.

3.2 NUCLEOTIDE EXCISION REPAIR AND CSA

In our paper I, we describe the identification of a new mutation in the CSA protein, and recapitulates the known CSA mutations at the time (Kleppa *et al.*, 2007). Crystal structures of CSA and CSB proteins are yet not available; however, the latter has been modelled. A recent mutation update for *CSA* and *CSB* doubles the number of known disease causing mutations and further discusses possible genotype-phenotype correlations and motif-function correlations (Laugel *et al.*, 2009). In the report, rare founder effects were identified in specific populations, including the mutation we reported, CSA10NO. This homozygous splice site mutation was confirmed in the *CSA* gene of three Somali patients in the UK (Laugel *et al.*, 2009). All known missense mutations in *CSA* are located in the WD motifs, of which half of them are contained in WD motif 4. The WD motifs are required for protein-protein interactions and to build the beta-propeller structure. Interestingly, the structure of another WD40-repeat protein, DDB2 in the UV-DDB complex, revealed that the DDB2 WD40 propeller exclusively binds the lesion, illustrated by either 6-4PP lesion or an abasic site in the DNA duplex bound to the DDB1-DDB2 complex (Scrima *et al.*, 2008). DDB1-DDB2 associates tightly with the CUL4A-RBX1 ubiquitin ligase complex (Scrima *et al.*, 2008). DDB2 is one of many specific substrate-recognizing DDB1-CUL4 associated factors (DCAFs), and CSA is one of the DCAFs taking the place of DDB2 in a similar ubiquitin ligase complex (Sugasawa, 2009;Abbas and Dutta, 2011). The p.Ala205Pro mutation located in the fourth WD motif has been shown to abolish binding of CSA to one of its interactors, DDB1 (Cao *et al.*, 2004;Jin *et al.*, 2006). The p.Trp361Cys mutation reported in a case of UV-sensitive syndrome (UVSS) correlates with defective TCR but normal repair of oxidative DNA damage, suggesting uncoupled roles for CSA in removal of UV-induced damage and oxidative damage (Nardo *et al.*, 2009). This mutation, positioned in the last putative WD repeat of CSA, does not disrupt or destabilize the overall functions of CSA as it renders a partially functional CSA in the UVSS proband. UVSS is a mild NER disorder characterized by photosensitivity and mild skin abnormalities, with normal growth and neurological development, and without any increased risk of cancer. Despite the mild symptoms, UVSS cells display the same TC-NER defect as CS cells(Nardo *et al.*, 2009). UVSS can be caused by the *CSA* mutation mentioned above, but also by the complete lack of CSB protein, or by a complementation group unknown to date (Fujiwara *et al.*, 1981;Itoh *et al.*, 1994;Horibata *et al.*, 2004;Nardo *et al.*, 2009). The broad spectrum of phenotypes associated with mutated *CSA* and *CSB* and the lack of a molecular explanation of the CS pathology emphasize the need of further research

in this field (Weidenheim *et al.*, 2009). Human *CSA* and *CSB* mutations have largely overlapping phenotypes and are generally clinically indistinguishable (Stefanini *et al.*, 1996). To date, mutated *CSA* has not been described in the most severe type II CS or in the prenatal cerebro-oculo-facio-skeletal (COFS) syndrome. Mutated *CSB* is identified in all types of CS and the very severe COFS, also, cataracts are often described in patients with mutated *CSB* and are associated with severe disease, but rarely found in patients with mutated *CSA* (Laugel *et al.*, 2009). Although the phenotypes resulting from *CSA* and *CSB* mutations might differ slightly, more patients would be needed to confirm any difference in clinical outcome among mutated *CSA* or *CSB*. *Csa* and *Csb* mouse mutants display a much milder phenotype than human CS patients. Yet, as for humans, the phenotypes of *Csa* and *Csb* mutants are impossible to separate (van der Horst *et al.*, 1997;van der Horst *et al.*, 2002).

CSA ubiquitylates CSB for degradation. Thus, it seems contradictory that both lack of CSA and lack of CSB cause CS (Groisman *et al.*, 2006). If *CSB* is mutated, there is no functional CSB, while no CSB degradation via the ubiquitin-proteasome pathway occurs when *CSA* is mutated. Recently, CSA ubiquitylation and CSB function have potentially been connected through the ubiquitin-binding domain (UBD) of CSB (Anindya *et al.*, 2010;Gray and Weiner, 2010). Anindya and co-workers show that TC-NER proteins assemble at the site of DNA damage but can not begin repair until CSB binds ubiquitin. Possibly, CSA ubiquitylates a target in the TC-NER complex, which is recognized by CSB-UBD as a signal to disassemble the initial TC-NER complex, leaving the core NER proteins to finish repair (Anindya *et al.*, 2010). Interestingly, the CSB homolog in yeast, Rad26, lacks the UBD domain. This correlates with the dispensable role of a yeast homolog of CSA. The best homolog of CSA in yeast, Rad28, is not required for TC-NER in budding yeast. Yet, CSA is absolutely required for TC-NER in mammalian cells, along with the UBD of CSB (Venema *et al.*, 1990;Bhatia *et al.*, 1996).

The mechanisms by which CSA and CSB influence repair of oxidative damage are puzzling. It has been speculated that CSA might be indirectly involved in oxidative damage repair. CSA is part of an ubiquitin ligase known to be involved in TCR of bulky lesions. The requirement of ubiquitylation for repair of oxidative damage is an interesting possibility, however, this lacks experimental evidence (Cramers *et al.*, 2011). CSB could have a general role in transcriptional control through chromatin maintenance or remodeling (Cramers *et al.*, 2011). CS-deficient cells are hypersensitive to several types of oxidative DNA damaging agents. This hypersensitivity is associated with accumulation of oxidative base modifications, including 8-

oxoG, 8-oxoA, and 5-hydroxy-2'-deoxycytidine in both *CSA* and *CSB* mutant genomic DNA (Tuo *et al.*, 2001;D'Errico *et al.*, 2007;Ropolo *et al.*, 2007;Pascucci *et al.*, 2011). Moreover, impaired host cell reactivation of plasmids containing a single 8-oxoG was observed in CS-deficient cells (Spivak and Hanawalt, 2006). SV-40-transformed *CSA* and *CSB* mutant cells did not confirm the hypersensitivity to oxidative DNA damaging agents seen in CS deficient primary cells (D'Errico *et al.*, 2007). Thus, one should be cautious in extrapolating data on oxidative stress sensitivity in transformed cells, where different cell responses are defective compared to normal cells. A possible role of the CS proteins in protection from oxidative damage was recently shown by functional increase of CSA and CSB inside mitochondria (mt), and complex formation with mtDNA, mtOGG-1, and mt single-stranded DNA binding protein (mtSSBP)-1 upon oxidative stress in human cells, linking BER and NER (Kamenisch *et al.*, 2010). Impaired repair of oxidative lesions throughout the genome is suggested to contribute to the CS phenotype, as reduced GGR was observed in CSA and CSB deficient human cells (Cramers *et al.*, 2011). TCR, on the other hand, is not involved in the processing of ionizing radiation induced oxidative lesions, and defective TCR is thus highly unlikely to underlie the radiation sensitivity of CS (Cramers *et al.*, 2011). The involvement of GGR, TCR and chromatin remodeling proteins in UV-induced repair has been proposed to depend on the developmental stage of cells (Lans *et al.*, 2010). GGR predominates in germ cells and dividing cells to keep the entire genome free of lesions, while in non-dividing somatic cells the priority is to maintain active genes through TCR (Lans *et al.*, 2010).

3.3 FEN1, - ITS ROLE AND REGULATION IN DNA REPLICATION AND DNA REPAIR

FEN1 is absolutely required for removal of RNA/DNA primers during Okazaki fragment maturation in lagging strand DNA synthesis and for cleavage of flap-substrates generated during LP-BER. In paper II and III we characterized three different FEN1 knock-in mice. The first mutated in the conserved FEN1 nuclease domain, the second mutated in the conserved PCNA-interacting protein (PIP) domain and the third encoding a fluorescently tagged FEN1, FEN1- YFPHis_6HA (entitled FEN1-YFP) fusion protein. The nuclease mutant is characterized with high incidence of lymphoma, whereas a mutation of the PCNA-interaction domain causes early embryonic lethality. In line with our results, Zheng and colleagues show that F343AF344A mutations in the PIP domain of FEN1 in mice disrupts FEN1's ability to interact with PCNA (Zheng *et al.*, 2007a;Zheng *et al.*, 2011a). The heterozygous F343AF344A mutant mice encompass both defect RNA primer removal and LP-BER, resulting in numerous DNA strand breaks (Zheng *et al.*, 2011a). Moreover, Zheng *et al.* show that heterozygous

F343AF344A mutant mice display a higher incidence of aneuploidy-associated cancer (Zheng *et al.*, 2011a). FEN1 is thought to be both a tumor suppressor protein and an enzyme upregulated in hyperproliferating cancer cells. In accordance with increased FEN1 expression in cancer cells, decreased CpG2 methylation of the *FEN1* promoter is associated with breast cancer (Singh *et al.*, 2008;Lahtz and Pfeifer, 2011). Thus FEN1 can promote cancer in two different ways; mutated FEN1 can increase genomic instability and initiate malignant transformation, while FEN1 overexpression gives the tumors a growth advantage (Zheng *et al.*, 2011b). Furthermore, the mutant mouse models suggest two different cancer causing mechanisms as presented by Zheng and Shen (Zheng and Shen, 2011). One mechanism which could lead to cancer is illustrated by the F343AF344A mutant, with aneuploid cancer arising from deficient RNA primer removal. The second mechanism is originating from impaired editing of polymerase α incorporation errors during Okazaki fragment maturation, exemplified by the E160D mutant. Mutated FEN1 with disrupted PCNA interaction is thought to cause retarded processing of RNA/DNA primers due to a partial defect in recruitment of FEN1 to replication foci (Zheng *et al.*, 2011a). Another role of the FEN1/PCNA interaction is for PCNA to ensure the appropriate dissociation of FEN1 from nicked DNA after flap cleavage (Zheng *et al.*, 2011a). F343AF344A mutant FEN1 may remove nucleotides from the 5' end nicked DNA ends, causing unwanted cycles of gap filling, DNA cleavage and ligation (Zheng *et al.*, 2011a). The defects in RNA primer removal and LP-BER result in frequent DNA breaks, activation of G2/M checkpoint protein Chk1, and induce aneuploidy (Zheng *et al.*, 2011a). While the F343AF344A mutant is not thought to result in a mutator phenotype, the FEN1 E160D mutant presented by Zheng *et al*. displayed a strong mutator phenotype, with a 25-fold increase in rates of base substitution in MEF cells carrying the FEN1 nuclease-defective mutation (Zheng *et al.*, 2007c). In normal cells, mis-incorporated bases may be proofread by DNA polymerase δ, removed by FEN1 if displaced into the 5' flap, or removed by 5' exonuclease activity of FEN1 if not displaced in the flap. Incorporation errors can also be removed by the mismatch repair pathway (Zheng and Shen, 2011). The sole contribution of BER deficiency to formation of spontaneous cancer in FEN1 E160D mice is difficult to assess, due to other DNA metabolic defects in these mice, including apoptotic DNA degradation deficiency (Zheng *et al.*, 2007c). Chemical-induced DNA damage by hydrogen peroxide (H_2O_2) and methylnitrosourea (MNU) treatment resulted in DNA strand breaks, chromosome instability and chromosomal breakage in the E160D nuclease deficient cells (Xu *et al.*, 2011). In mice treated with MNU, an alkylating agent similar to a tobacco-specific carcinogen, FEN1 E160D mice were significantly more susceptible to MNU exposure and developed lung

adenocarcinoma (Xu *et al.*, 2011). Using nuclear extracts and reconstituted purified proteins, Xu *et al*. demonstrate that the E160D FEN1 mutant is deficient in processing LP-BER intermediate structures (Xu *et al.*, 2011). Thus, the E160D mutation results in a nuclease-deficient FEN1 impaired in processing of intermediate DNA substrates formed during LP-BER, which again lead to DNA breaks, leaving E160D cells susceptible to DNA damage (Xu *et al.*, 2011).

Recent biochemical and genetic studies provide evidence that post-translational modifications of the FEN1 protein are involved in regulation of protein-protein interactions and also determine the cellular localizations of FEN1 (Zheng *et al.*, 2011b). Acetylation (Hasan *et al.*, 2001), phosphorylation (Henneke *et al.*, 2003) and methylation (Guo *et al.*, 2010) are three modifications found to regulate FEN1 *in vivo*, where methylation of residue R192 in FEN1 prevents phosphorylation at its residue S187 (Guo *et al.*, 2010). Methylated FEN1 interacts with PCNA, whereas phosphorylated FEN1 dissociates from PCNA (Guo *et al.*, 2010). This provides novel insight into the mechanism for the FEN1 nuclease to dynamically associate with and dissociate from PCNA and the DNA substrate. It has been proposed a model of sequential actions where methylated FEN1 replaces the DNA polymerase and access PCNA and the flap structure, upon flap cleavage FEN1 is demethylated by an unknown mechanism, allowing phosphorylation of the nuclease which then falls off PCNA leading to ligase recruitment (Zheng *et al.*, 2011b). FEN1 acetylation has an inhibitory effect on its enzymatic activity (Hasan *et al.*, 2001;Friedrich-Heineken *et al.*, 2003). Acetylation of FEN1 and DNA2 nuclease/helicase promotes the formation of longer flaps thought to involve further processing by RPA and DNA2 (Balakrishnan *et al.*, 2010). Recent findings indicate that the cell use acetylation of BER and replication proteins to allow controlled removal of a greater number of nucleotides with high accuracy (Hasan *et al.*, 2002;Bhakat *et al.*, 2003;Bhakat *et al.*, 2006;Balakrishnan *et al.*, 2010). Our kinetic study on the FEN1-YFP fusion protein to sites of DNA damage in live cells (paper III), revealed a fast accumulating FEN1 with high turnover, in line with the dynamical regulation of FEN1 by post-translational modifications.

If a BER complex exists remain to be elucidated, however, Jaiswal and Narayan argue in a recent study that a complex with all the essential BER-components, including FEN1, assembles on abasic DNA in nuclear extracts from human colon cancer cells (Jaiswal and Narayan, 2011). FEN1 recruitment onto the AP-site was found to be as fast as that of APE1,

and did not change with the time course of the assembly of the complex (Jaiswal and Narayan, 2011). Further in support of FEN1 in a BER multiprotein complex, Hanssen-Bauer *et al.* suggest that XRCC1 organizes BER into multiprotein complexes of varying size, depending on the nature and context of the DNA damage (Hanssen-Bauer *et al.*, 2011). A model of three modes of BER is presented, FEN1 is thought to take part in the BER complex at high levels of DNA damage and in replication-associated BER, but not in the classic BER at endogenous and low levels of induced DNA damage (Hanssen-Bauer *et al.*, 2011). It has also been proposed that LP-BER function by sequential enzyme actions in the context of a multienzyme complex that remains structurally intact during the repair process (Balakrishnan *et al.*, 2009). Yet, another suggestion is that preformed BER complexes predominantly repair endogenous base lesions, while repair via hand-off mechanism by sequential recruitment could occur with induced DNA damage (Hegde *et al.*, 2010).

3.4 METHODOLOGICAL ASPECTS

To examine the mobility and kinetics of FEN1 *in vivo* we generated a knock-in mouse expressing EYFP-tagged FEN1 from the endogenous *Fen1* locus (Paper III). When evaluating the results, we have to take into account that we observe a tagged FEN1 protein, which possibly could be disturbed as compared to its wild-type state. The aim when generating a fluorescently labeled protein was expression of the fusion protein at levels comparable to those for the native unlabeled protein, which targets to the correct compartment in the cell, and exhibits a behavior similar to that of the native protein in terms of half-life, dynamics, and protein-protein interactions. EYFP and other fluorescent proteins have the ability to fold even after fusion to another protein, thus enabling the study of proteins *in vivo*. In addition, EYFP gives a bright fluorescent signal, and it can be used in conjunction with far red fluorescent labeling, enabling simultaneous imaging of two fluorescent proteins, possibly colocalizing, without false positive signal or bleed-through fluorescence. The size of EYFP, 27 kDa, with a β–barrel 3nm in diameter and 4nm long, represents a significant addition to a protein and thus may have steric consequences for protein folding, function, or targeting (Yang *et al.*, 1996). Therefore, one needs to be cautious when evaluating results of the fusion protein when it comes to localization, protein-activity and protein-protein interactions, and compare to, if known, these characteristics of the native untagged protein.

Although genome maintenance mechanisms in mice and humans are highly conserved, differences exists in certain aspects of DNA repair, metabolic rate, immune system, telomere

length and life span (Hasty *et al.*, 2003;Mestas and Hughes, 2004;Demetrius, 2005). While mouse models provide a unique biological perspective for tissue-specific effects of DNA damage, some limitations exist in modeling human disease in the mouse. This is particularly problematic in the nervous system, in which the mouse appears to be more resistant to DNA damage compared to humans (Niedernhofer, 2008). For example, *Csa* or *Csb* mutations do not appear to lead to demyelination and other severe neurological decline in mice, in contrast to the detrimental neurological abnormalities seen in human. Although no good model of the main CS phenotype exists, the single knock-out mouse model seems to correspond to mild UVSS, while TCR/GGR double knock-out mouse models, deficient for either *Csb* and *Xpa* or for *Csb* and *Xpc*, seem to correspond to the severe COFS (Niedernhofer, 2008;Cleaver *et al.*, 2009). Further analysis is needed to find out whether the primary defect is neurodegeneration (XP) or demyelination (CS). As mouse models become more refined, current limitations could be overcome by for example using combinatorial gene inactivation approaches to mimic the neuropathology seen in human diseases. The general observation of milder phenotypes, especially less neuropathology, of NER-deficient mice versus humans deprived of functional NER may mean that the level of damage is influenced by environmental factors. The most pronounced hallmark of NER-deficient mice is elevated rates of UV-induced skin cancer, regardless of which NER gene being deficient. In humans, on the other hand, there is a clear distinction between gene deficiencies that result in cancer and those that result in neurological defects (Niedernhofer, 2008). This discrepancy could well be due to the short lifespan of mice, and by the high UV doses assessed. Conversely, unprotected XP-patients develop skin cancer at an early age (Cleaver *et al.*, 2009). While NER knock-outs provide several mouse models of human disease, the BER knock-outs are mostly embryonic lethal, with the exception of mice lacking individual DNA glycosylases which initiate the BER pathway. However, work with BER SNPs and meta-analysis have provided evidence for a role of BER in human disease (Maynard *et al.*, 2009). Through heterozygous mouse models of the essential BER-proteins, and viable homozygous nulls for the glycosylases, BER-deficiency and its influence on lifespan and health is modeled in rodents (Xu *et al.*, 2008). Mice deficient in BER-function display severe phenotypes, including cancer, premature aging and metabolic defects (Mostoslavsky *et al.*, 2006;Vartanian *et al.*, 2006;Chan *et al.*, 2009). Tissue-specific knockdowns and combination of mouse models heterozygous or homozygous for BER-genes with mouse models deficient in cell cycle check point or apoptosis genes may reveal variability among tissues, impact of BER on aging and on fitness, and the corresponding molecular mechanisms.

4. FUTURE PERSPECTIVES

Increasing the genotype-phenotype knowledge through diagnosis of CS patients both at the molecular and clinical level will possibly reveal distinct biological roles of the CSA and CSB proteins. However, the missing correlation seen between mutation and disease-outcome to date necessitates investigations at the protein level, to clarify the functional and coordinating roles of CSA and CSB alone and in the TCR-complex. Fluorescently tagged proteins in live cells could be an important tool in the study of mutated CS proteins and their interactions and involvement in DNA repair, elegantly shown by Anindya and coworkers (Anindya *et al.*, 2010). Combining the fluorescent CSB with fluorescent CSA or another TCR-protein to image recruitment and colocalization to stalled RNAPII, or failure of it, in mutants as compared to wild-type protein, could thus elucidate which protein(s) is bound by the CSB-UBD and who is ubiquitylating whom.

The FEN1-YFP mouse has the potential of use in several fields, due to FEN1's involvement in many metabolic pathways, and its two-sided role in cancer. Further characterization of the FEN1-expression levels in different tissues and during development, from early two-cell stage via blastocyst and embryogenesis, until differentiated and eventually post-mitotic cells could be done *in vivo*, and the expression compared among tissue-types, developmental stages and other repair proteins. Crossing FEN1-YFP mice with other fluorescently tagged LP-BER proteins could be used for *in vivo* studies of protein-protein interactions and colocalization at DNA damage sites. To investigate further the role of FEN1 in proliferative cells, partial hepaectomy could be performed, and FEN1-YFP expression monitored in the rapid regenerating liver tissue. Also, tumor cells or tissues expected to have upregulated FEN1 in the hyperproliferating cells, would be interesting to evaluate and visualize. FEN1-YFP is not by itself expected to increase the risk of cancer development, so either spontaneous or chemically/radiation induced tumor would be prerequisite. Another option is to study heterogenous mice, by crossing FEN1-YFP either with a heterogenous BER-deficient mouse or a cancer mouse model. Moreover, the kinetic studies of FEN1 initiated in our paper III could be expanded, including measurements to obtain K_{on} and K_{off} data, increase number of cells monitored for long-time fluorescence recovery, treat cells with more DNA-damaging agents and do FRAP, compare FRAP on LD for FEN1 in different tissues, e.g. brain and skin, both in cell cultures and in tissue slices. By combined effort of computational modeling and quantitative analysis of the mammalian BER components in live cells, similar to what was

done for the NER-machinery (Luijsterburg *et al.*, 2010), one might be able to tell if there is a BER complex, and if so, how it assembles and function on DNA repair intermediates.

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