Karin Margaretha Gilljam

DNA repair protein complexes, functionality and significance for repair efficiency and cell survival

Thesis for the degree of Philosophiae Doctor

Trondheim, August 2010

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I dedicate this thesis to my mother who died too young from cancer.

NORGES TEKNISK-NATURVITENSKAPELIGE UNIVERSITET
DET MEDISINSKE FAKULTET

DNA-reparasjons-protein-komplekser, funksjonalitet og signifikans for reparasjonseffektivitet og skadetoleranse

Karin Margaretha Gilljam

All informasjon om en organisme er lagret i vår arvestoff, DNA. DNA er et relativt ustabilt makromolekyl som konstant blir utsatt for farer som truer dets integritet, både fra omgivelsene og fra kjemiske prosesser inne i selve cellen. I tillegg kan baser spontant bli mistet uten noe form for påvirkning. Selve kopieringen av DNA, den såkalte DNA-replikasjonen er svært rask og er en kritisk prosess i cellen hvor mye kan gå galt. I tillegg kan ureparerte DNA-skader ved replikasjonen foreviges i form av mutasjoner. Mutasjoner i gener som koder for proteiner som regulerer cellens vekst og død kan resultere i ukontrollert cellevekst og dermed kreft. En av cellens strategier for å sikre effektiv og trygg replikasjon og reparasjon av DNA'et er å samarbeide ved å danne proteinkomplekser, hvorav PCNA ofte spiller en sentral rolle. PCNA sitter som en homotrimerisk ring rundt DNA-tråden som replikeres, og fungerer som en plattform for binding av mange proteiner. I tillegg til binding av DNA-replikasjonsproteiner, bindes også mange DNA-reparasjonsproteiner til PCNA, og sørger for effektiv reparasjon av skadet DNA både før og etter selve replikasjonen. I tillegg er PCNA involvert i DNA-syntese ved reparasjon som ikke er assosiert med replikasjon.

I 1998 ble det funnet et motiv (en peptid-sekvens) som er ansvarlig for at mange proteiner bindes til PCNA, kalt PCNA Interacting Peptide (PIP). I artikkel 1 fant vi ved hjelp av blant annet fluorescerende proteiner og konfokal mikroskopi et nytt motiv som er viktig for proteiners binding til PCNA. Dette motivet fant vi først i det direkte alkyleringsreparasjons-proteinet; human AlkB homologue 2 og derfor kalte vi motivet AlkB homologue 2 PCNA Interacting Motif (APIM). I denne artikkelen verifiserer vi et funksjonelt APIM motiv i fem proteiner og viser at over-uttrykk av dette motivet gjør celler mer sensitive for alkylerende skade. Dette tyder på at overuttykk av APIM hemmer bindingen mellom APIM-inneholdende DNA reparasjons-proteiner og PCNA slik at de ikke reparerer DNA-skadene optimalt.

I samme artikkel viser vi også at APIM er konservert i mer enn 200 proteiner, blant annet i nukleotideeksisjonsreparasjons (NER) proteinet Xeroderma Pigmentosum group A (XPA), og i artikkel 2 verifiserer vi at APIM også er et funksjonelt PCNA bindende

motiv i XPA. Vi viser og at overuttrykk av APIM-peptidet gjør celler mer sensitive for skade fra UV-lys, en type DNA-skade som hovedsakelig blir reparert av NER. I tillegg finner vi bevis som støtter at det er redusert funksjon av XPA som er årsak til at cellene er mer UV-sensitive ved overuttrykk av APIM, antagelig pga. svekket binding til PCNA.

I artikkel 3 ser vi nærmere på baseeksisjonsreparasjons- og singeltrådbrudds-reparasjons-proteinet XRCC1. Dette er i likhet med PCNA og XPA et protein uten enzymatisk funksjon, men med mange bindingspartnere, blant annet PCNA. Hvilken del av XRCC1 som er viktig for dens funksjon i cella er derimot ikke helt klarlagt, noe vi undersøker nærmere i denne artikkelen. Det viser seg at den delen av XRCC1 som har evnen til å binde PCNA og alkyleringsreparasjons-proteinet MPG er den eneste XRCC1 mutanten som kan stimulere reparasjon av alkyleringsskader, noe som igjen bekrefter viktigheten av å binde seg til PCNA.

Oppsummert tar dette arbeidet for seg hvordan DNA-reparasjonsproteiner binder seg til hverandre og PCNA, og hvordan dette påvirker evnen til å reparere DNA og dermed tåle DNA-skade.

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Ovennevnte avhandling er funnet verdig til å forsvares offentlig
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LIST OF PAPERS

Paper 1:
Identification of a novel, widespread, and functionally important PCNA-binding motif.

Karin M. Gilljam[†], Emadoldin Feyzi[†], Per A. Aas[†], Mirta M.L. Sousa, Rebekka Müller, Cathrine B. Vågbø, Tara C. Catterall, Nina B. Liabakk, Geir Slupphaug, Finn Drabløs, Hans E. Krokan, and Marit Otterlei

[†] These authors contributed equally to this work

Journal of Cell Biology, 2009

Paper 2:
Proper functioning of the Xeroderma Pigmentosum group A protein is dependent on interaction with PCNA

Karin M. Gilljam, Rebekka Müller, and Marit Otterlei

Manuscript submitted May 2010

Paper 3:
The NLS to BRCT1 region of XRCC1, harboring the three most common single nucleotide variations, is essential for the scaffolding function of XRCC1.

Audun Hanssen-Bauer[†], Karin Solvang-Garten[†], Karin M. Gilljam, Kathrin Thorseth, Mansour Akbari, and Marit Otterlei

[†] These authors contributed equally to this work

Manuscript

ABBREVIATIONS

A	Adenine
A	Alanine, Ala
2OG	2-oxoglutarate
AAG	3-alkyladenine DNA glycosylase
ABH	AlkB Homologues
AGT	O^6-alkylguanine DNA alkyltransferase
AOA1	Ataxia-oculomotor apraxia 1
AP	Apurinic/apyrimidinic sites
APE1	AP endonuclease 1
APIM	AlkB homologue 2 PCNA-interacting motif
ATM	Ataxia telangiectasia mutated
BER	Base excision repair
BRCT	BRCA1 carboxyl-terminal
BRCA1/2	Breast and ovarian cancer type 1/2 susceptibility protein
C	Cytosine
CHO	Chinese hamster ovary
CFP	Cyan fluorescent protein
CPD	Cyclobutane pyrimidine dimer
CS	Cocayne syndrome
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxyribonucleotides
dRP	Deoxyribosephosphate
dRPase	Deoxyribosephosphate phosphodiesterase
ds	Double strand
DSB	Double strand break
DSBR	Double strand break repair
F	Phenyl alanine
FA	Fanconi anemia
FEN1	Flap endonuclease 1
FRET	Fluorescence resonance energy transfer
FTO	Fat mass and obesity associated protein
G	Guanine
GGR	Global genome nucleotide excision repair
GFP	Green fluorescent protein
HeLa	Henrietta Lacks

HNPCC	Hereditary non-polyposis colon cancer
HR	Homologous recombination
HR23B	Homologue of yeast Rad23
HU	Hydroxy urea
ICL	Interstrand crosslink
IR	Ionizing radiation
MBD4	Methyl-binding domain glycosylase 4
meA	Methyladenine
meC	Methylcytosine
meG	Methylguanine
meT	Methylthymine
MGMT	O^6-methylguanine DNA methyltransferase
MMR	Mismatch repair
MMS	Methyl methanesulphonate
MPG	N-methylpurine DNA glycosylase
MRN	MRE11/RAD50/NBS1
MSH	MutS homologue
MLH	MutL homologue
MYH	mutY homologue
NEIL2	Nei endonuclease VIII-like 2
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
OGG	8-oxoguanine DNA glycosylase
OH	Hydroxyl
p21	Cyclin-dependent kinase inhibitor
PCNA	Proliferating cell nuclear antigen
PIP	PCNA interacting peptide
PMS2	Post-meiotic segregation 2
PNK	Polynucleotide kinase 3'-phosphatase
Pol	Polymerase
Pro-BRE	BRCA1/2-containing complex subunit
ROS	Reactive oxygen species
RPA	Replication protein A
SAM	S-adenosylmethionine
SCAN1	Spinocerebellar ataxia with axonal neuropathy 1
SNP	Single-nucleotide polymorphism
SMC5	Structural maintenance of chromosomes 5

S-phase	Synthesis-phase
ss	Single stranded
SSB	Single strand break
SSBR	Single strand break repair
T	Thymine
TCR	Transcription coupled nucleotide excision repair
TFIIH	Transcription factor II H
TFII-I	Transcription factor II-I
TFIIS-L	Transcription factor II S-Like
TDP1	Tyrosyl DNA phosphodiesterase
Topo	Topoisomerase
TLS	Translesion synthesis
TTD	Trichothiodystrophy
UDG	Uracil-DNA glycosylase
UNG	Uracil-DNA glycosylase
UV	Ultra violet
W	Tryptophan, Trp
Y	Tyrosine, Tyr
YFP	Yellow fluorescent protein
XAB1/2	XPA-binding protein 1/2
XLF	XRCC4-like factor
XP	Xeroderma pigmentosum
XPA-G	Xerodemra pigmentosum group A-G
XPV	Xeroderma pigmentosum variant
XRCC1	X-ray repair cross-complementing protein 1

INTRODUCTION

Keeping the genome intact is a challenge due to the unstable nature of DNA and the threat from endogenous and environmental agents. The acute effects of DNA damage are cell cycle arrest, transcription block, and apoptosis, but DNA damage left unrepaired may result in mutations. From an evolutionary perspective, mutations are essential for generation of genetic variation, however, with respect to human health, mutations are harmful as they are the driving force for oncogenesis, leading to the formation of cancer.

DNA replication

DNA replication is a critical event in the cell; errors can be made and importantly, DNA damage unrepaired upon DNA replication may give rise to mutations. Thus, many DNA repair proteins interact with the replication machinery in order to be at the right place at the right time.

All organisms must duplicate their entire DNA before the cell can divide. In mammals, DNA replication occurs at a polymerisation rate of 20-50 nucleotides per second (Burgers, 2009). The mammalian genome consists of 3×10^9 base pairs, but only 1 error in every 10^9 base pair is made (Alberts, 1994). To achieve such low error-rate during the rapid polymerization, mechanisms for reliable and efficient DNA replication have evolved: The DNA polymerases catalyze a nucleophilic attack on the 3' hydroxyl (OH) terminus from the sugar backbone only when the base on the incoming nucleotide is complementary, i.e. forms a Watson-Crick base pair with the opposite base (Stryer, 1995). Furthermore, the few errors made by the replicative polymerases are repaired by the 3' to 5' exonuclease proofreading activity within the polymerase itself (Alberts, 1994). Finally, many proteins participate in the formation of large replication protein complexes, in order to ensure efficient and reliable DNA duplication.

Since the DNA polymerases attack the 3' terminus and both strands serve as templates, the so-called replication fork is asymmetrical. One strand, the leading strand, is continuously duplicated, while the opposite, the lagging strand, is discontinuously duplicated. Despite the differences of the two strands, they are held together by large multiprotein complexes that move rapidly along the DNA. For the leading strand, Pol ε is suggested to be responsible for the continuous polymerization, at least in yeast (Pursell et al., 2007). For the lagging strand, however, there is a constant need of RNA primers formed by the Pol α/primase. Pol α elongates the RNA primers for a few nucleotides, and is replaced by Pol δ. Pol δ continues polymerization until it runs into the next RNA primer. Pol δ displaces the RNA, and the flap is cleaved off by Flap endonuclease I (FEN1) followed by sealing of the gap by DNA ligase I (LIG1) (Burgers, 2009). Other proteins involved in the replication machinery complex are: Replication protein A (RPA) which stabilizes the ssDNA; DNA helicase which unwinds the template; and topoisomerases which reveal the supercoil. Importantly, the proliferating cell nuclear antigen (PCNA) forms a sliding clamp around DNA, constituting a platform for binding and organization of the DNA polymerases, FEN1, LIG1 and more (Bowman et al., 2004; Maga and Hubscher, 2003). Many core replication proteins bind to PCNA through a conserved motif called the PCNA interacting peptide (PIP) box (Warbrick, 2000), discussed later. The replicating protein complex is illustrated in figure 1.

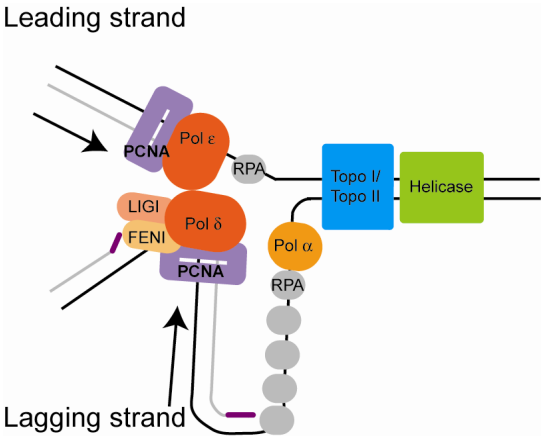

Figure 1: Cartoon of the replication fork. Black lines illustrate the template DNA, the grey lines the newly formed DNA and the purple lines the RNA primers.

There are essentially two forms of topoisomerases solving the topological problems caused by the DNA unwinding during replication, Topoisomerase (Topo) I and II. Topo I cuts one strand while Topo II cuts both strands and can therefore be a threat to the cell as it has the potential to cause lethal double strand breaks (DSBs) (Agostinho et al., 2004; Nitiss, 2009a). The positive supercoiled DNA can be relaxed by either Topo I or Topo II in front of the replication fork as illustrated in figure 1. However, only Topo II seems to be able to solve the topological problem in late S-phase by decatenation. When two replication forks meet, completion of replication leads to formation of two interlinked catenanes (figure 2). Resolution of this catenan requires Topo II. There are two isoforms of Topo II, called Topo II α and β, where Topo II α appears to be essential for cell survival (Agostinho et al., 2004). In addition to replication, Topo II also has a pronounced role in chromosome separation and transcription which encounter similar topology issues (Nitiss, 2009a).

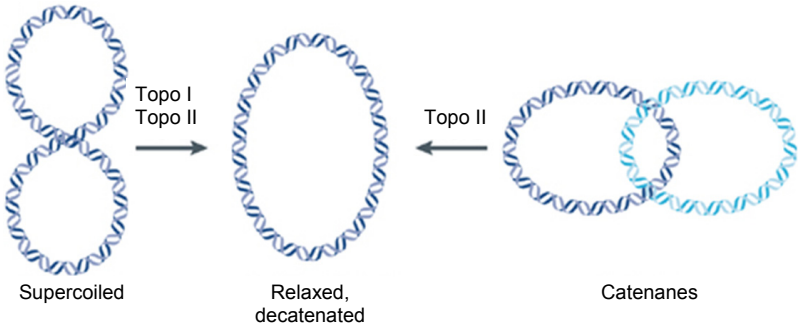

Figure 2: Resolving of topological problems including decatenation by Topo II. Adapted from (Nitiss, 2009a).

Topo II α is one of the proteins containing the newly identified PCNA interaction motif; AlkB homologue 2 PCNA interacting motif (APIM), published in paper 1. Topo II α has previously been suggested to interact with PCNA through a PIP-box like sequence (Niimi et al., 2001); however, our studies indicate that it is the APIM motif, rather than the proposed PIP-box, that is responsible for the interaction between Topo II α and PCNA.

Proliferating cell nuclear antigen

PCNA consists of three similar subunits forming a donut with an overall negative charge. The inner surface; however, is positively charged due to many lysine and arginine residues, enabling DNA to pass through the donut without electrostatic repulsions. PCNA consists of a "front" and a "back" side, and it is suggested that PCNA forms a double trimer with a back-to-back interaction (Naryzhny et al., 2005). The level of PCNA molecules is cell cycle regulated and reaches a peak during S-phase. The average number of PCNA molecules in a normal cell is 500 000 monomers. However, in a rapidly dividing cancer cell, the average is 4 000 000 PCNA molecules (Naryzhny, 2008). PCNA has therefore been suggested as a prognostic marker for cancer (Naryzhny, 2008; Stoimenov and Helleday, 2009).

Numerous proteins bind to PCNA, and all identified protein - PCNA interactions are on the "front" side of PCNA (Jonsson et al., 1998). Many proteins bind through the conserved PIP-box (Qxx(L/I/M)xx/(F/Y)(F/Y)) identified in 1998 (Warbrick, 1998). Since then, a second PCNA binding motif, termed the KAx-box, was suggested (Xu et al., 2001); however, the biological significance of this motif is not verified. In 2009 we identified a new PCNA binding motif, first identified in the AlkB homologue 2, thereby its name APIM: AlkB homologue 2 PCNA Interacting Motif. This motif is functionally verified in six human proteins and a conserved motif is found in more than 200 other proteins. The discovery of this motif is described in paper 1.

PCNA plays important roles in many cellular processes in addition to DNA replication. These include involvement in regulation of replication bypass, prevention of sister chromatid recombination, DNA repair, chromatin remodeling and epigenetics, sister chromatin cohesion, cell cycle control and cell survival (Moldovan et al., 2007). With such diverse roles, and with a growing number of binding partners, a tight regulation is needed. This regulation occurs at several levels; one level is through different affinity to PCNA for proteins which bind to the same part of PCNA. An example of this is the PIP-containing protein cyclin-dependent kinase inhibitor 1 (p21) which interacts with

PCNA with a higher affinity than other PIP-box containing proteins. The PCNA - p21 binding thereby result in replication arrest by blocking the binding of Pol ε and δ to PCNA (Podust et al., 1995). Also, post translational modifications (PTMs) on either PCNA or its binding partners can regulate the affinity. PCNA ubiquitylation is the most documented PTM on PCNA and is involved in the polymerase switch in translesion synthesis described later (Lee and Myung, 2008; Moldovan et al., 2007). Furthermore, PCNA is reported to be SUMOylated, phosphorylated, acetylated and deacetylated (Hoege et al., 2002; Naryzhny and Lee, 2004; Prosperi et al., 1994). Also, a cancer specific modification on PCNA; methyl esterification, has been identified (Hoelz et al., 2006). PCNA is also know to be proteolyzed, but the biological relevance of this is unclear (Naryzhny, 2008). The PCNA form which binds to the APIM motif seems to have a PTM modification; however, the nature of this modification is not known (paper 1).

Numerous PCNA molecules, replication factors, cell cycle regulating proteins, DNA repair proteins etc. cluster to form so-called replication factories in the nucleus. These clusters give foci which can be visualized by fluorescent microscopy. When PCNA is fused to a fluorescent protein such as Green fluorescent protein (GFP) these foci can also be detected in live cells and this has been employed in the work for this thesis. In early S-phase the foci are small and localized throughout the nucleus, in mid S-phase the foci localize around the nucleoli and close to the membrane, and in late S-phase the foci are large and localized around the nucleoli (Leonhardt et al., 2000).

DNA damage

Damage to our DNA happens continuously, both by spontaneous reactions, most frequently from reactions with oxygen and water, and by influence from endogenous and exogenous agents. Quantitatively, most damage occurs as a result of spontaneous reactions and from endogenous agents, however, exogenous agents also pose a threat to the genome integrity. The exogenous agents include different chemicals that severely damage the DNA, including chemicals used in chemotherapy for cancer patients

Endogenous DNA damage

Reactive oxygen species (ROS) are generated during normal cellular metabolism and are among the most important sources of endogenous damage (De Bont and van Larebeke, 2004). The superoxide anion radical ($\cdot O_2^-$) is abundant in the cell, but its reactivity is low. $\cdot O_2^-$ can, however, be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase, which in turn can be reduced to the extremely reactive hydroxyl radical (·OH). Hydrogen peroxide is used as a source for generation of oxidative damage in paper 3. The DNA damage from ROS includes oxidized bases, single strand breaks (SSBs) and DSBs (De Bont and van Larebeke, 2004). Furthermore, oxygen radicals can abstract electrons from organic macromolecules such as lipids in a reaction called lipid peroxidation. The oxidized lipid products react with DNA and can result in the severe crosslinking between opposite DNA strands, so-called interstrand crosslinks (ICLs) (Friedberg, 2006).

Alkylating agents are electrophilic compounds with affinity for nucleophilic centers in organic macromolecules (Friedberg, 2006). They may arise from endogenous and exogenous sources and are widely used in cancer treatment. The main source of endogenous alkylation is S-adenosylmethionine (SAM), a small molecule with a reactive methyl group. SAM is involved in execution of physiologically enzymatic DNA methylation which is important in gene expression regulation. However, due to its reactivity, mutagenic and cytotoxic adducts can also be formed (De Bont and van Larebeke, 2004).

The glycosidic bond between the base and the deoxyribose is labile under certain conditions and may be cleaved off forming apurinic/apyrimidinic (AP) sites. This process can occur both spontaneously or as a consequence of ROS, and constitutes one of the most frequent lesions in DNA with 10 000 to 20 000 estimated AP sites per cell per day (Friedberg, 2006). The AP sites are cytotoxic as they stall replication and may lead to collapse of the replication fork forming DSBs. AP sites are also mutagenic, as the polymerase lacks a template, thus a random base is inserted, which in 54% of the incidents are adenines (De Bont and van Larebeke, 2004; Lawrence et al., 1990).

Hydrolytic deamination occurs more frequently in ssDNA than in dsDNA, and more frequently of pyrimidines than of purines. 100 to 500 cytosines per cell per day are estimated to deaminate forming uracil (De Bont and van Larebeke, 2004). Uracil, not normally present in DNA, resembles thymine and forms a base pair with adenine during replication. Thus, deaminated cytosines in DNA are mutagenic as they give C:G to T:A transition mutations. Uracil may also be mis-incorporated during DNA synthesis as substitute for thymine. In this case, uracil will not be directly mutagenic; however, the AP sites generated during removal of these uracils may be potentially mutagenic and cytotoxic (De Bont and van Larebeke, 2004; Friedberg, 2006).

Exogenous DNA damage

In general, exogenous DNA damage is more bulky than endogenous DNA damage, and is the main source for DSBs (De Bont and van Larebeke, 2004). Damage to DNA caused by ultraviolet (UV) light irriadiation was the first template for study of DNA repair (Friedberg, 2006). The UV light is divided into UV-A (320-400 nm), UV-B (295-320 nm) and UV-C (100-295 nm). The DNA absorption peak is at 260 nm (UV-C specific), however, not much UV-C radiation reaches the earth since wavelengths below 300 nm have low penetration through ozone. Thus, the solar UV light at the earth mainly consists of UV-A and UV-B (Cadet et al., 2005; Pfeifer et al., 2005). UV-B (used in the work for paper 2) and UV-C mostly induce DNA damage directly by covalent linkage between adjacent pyrimidines, forming most frequently cyclobutane pyrimidine dimers (CPDs), and to a smaller extent (6-4) photoproducts (Yoon et al., 2000) (illustrated in figure 3). Particularly the (6-4) photoproducts, but also the CPDs distort the DNA helix and both interfere with DNA replication (Kaufmann, 2007). UV-A radiation is not readily absorbed by DNA and mostly damages DNA indirectly through formation of reactive species, most frequently by formation of ROS (Cadet et al., 2005; Pfeifer et al., 2005).

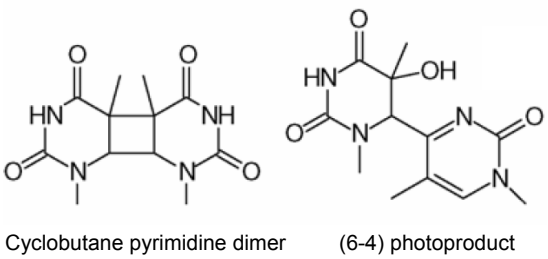

Figure 3: Cartoon of the most common DNA damage from UV; Cyclobutane pyrimidine dimer (CPD) and (6-4) photobroduct. Adapted from (Cadet et al., 2005).

Ionizing radiation (IR) is naturally occurring cosmic radiation, and has always been present. Damage from IR can take place either through direct absorption of the radiation energy by DNA, or indirectly as for the UV damage. IR may damage the base and form strand breaks (Friedberg, 2006). Moreover, DNA can be damaged by a wide range of chemicals. In addition to the chemotherapeutic anti-cancer drugs, carcinogenic chemicals are present in pollution, food, industrial waste, tobacco smoke, and more (Poirier, 2004).

Anti-cancer chemotherapy

The aim of anti-cancer therapy is to kill the cancer cells more efficiently than normal cells. To achieve this, cancer therapy must exploit the molecular and cellular features, characteristic for the target cancer cells. Since most cancer cells have a higher proliferation rate than normal cells, most cancer drugs target the cell cycle. Entry into the cell cycle can be inhibited by hormonal manipulation, therapeutic antibodies or drugs that inhibit the growth signals (Helleday et al., 2008). However, the use of DNA damaging chemotherapy which inhibits the cell cycle is more common and will be emphasized in this thesis. There are many cancer drugs, and the main groups are listed in table 1. Their general trait is their ability to produce excessive amounts of DNA damage causing cell death, either directly or following DNA replication.

Table 1: The main groups of anti-cancer chemotherapy and the various lesions they may form. Modified from (Helleday et al., 2008). * Chemotherapy used in the work for this thesis.

ANTI-CANCER CHEMOTHERAPY	DNA LESION
Radiotherapy and radiomimetics Ionizing radiation Bleomycin	Single-strand breaks Double-strand breaks Base damage
Monofunctional alkylators Alkylsuphonates Nitrosurea compounds Temozolomide *	Base damage Bulky adducts
Bifunctional alkylators Nitrogen mustard Mitomycin C * Carmustine/BCNU * Cisplatin (*)	Double-strand breaks DNA crosslinks Bulky adducts
Antimetobolites 5-Fluorouracil Hydroxyurea Folate analogues	Base damage Replication lesions
Topoisomerase inhibitors Camptothecins Etoposide	Double-strand breaks Single-strand breaks

Inhibitors of DNA replication impair replication fork progression which may cause DNA lesions including DSBs, and are therefore regarded as DNA damaging agents. Antimetabolites interfere with DNA replication by inhibiting nucleotide metabolism pathways, thereby depleting the cells of deoxyribonucleotides (dNTPs), or by being incorporated into the DNA. One of these antimetabolites, hydroxyurea (HU) is a clinically important anti-cancer drug and is also commonly used in the laboratory for study of arrested replication forks. HU acts by inhibiting the enzyme ribonucleotid reductase, preventing the conversion of ribonucleotides into dNTPs, thereby depleting the cells for dNTPs and stalling replication (Saban and Bujak, 2009). Inhibitors of the topoisomerases exploit the naturally occurring strand breaks. As already mentioned,

Topo I introduces SSBs and Topo II introduces DSBs while resolving torsional strains during DNA replication. Inhibitors of Topo I cause positive supercoils in front of the replication forks and replication associated DSBs, whereas inhibitors of Topo II trap the enzyme in complex with DNA leaving DSBs (Nitiss, 2009b; Pommier, 2006).

Alkylating agents are the oldest group of anti-cancer drugs, and remain among the most important group of chemotherapeutics in cancer treatment. In fact, alkylating drugs were first introduced as mustard gas during World War I as an agent for chemical warfare. Unexpectedly, beneficial traits of this horrifying drug were observed by the US Chemical Defense Research Department (Biesele et al., 1950). They found that the mustard gas interfered with mitosis and gave chromosomal aberrations, and by dissolving mustard gas in alcohol in the late 1920s, the first anti-cancer cytostatic with promising effect on superficial tumors was invented (Biesele et al., 1950; Joensuu, 2008). Although this drug was abandoned from clinical use, it constituted the foundation for future research resulting in the crosslinking alkylators used today.

Alkylating drugs exert their cytotoxic effect by modifying the DNA bases by covalently binding to DNA, either directly or after being metabolized in the body. The alkylating agents can be either monofuncitonal, with one reactive site modifying single bases, or bifunctional, with two reactive sites, capable of crosslinking two DNA bases in the same (intra-) or the opposite (inter-) strand. Temozolomide used in paper 1 is an example of a monofunctional alkylator. Temozolomide is believed to introduce methyl adducts on N^7G and O^6G, with the O^6meG as the most toxic lesion, inhibiting the DNA, RNA, and protein synthesis (Marchesi et al., 2007). The alkylators Mitomycin C, used in paper 1, and cisplatin mentioned in paper 2 are examples of bifuncional agents and are frequently used in treatment of several malignancies. Cisplatin has no alkyl group, but its mechanism is similar to that of alkylating drugs. Cisplatin primarily forms adducts on GG resulting in intrastrand crosslinks (95%). However, the minor ICLs are believed to constitute its anticancer effect by forming a total block of the DNA synthesis, thus interfering with the cell cycle (Chaney et al., 2005; Siddik, 2003; Wang and Lippard, 2005).

Cellular response to DNA damage

DNA damage can be both mutagenic and cytotoxic to the cell. Left unrepaired upon DNA replication, damage to DNA can give rise to mutations, and accumulation of mutations in genes coding for proteins involved in the cell's regulation of growth and death may in the worst case give rise to immortal cancer cells. DNA damage may also impair the protein synthesis, arrest the cell cycle, and lead to cell death - thus the cytotoxicity of the DNA damage. To render life possible, cells have evolved a natural defense to combat these threats. One of these defenses is through DNA repair, which counteracts most of the DNA damage before they harm the cells.

DNA repair

Traditionally, DNA repair has been divided into distinct pathways, such as lesion bypass, mismatch repair, direct repair, nucleotide and base excision repair, single and double strand break repair and the newly identified Fanconi anemia (FA) pathway. Recent research has, however, shown that the pathways are not always distinct and crosstalk between the different repair pathways is common. This is demonstrated by the Fanconi anemia (FA) ICL repair protein; FANCD1, which is identical to the DSB repair protein; breast and ovarian cancer type 2 susceptibility protein (BRCA2) (Wang, 2007). In the work for the papers presented in this thesis, we have studied the repair efficiency and damage tolerance after induction of certain types of DNA lesions. Most DNA lesions can be repaired by several DNA repair pathways, thus; in this thesis, the repair is described dependent on which type of DNA damage is repaired. Also, many DNA repair pathways are involved in the repair of more than one type of DNA damage, and will be briefly described the first time mentioned. To simplify, the focus will be on human, nuclear DNA repair. Proteins involved in direct repair as well as nucleotide and base excision repair are studied in paper 1, 2 and 3 respectively. Common for all three papers, however, is the importance of complex formation, especially with the "Maestro of the replication fork" PCNA.

Repair of damaged bases and single strand breaks

DNA damage from endogenous impact such as ROS and alkylation as well as spontaneous hydroxylation causes loss or damage of the DNA base. Loss of the base resulting in AP sites has the potential to form single strand breaks (SSBs). This kind of DNA damage is predominantly repaired by the base excision repair (BER)/single strand break repair (SSBR) pathways, however, mechanisms of direct reversal of the damaged base, such as bases alkylated by SAM, are also important. Moreover, the mismatch repair (MMR) pathway, normally regarded as a repair pathway for mis-incorporated bases, is involved in the repair of certain types of base damage.

Mismatch repair

The main function of MMR is to execute post-replicative repair of errors that have escaped the 3'- 5' exonucleolytic proofreading activity by replicative DNA polymerases, but it can also recognize base damage such as O^6meG caused by the anti-cancer drug temozolomide used in paper 1. Mis-incorporated bases are identified due to their failure to form Watson-Crick base pairs, while the base damage is identified due to a weakened base pairing as well as a slightly distorted helix (Dalhus et al., 2009). Mis-incorporated bases can give rise to mutations, while the methylated guanine can form base pairs with both cytosine and thymine; however, both meG:C and meG:T recruits the MMR machinery (Jiricny, 2006; Jun et al., 2006).

There are still some disputes regarding the exact mechanisms for MMR. However, it is commonly agreed that base - base mispairs and small insertion/deletion loop mispairs are recognized by a heterodimer of MutS homologues MSH2 and MSH6 (also-called MutSα), whereas a heterodimer containing MSH2 and MSH3 (also-called MutSβ) recognizes larger insertion/deletion loop mispairs (Jiricny, 2006; Jun et al., 2006). The MutSα or β complex binds to the mismatch and recruits a second heterodimer composed of two MutL homologues; MLH1 and post-meiotic segregation 2 (PMS2) (also-called MutLα). MutSα/β and MutLα are converted into sliding clamps by an ATP-driven conformation switch. The MutSα/β and MutLα complexes translocate in both directions in search for strand discontinuity. EXO1, a 5' to 3' exonuclease, subsequently binds

MSH2 and MLH1 (Jager et al., 2001; Tishkoff et al., 1998). This complex is believed to degrade a stretch of several hundred nucleotides, while RPA stabilizes the ssDNA. The degeneration ceases as the complex encounters the miss-pair. Replicative DNA polylmerases can then resyntesize the degraded region and DNA ligase seals the nick (Genschel and Modrich, 2003; Jiricny, 2006; Jun et al., 2006). Since only the newly synthesized strand is degraded, damaged bases such as O^6meG are not removed. Instead, MMR is believed to act as a damage sensor, signaling cell cycle arrest due to the so-called futile cycle, leaving other repair pathways to remove the damage (Jiricny, 2006; Meyers et al., 2003). The futile cycle is a result of MMR removal of the mismatch leaving the damaged base intact. The remaining lesion makes the MMR pathway to restart again and again, leading to cell cycle arrest. This allows time for other DNA repair mechanisms to remove the erroneous base, or alternatively formation of strand breaks and subsequently cell death (Jiricny, 2006; Meyers et al., 2003).

PCNA appears to function at several steps in MMR. It is essential from the start during mismatch recognition by binding to MSH3 and MSH6 (Flores-Rozas et al., 2000; Iyer et al., 2008; Kleczkowska et al., 2001), during translocation by binding of MLH1 (Lee and Alani, 2006), during digestion by binding to EXO1 (Nielsen et al., 2004), and throughout the completion of the repair pathway by binding the polymerase during DNA resynthesis (Moldovan et al., 2007). Notably, MSH3, MSH6 and MLH1 contain the conserved PIP-box motif (Kleczkowska et al., 2001; Lee and Alani, 2006). The importance of PCNA in the MMR pathway was demonstrated by Clark and colleagues by mutating the PIP-box in MSH6, which resulted in increased mutation rate (Clark et al., 2000). When using a p21 peptide which binds PCNA tightly by its PIP-box, the binding site on PCNA for other PIP-box containing proteins is blocked (Podust et al., 1995). This was utilized by Maish and colleagues who found that p21 prevents the binding of the MMR factors to the replication fork, demonstrating that PCNA is required for recruitment of the MMR machinery (Masih et al., 2008).

Direct repair

Direct repair is a mechanism for removing the lesion, without removing the nucleotide. There are essentially two mechanisms of direct repair of alkylated bases in humans, repair by transfer and by oxidative demethylation. In humans, there is one alkyl transferase called O^6-methylguanine-DNA methylatransferase (MGMT) also-called O^6-alkylguanine-DNA alkylatransferase (AGT), removing O-alkyl lesions from DNA (Kaina et al., 2007). This is a so-called suicide enzyme as it transfers the alkyl group to a cysteine acceptor within itself resulting in its inactivation followed by ubiquitylation and degradation (Kaina et al., 2007). Inactivation after alkyl transfer has also been reported to facilitate a switch of MGMT from a DNA repair protein to a transcription regulator, enabling the cell to sense, as well as respond to, mutagens (Teo et al., 2001).

The N-alkyl lesions are removed by the oxidative demethylases. There are 9 oxidative demetylases in humans. These are homologues of the bacterial AlkB protein, and members of a large superfamily of enzymes known as iron (II) and 2-ketoglutarate-dependent dioxygenases (Aravind and Koonin, 2001; Gerken et al., 2007). They are identified based on their homology, but little is know about the AlkB homologue 4 to 9 (ABH4 to 8 and FTO; fat mass and obesity associated protein). ABH8 has been shown to contain an RNA binding motif (Osada et al., 2002), and to be associated with generation of intracellular ROS and development of bladder cancer (Shimada et al., 2009), and FTO is known to remove 3meU and 3meT from ssDNA and RNA (Gerken et al., 2007; Jia et al., 2008). FTO was recently identified as an AlkB homologue based on sequence similarity, and variants of this protein are associated with obesity in humans (Frayling et al., 2007). Besides DNA and RNA repair, the homologues are believed to be involved in normal RNA methylation and demethylation, as well as demethylation of proteins (Sundheim et al., 2008).

At this date, most is known about ABH1, 2 and 3. Biochemical studies have shown that these homologues can demethylate bases in DNA (ABH1, 2 and 3) and RNA (ABH1 and 3) by an oxidative demethylation mechanism shown in figure 4 (Duncan et al., 2002; Westbye et al., 2008). Similar to the bacterial AlkB, the enzymes use oxygen, 2-

oxoglutarate (2OG) and iron (Fe^{2+}) to hydroxylate the methylated base (Falnes et al., 2002; Trewick et al., 2002; Aas et al., 2003). Hydroxymethyl is unstable, and is spontaneously released as formaldehyde (Sedgwick, 2004).

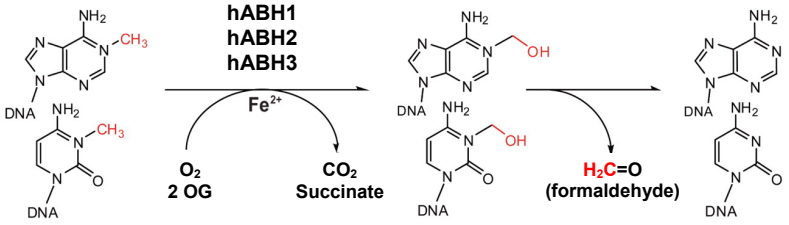

Figure 4: The general mechanism of the human AlkB homologues.
Adapted from (Roy and Bhagwat, 2007).

The first homologue discovered; ABH1 has the highest sequence similarity to AlkB (Aravind and Koonin, 2001; Wei et al., 1996). This homologue is predominantly localized in mitochondria where it repairs 3meC in ssDNA as well as in RNA (Westbye et al., 2008). ABH3 is localized to the nucleus as well as the cytoplasm and repairs 1meA and 3meC, preferentially from ssDNA and RNA (Aas et al., 2003).

The human AlkB homologue studied in this thesis is hABH2, which preferentially reverses 1meA and 3meC in nuclear dsDNA (Falnes et al., 2004; Koivisto et al., 2004; Aas et al., 2003). Furthermore, hABH2 was recently shown to reverse 1,N^6-ethenoadenine in DNA (Ringvoll et al., 2008). In 2003, our group published that hABH2 colocalizes with PCNA in replication foci, suggesting a role for this protein close to the replication fork (Aas et al., 2003). Removal of the alkylated lesions by hABH2 is therefore believed to act in front of the replication fork, preventing the alkylated bases to give rise to mutations. Subsequent studies in knock-out mice showed that cells deficient in mABH2 accumulated 1meA in DNA (Ringvoll et al., 2006). Embryonic fibroblast cells from these mice were sensitive to MMS, and the removal of 1meA was impaired, particularly in cells arrested in the S-phase (Ringvoll et al., 2006). This supports the importance of hABH2 during replication, suggested by Aas and

colleagues (Aas et al., 2003). hABH2 does not, however, contain the PIP-box. When searching for the sequence responsible for hABH2's colocalization with PCNA, we found that this sequence, which we called APIM also was responsible for a direct interaction between these proteins. The discovery of APIM is described in paper 1.

Base excision and single strand break repair

BER is a multistep DNA repair pathway removing damaged bases from DNA. As an intermediate in BER, SSBs are formed, and repair of these (SSBR) can be considered to be the same pathway as BER after excision of the damaged base. However, SSBs can also be formed directly by disintegration of the oxidized sugar from ROS attack, or as a result of erroneous or abortive activity of Topo 1 (Caldecott, 2008). The BER/SSBR pathway can be both replication coupled and replication independent, described in more detail later. SSBs left unrepaired upon replication may be fatal to the cells as they can lead to DSBs, demonstrating the importance of replication coupled repair.

Except for the initial base excision and strand excision steps, BER is essentially the same as SSBR illustrated in figure 5. The BER pathway is initiated by DNA glycosylases which recognize and cleave the N-glycosylic bond between the sugar backbone and the damaged base forming an AP site. There are several DNA glycosylases more or less damage specific. Roughly they can be divided into mono- and bi-functional glycosylases. The mono-funtional glycosylases simply cut out the damaged base, leaving the sugar backbone intact. The bi-functional glycosylases however, display an associated lyase function incising 5' and/or 3' to the AP site (Dalhus et al., 2009).

SSBs need another detection mechanism to recruit the repair machinery since there are no erroneous bases to be recognized. Instead, SSBs are recognized by Poly (ADP-ribose) polymerase 1 (PARP1), which rapidly binds and modifies itself and the target proteins with chains of PAR (Drew and Plummer, 2009; Hakme et al., 2008). PARP1 is also activated by SSBs formed indirectly during BER (Durkacz et al., 1980); however, the biological significance of this remains elusive since the damage has arisen during a coordinated controlled process where the intermediates are passed on from one enzyme

to another (Caldecott, 2008). Recent data indicate; however, that PARP1 is needed for recruitment of downstream BER/SSBR proteins, particularly the proteins involved in the long patch repair described later (Akbari et al., Unpublished). Proteins are recruited by the negative charge of the PAR-chain, through interaction by dedicated PARP binding motifs (Hakme et al., 2008).

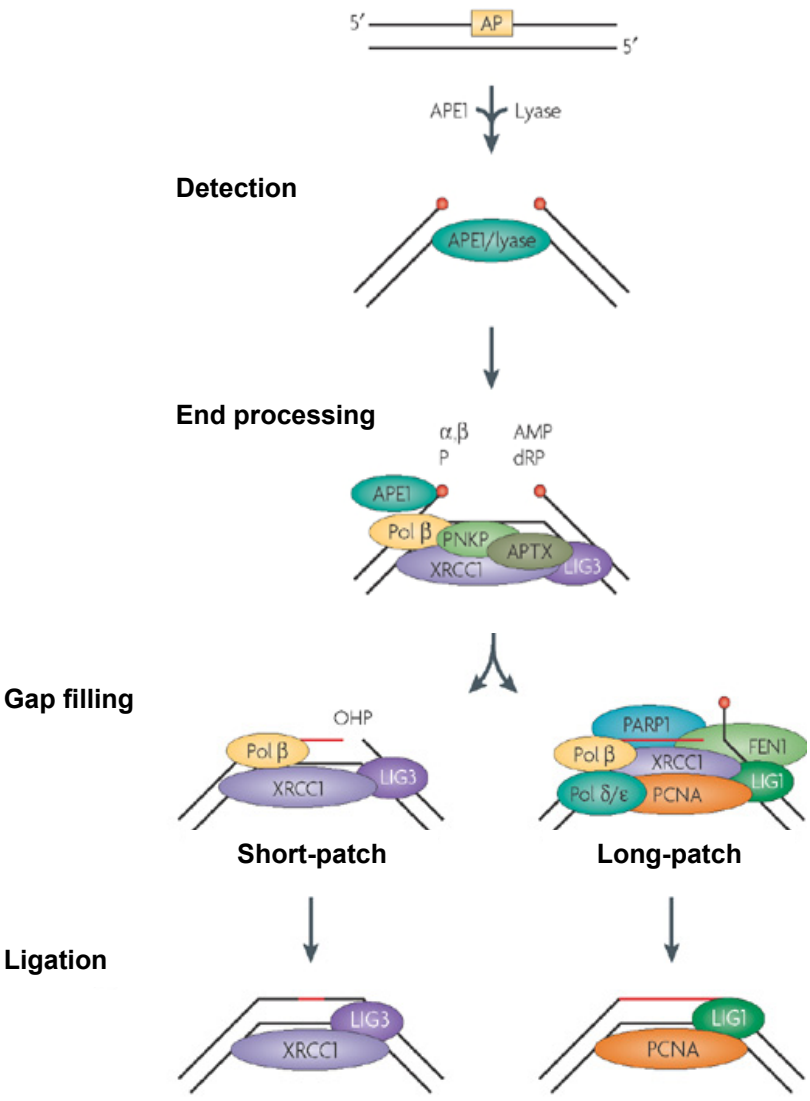

Figure 5: Sketch of the BER/SSBR pathway after base damage excision by the glycosylase. Adapted from (Caldecott, 2008).

After removal of the damaged base by the DNA glycosylase, the AP site formed is incised by an AP endonuclease (APE1) 5' to the AP site creating an SSB. For further repair, the ends need to be restored to the conventional 3'-OH and 5'-phosphate. Enzymatically, this is the most diverse step in BER (Caldecott, 2008). An example is DNA damage from ROS which can leave both phosphate and phosphoglycolate at the 3' end. These are processed by polynucleotide kinase 3'-phosphatase (PNK) and APE1 respectively (Evans et al., 2000; Wiederhold et al., 2004), both binding to the scaffolding protein X-ray repair cross-complementing protein 1 (XRCC1) (Caldecott, 2008; Horton et al., 2008). The gap can be sealed by a single nucleotide (short patch BER), or up to 10 nucleotides (long patch BER). Pol β is believed to be the main polymerase for gap sealing during BER, at least in short patch BER (Podlutsky et al., 2001). Pol β is shown to interact with XRCC1 (Caldecott et al., 1994), and to interact and colocalize with PCNA and XRCC1 in replication foci (Akbari et al., Unpublished; Akbari et al., 2010; Kedar et al., 2002). Notably, Pol β also possesses deoxyribosephosphate phosphodiesterase (dRPase) activity (Podlutsky et al., 2001), generating a ligatable 5' end which can be sealed by DNA liagase III (LIG3) which forms stable complexes with XRCC1 (Parsons et al., 2005). If the dRP-fragment is modified in such a way that it becomes resistant to the dRPase activity of Pol β, the dRP-fragment is removed as part of a single strand flap generated by strand displacement synthesis in the long patch BER (Akbari et al., 2009; Kubota et al., 1996). In long patch BER, Pol β, δ and ε in conjunction with PCNA incorporate nucleotides while displacing the old strand generating a flap. This flap is removed by FEN1 and the gap is sealed by LIG1 (Pascucci et al., 1999).

It is still disputed what determines whether BER/SSBR ends up in the short- or the long patch pathway. It has been reported to be dependent on the cell cycle, the presence of FEN1, PCNA and PARP1, and the type of damage (Akbari et al., 2009; Caldecott, 2008; Fan and Wilson, 2005). Recent studies by our group, however, have shown that after DNA insult by low dose of near UVA light, XRCC1 recruits its "core" complex containing PNK, Pol β, and likely LIG3 and other short patch BER proteins (Akbari et al., Unpublished). Higher dose of the near UVA light; however, recruits PCNA and FEN1 which are involved in the long patch BER, indicating that the amount or the

nature of the damage determines which path to be activated. Higher accumulation of PARP1 at the damaged area by addition of PARP inhibitor recruited long patch proteins to micro-irradiated regions also at low UVA doses, suggesting a role for PARP1 in signaling long patch BER (Akbari et al., Unpublished). There are several commercially available PARP-inhibitors, and inhibition of the PARP1-dependent SSBR has shown promising results in the treatment of cancers in patients with defective DSB repair (DSBR). PARP1 has therefore been the subject of extensive research the last few years (Bryant et al., 2005; Drew and Plummer, 2009; Helleday et al., 2008).

In addition to PARP1, the scaffolding protein XRCC1 plays a major role for complex formation in BER/SSBR. XRCC1 was first identified due to a mutant in Chinese hamster ovary cell (CHO, EM9) (Thompson et al., 1982). The XRCC1 mutant was isolated due to its hypersensitivity to MMS and IR, and the cells displayed defective SSBR, increased sister chromatid exchange as well as reduced homologous recombination (HR) (Hoy et al., 1987; Thompson et al., 1982). Later, the same group cloned the gene, and by expressing the XRCC1 protein in EM9 mutant cells, they found that this protein could restore the SSBR to the same level as the XRCC1 wild type (CHO AA8) cells (Thompson et al., 1990). The CHO EM9 and AA8 cells are used for studies performed for paper 3.

XRCC1 has no known enzymatic activity, but exerts its effect as a scaffolding protein (Caldecott, 2008; Horton et al., 2008). XRCC1 is important for efficient recruitment of proteins to BER/SSBR from the early damage recognition in BER by binding to DNA glycosylases such as Uracil-DNA glycosylase 2 (UNG2), N-methylpurine DNA glycosylase (MPG) also-called 3-alkyladenine DNA glycosylase (AAG), Nei endonuclease VIII-like 2 (NEIL2) and 8-oxoguanine DNA glycosylase (OGG1) (Akbari et al., 2010; Campalans et al., 2005; Das et al., 2006; Marsin et al., 2003) and by binding to PARP1 in SSB recognition (Caldecott, 2008; Horton et al., 2008; Masson et al., 1998). Furthermore, XRCC1 is reported to interact with the downstream BER proteins Pol β, PNK, PCNA, APE1, LIG3, and PARP1 and 2 (Caldecott, 2008; Caldecott et al., 1994; Fan et al., 2004; Masson et al., 1998; Schreiber et al., 2002; Vidal et al., 2001). Importantly, XRCC1 binds to itself forming multimers (Akbari et al., 2010;

Fan et al., 2004). Also Aprataxin and tyrosyl DNA phosphodiesterase (TDP1) binds to XRCC1 (Caldecott, 2008; Plo et al., 2003). Aprataxin and TDP1 are not essential for BER; however, congenital defects in these proteins give rise to neurological disorders described later.

Fully competent short patch and long patch BER complexes can be isolated by immunoprecipitation of XRCC1 (Akbari et al., Unpublished; Akbari et al., 2010). There seems to be at least three distinct BER/SSBR complexes. One complex is present in unthreated cells independent of the cell cycle, one is formed upon DNA insult, and one is bound to PCNA at sites of DNA replication (Akbari et al., Unpublished; Akbari et al., 2010). Results from pull-down followed by BER activity assays by Akbari and colleagues lead to suggestion of a model where there are pre-replicative BER/SSBR complexes containing UNG2 and XRCC1 proteins and a post-replicative BER/SSBR divided into two steps. Post-replicatively, UNG2 is believed to bind PCNA at the site of the replication excising the mis-incorporated uracil, forming an AP site which is repaired by a tightly followed, but non-interacting, XRCC1 complex (Akbari et al., 2010).

XRCC1 consists of tree functional domains, one N-terminal DNA binding domain, one internal BRCA1 carboxyl-terminal (BRCT) 1 domain and one C-terminal BRCT2 domain (Horton et al., 2008). Which domain is important for the scaffolding properties of XRCC1 has, however, been somewhat elusive. In paper 3, the relative contribution of each of these domains for intra-nuclear localization, recruitment to DNA damage, capacity for recruitment of the other BER/SSBR proteins as well as their capacity to form functional BER/SSBR complexes are described. The nuclear localization signal (NLS) to BRCT1 part of XRCC1 which turned out to be the key region for the function of XRCC1, contains three common single-nucleotide polymorphisms (SNPs). These SNPs have been the subjects to massive epidemiological studies in the search for correlations between the different XRCC1 SNPs and the capacity of DNA repair and cancer risk (Vineis et al., 2009). Thus, the repair capacity of XRCC1 with the various SNPs was also examined in paper 3.

For replication coupled BER/SSBR, PCNA is involved from damage recognition through binding of the DNA glycosylases to the final ligation, suggesting a PCNA-guided ordered reaction (Moldovan et al., 2007). UNG2 is up-regulated during the S-phase of the cell cycle (Hagen et al., 2008), and colocalizes with PCNA in replication foci through its PIP-box (Otterlei et al., 1999). This brings UNG2 to the replication fork where it is believed to perform pre- and post-replicative excision of uracil as described previously. The glycosylase mutY homologue (MYH) involved in the repair of mis-incorporated adenine opposite of 8oxoG (Slupska et al., 1996), also interacts with PCNA through its PIP-box (Chang and Lu, 2002). MYH is likely involved in post-replicative removal of mis-incorporated adenine similar to UNG2's removal of mis-incorporated uracil (Akbari et al., 2010). In addition, MPG removing methylated purine bases, mainly 3meA (O'Connor and Laval, 1991), interacts with PCNA through an inverted PIP-box (Xia et al., 2005). 3meA is a replication blocking lesion, thus MPG is likely repairing this lesion in a pre-replicative process. PCNA is shown to not only bind, but also to stimulate UNG2, NEIL1 and possibly MPG (Dou et al., 2008; Ko and Bennett, 2005; Xia et al., 2005). Furthermore, XRCC1, APE1, FEN1, Pol β/ε/δ and finally LIG1 interact with PCNA (Dianova et al., 2001; Fan et al., 2004; Moldovan et al., 2007). In BER/SSBR independent of replication, PCNA is believed to be mainly involved in the long patch gap filling (Caldecott, 2008).

The involvement of PCNA in BER/SSBR is undisputable, and also PARP1 has been reported to interact with PCNA (Simbulan-Rosenthal et al., 1999). PCNA is known to be poly (ADP) ribosylated by PARP1, and there seems to be a correlation between S-phase associated expression of PCNA and PARP1. This suggests a role for PARP1 in PCNA expression, possibly by interacting with its promoter (Simbulan-Rosenthal et al., 1999). Interestingly, conserved APIM motifs are found in PARP1 as well as PARP2 and 4 (paper 1); however, the biological significance of these motifs in PARP remains to be confirmed.

Repair of double strand breaks

DSBs constitute the most serious DNA damage as they can lead to mutations, chromosome instability and cell death (Bernstein and Rothstein, 2009). DSBs can be formed directly from IR or ROS or indirectly from incomplete BER/SSBR, from inhibited Topo II, stalled and collapsed replication fork, low pH and more (Ohnishi et al., 2009). To counteract the serious threat of DSBs, cells have evolved two distinct pathways, HR and non-homologous end joining (NHEJ). Defects in either one of these pathways lead to genetic instability and tumorgenesis (Delacote and Lopez, 2008). HR is most efficient when the sister chromatid is close by, thus post replication. NHEJ has generally been believed to act during the G1-phase; however, studies have shown that NHEJ can act in all stages of the cell cycle (Rothkamm et al., 2003). This enables the pathways to complement for each other in all stages of the cell cycle, except in G1 where there is no template for HR.

Homologous recombination

HR is mostly an error-free repair pathway that uses the homology of the sister chromatid to direct DNA synthesis across the damaged region. Roughly, Ataxia telangiectasia mutated (ATM) and the MRE11/RAD50/NBS1 (MRN) complex mediate the cell's initial response to DSBs. The damaged ends are then modified to generate 3'ssDNA tails which are substrates for HR. The key proteins RAD51 and RPA bind to the 3'ssDNA, forming a nucleoprotein filament. When homology is found, this nucleoprotein filament attaches to the sister chromatid invading the double strand, forming a so-called D-loop, constituting the template for DNA polymerase (Ohnishi et al., 2009). The strand invasion is dependent on RAD51, which is upregulated in many cancer types (Richardson, 2005). Another key protein in HR is the BRCA2 protein which is the same protein as FANCD1. BRCA2 binds RAD51, thereby restricting its action to the sites of DSBs where RAD51 accumulates. BRCA1 is also involved in HR repair by binding to the RAD51 and BRCA2; however, the exact mechanism for this contribution is not clear (Huen et al., 2010). Other proteins participating in HR are RAD51B, RAD51C, RAD51D, XRCC2, XRCC3, RAD54 and RAD54B (Dudas and Chovanec, 2004; Ohnishi et al., 2009). The importance of BRCA1 and 2 is visualized

by women with BRCA1 and 2 defects. These women are highly predisposed to breast and ovarian cancer (Huen et al., 2010; Yu et al., 2000), thus the full name for these proteins; breast and ovarian cancer type 1/2 susceptibility protein (BRCA1/BRCA2) (Huen et al., 2010).

PCNA has not so far been reported to directly participate in the HR pathway. However, in paper 1, we show that the RAD51 paralog, RAD51B, interacts with PCNA via the newly identified APIM motif. RAD51B is thought to assist RAD51 in the early stages of HR, and is unique amongst the RAD51 paralogs in that its deficiency results in hypersensitivity to DNA damaging agents, chromosomal instability and impaired RAD51 foci formation (Date et al., 2006). Furthermore, APIM was also identified in the BRCA1/BRCA2-containing complex subunit 45 (pro-BRE) and in the human structural maintenance of chromosomes 5 (hSMC5), both involved in HR (paper 1). Interestingly, the hSMC5 in complex with hSMC6 has been reported to be necessary for post-replicative repair of DSBs (Potts et al., 2006). The functionality of APIM in pro-BRE and hSMC5, however, remains to be investigated.

Non-homologous end joining

This DNA repair pathway is a straight forward re-ligation of the DNA ends without requirement of template, in a manner believed to be error-prone. The fidelity of NHEJ, however, depends on the nature of the DSB. DSBs from collapsed replication forks mostly generate one-ended DSBs. NHEJ requires two ends, and will ligate the one-ended DSB with a distal end causing gene rearrangements (Delacote and Lopez, 2008). The DSBs formed during G1 such as from IR and ROS leave two proximal ends, readily re-ligated by NHEJ (Delacote and Lopez, 2008). Since the main part of the genome is non-coding, loss or gain of a few nucleotides may not affect the genetic stability.

Roughly, the Ku70/80 heterodimer binds the DNA ends recruiting and activating DNA-dependent protein kinase (DNA-PK). DNA-PK phosphorylates the Ku proteins, XRCC4, XRCC4-like factor (XLF), Artemis, and itself (Pastwa et al., 2009). Blunt DNA ends are ligated by a complex of XLF, XRCC4 and DNA ligase IV (LIG4) (Ahnesorg et al.,

2006). However, when loss or gain of nucleotides is required, additional nuclease and polymerase activities take place. In humans, the endonuclease Artemis and the DNA polymerases Pol γ and Pol λ are reported to have roles in formation of blunt ends in the NHEJ pathway (Lieber, 2008; Povirk et al., 2007). Interestingly, an alternative DNA-PK/LIG4/XRCC4- independent pathway has been identified (Audebert et al., 2004). By using the DNA strand break chemical Calichemaicin γ1, PARP1 inhibitor, and cells proficient and deficient in PARP1, Ku80 and XRCC1, Audbert and colleagues found that this alternative pathway requires PARP1 and the ligation activity of XRCC1 - LIG3 (Audebert et al., 2004). Moreover, the XRCC1 partner PNK is reported to be involved in restoration of ligatable ends (Chappell et al., 2002). These are proteins traditionally referred to as BER/SSBR proteins, demonstrating the growing evidence of crosstalk between the DNA repair pathways.

As mentioned previously, XRCC1 binds to PCNA (Fan et al., 2004). Moreover, PARP1 contains the APIM motif described in paper 1 and may also bind to PCNA. Involvement of XRCC1 and PARP1 in the alternative NHEJ pathway may therefore recruit PCNA. However, so far, there are no reports of PCNA interference with NHEJ, but due to the multiplicity of PCNA, a role of PCNA in NHEJ would not be surprising.

Repair of intrastrand crosslinks

Intrastrand crosslinking can be formed as a consequence of exogenous insult such as UV-B and UV-C and from bifunctional chemical agents commonly used in cancer treatment. Furthermore, crosslinks can be formed by endogenous insults such as from peroxidized lipids (Friedberg, 2006). The intrastrand crosslinks kink the DNA helix, serving as templates for the nucleotide excision repair (NER) pathway. Furthermore, specialized DNA polymerases can bypass these intrastrand crosslinks by the so-called translesion synthesis (TLS) during replication.

Nucleotide excision repair

NER is the most versatile of the repair pathways as it repairs the diverse group of helix-distorting DNA lesions, mainly generated by environmental agents (Altieri et al., 2008; Hoeijmakers, 2009). These lesions interfere with base pairing, and disrupt transcription as well as DNA replication (Kaufmann, 2007; Unsal-Kacmaz et al., 2007). Even so, this pathway has not yet been reported to be coupled to replication. The lesions repaired by NER include pyrimidine dimers (mainly (6-4) photoproducts) caused by UV radiation (figure 3) and "bulky" chemical adducts that are incorporated in the DNA disrupting the folding (Nouspikel, 2009). Dependent on whether the damage occurs in the actively transcribed domains or elsewhere in the genome, repair is initiated by either of the two sub pathways; transcription coupled nucleotide excision repair (TCR), dealing with damage blocking the RNA polymerase or global genome nucleotide excision repair (GGR) recognizing damage throughout the genome (Nouspikel, 2009). Figure 6 shows a simplified sketch of TCR and GGR.

In TCR, the stalled RNA polymerase complex recruits Cockayne syndrome A and B (CSA and CSB) proteins. CSA has been shown to polyubiquitylate CSB, causing its release and degradation (Groisman et al., 2006). In GGR, recognition of the DNA damage is dependent on the kink. In the cases where the DNA lesions are causing a strong kink, e.g. (6-4) photoproducts, Xeroderma pigmentosum (XP) group C in complex with human homologue of yeast Rad23 protein (HR23B) recognize the lesion (Nouspikel, 2009; Sugasawa et al., 1998). DNA lesions causing a more modest kink, e.g. CPDs; however, are first recognized by damage DNA binding protein 1 (DDB1) together with an XPE/DDB2 complex. This heterodimer increases the kink, making it readily recognizable for the XPC complex (Chu and Chang, 1988; Nouspikel, 2009; Tang and Chu, 2002). After damage recognition, GGR and TCR are identical. The ten-component basal transcription factor II H (TFIIH) interacts with XPC from GGR or the stalled transcription apparatus from TCR. XPB and XPD, components of TFIIH are DNA helicases unwinding the DNA (Evans et al., 1997; Sarker et al., 2005). This makes space for binding of XPA and RPA, preventing reannealing (Evans et al., 1997). The

strand is cleaved by ERCC1-XPF and XPG, and the damaged oligonucleotide (25-30 bases) is removed (Mu et al., 1996; O'Donovan et al., 1994). Finally, the remaining gap is filled by the DNA replication machinery (Popanda and Thielmann, 1992; Shivji et al., 1992) and sealed by DNA ligase, presumably by LIG3 in complex with XRCC1 (Moser et al., 2007; Ogi et al., 2010).

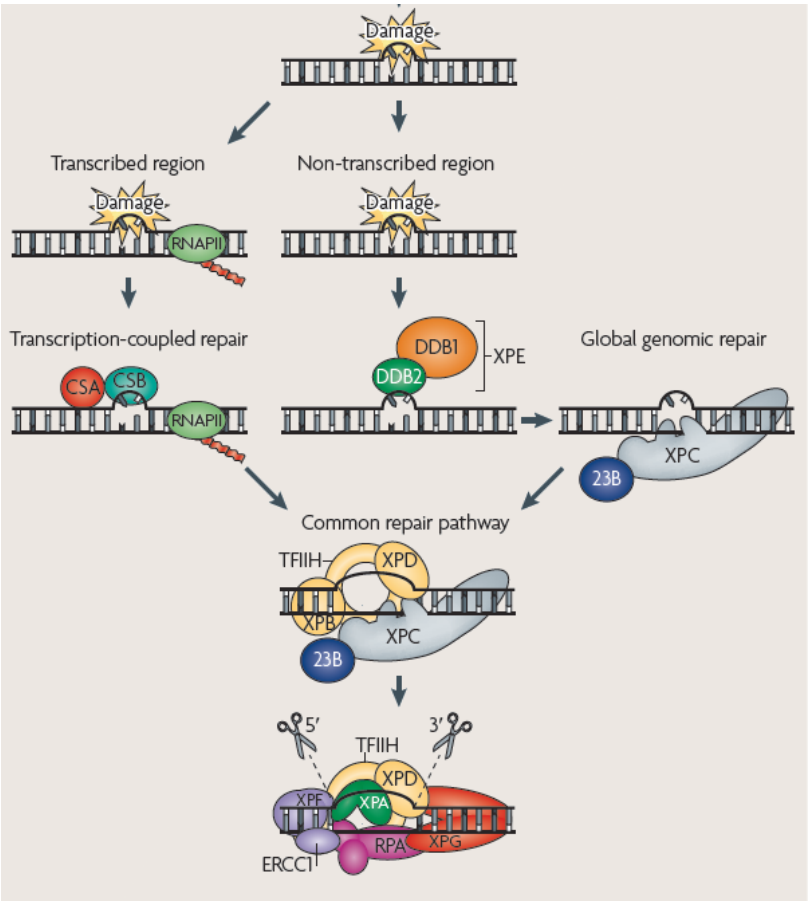

Figure 6. Simplified sketch of NER, from damage recognition to incision (Cleaver 2009).

All together, more than 30 proteins participate in the NER pathway (Hoeijmakers, 2009). XPA has an essential, but not yet fully understood role in the core incision complex, where it is believed to be involved in damage verification and tethering of DNA and the other NER subunits (Camenisch and Nageli, 2008; Nouspikel, 2009).

Furthermore, a recent publication suggests a presence of XPA in the process all the way from damage verification to the repair synthesis (Luijsterburg et al., 2010). Despite the small size (273 amino acids), XPA has many binding partners. XPA is reported to interact with RPA, XPC-HR23B, TFIIH, ERCC1, XPC, and XPA-binding protein 1 and 2 (XAB1 and XAB2) (Camenisch and Nageli, 2008; He et al., 1995; Krasikova et al., 2008; Matsuda et al., 1995; Nakatsu et al., 2000; Nitta et al., 2000; Tsodikov et al., 2007; You et al., 2003). Finally, XPA is reported to interact with DNA (Kuraoka et al., 1996), and itself forming dimers (Yang et al., 2002). Unlike the other NER proteins, XPA has no other known biochemical functions, thus impairing XPA will specifically affect NER. This makes XPA a potential target for improving cancer chemotherapy, and certain studies have shown that by impairing XPA directly, cells are sensitized to the crosslinking agent cisplatin (Cummings et al., 2006; Wu et al., 2003).

The presence of PCNA is indispensable for functional NER (Moldovan et al., 2007; Shivji et al., 1992). In 1997 PCNA was shown to participate in the DNA repair synthesis step in NER, through binding to the N-terminal of XPG. They also showed that this domain was essential for PCNA binding and NER activity (Gary et al., 1997). Subsequent studies showed that this N-terminal PCNA binding domain in XPG was the conserved PIP-box motif (Warbrick, 2000). Moreover, in paper 2, we show that also the scaffolding NER protein XPA binds to PCNA mediated by the APIM motif, and that this interaction is required for proper function of the NER pathway.

Lesion bypass

Arrested replication fork is a challenge for the cell, and prolonged stalling can result in fatal DSBs. Conserved from bacteria to humans, cells possesses mechanisms for replicating past certain types of lesions, by using specialized TLS DNA polymerases. In humans, there are currently 9 known specialized polymerases, termed Pol η (eta), ι (iota), κ (kappa), λ (lambda), μ (mu), θ (theta), ζ (zeta, Rev3), Rev1, and ν (nu) (Loeb and Monnat, 2008). These polymerases can temporarily switch with the blocked replicative DNA polymerases and polymerize over the damaged DNA. This is possible because these TLS polymerases have more flexible base-pairing properties enabling

them to bypass the lesions. The flexibility, however, makes them "sloppy" and not as accurate as the replicative DNA polymerases. Moreover, unlike the replicative polymerases (Pol δ,ε,γ) the TLS polymerases lack proofreading activity. Together with the flexibility, this contributes to a common reference of these polymerases as error-prone, thereby mutagenic (Loeb and Monnat, 2008; Wang, 2001). Some polymerases have distinct biological roles, and Pol η replicates accurately past T-T CPDs from UV irradiation. In the absence of Pol η, the TLS polymerases Pol ι, Pol κ, Pol ζ, and Rev1 may bypass CPDs, however, in an error-prone manner. Thus, cells lacking Pol η are hypermutable following UV exposure, visualized by the skin cancer predisposition syndrome XP variant (XPV) (Huang and D'Andrea, 2006; Inui et al., 2008; Pfeifer et al., 2005; Vaisman et al., 2003). Also, Pol κ, Pol ι, and Rev1 can copy past bulky DNA adducts, while Pol ζ and Pol θ are believed to be important for bypass of DNA crosslinks (Loeb and Monnat, 2008). Furthermore, Pol η, Pol ζ, Pol μ, and possibly Pol ι, may also participate in somatic hypermutation involved in antibody maturation (Wang, 2001; Zhu and Zhang, 2003).

Switching from the replicative DNA polymerase and deciding which of the TLS DNA polymerases to use, is probably to a large extent determined by PTMs on PCNA. Ubiquitylation of PCNA is one of the best characterized examples of how PTMs on PCNA regulate its action (Lee and Myung, 2008; Moldovan et al., 2007). Monoubiquitylation on PCNA at K164 is reported to increase the affinity towards the TLS polymerases (Huang and D'Andrea, 2006). The ubiquitin dependent binding of the TLS polymerases to PCNA is widely believed to be physically coupled to stalled replication forks (Davies et al., 2008; Ulrich, 2009; Yang and Zou, 2009). This has recently been challenged by Karras and Jentsch. They suggest that TLS triggered by monoubiquitylation of PCNA not normally mediates bypass at stalled replication forks, but rather mediates bypass post replication or even outside of the S-phase, at least in yeast (Karras and Jentsch, 2010). Pol η, Pol ι and Pol κ all bind PCNA through their PIP-boxes (Moldovan et al., 2007). Pol ζ on the other hand, contains the newly discovered APIM motif (paper 1), however, whether this motif is functional in Pol ζ, remains to be determined.

Repair of interstrand crosslinks

Most crosslinking agents generate both intrastrand crosslinks and ICLs, however, the ICLs are believed to be more severe as they pose a complete block of both transcription and DNA replication (McCabe et al., 2009). The repair of ICL is complex and not yet fully understood, however, it is believed to be both replication dependent, relying on the FA, HR and NER pathways and replication independent, relying on NER and TLS (Karras and Jentsch, 2010; Wang, 2007; Wang et al., 2001).

The Fanconi anemia pathway

The FA pathway was identified due to the rare cancer predisposition syndrome with the same name caused by mutation in any of the 13 hitherto known *FANC* genes. Little is known about the mechanism of the FA pathway, but it is known to be crucial for replication-coupled repair of ICLs. The FA proteins form complexes with each other as well as with proteins from the HR pathway (Bagby and Alter, 2006; Knipscheer et al., 2009). The FA pathway is believed to act in front of the replication fork, where it is somehow involved in the repair of ICLs, enabling the replication fork to progress (Wang, 2007). The 13 FANC proteins can be divided into three groups based on their function. Group I consists of 8 FA proteins constituting the core complex (FANCA, B, C, E, F, G, L, and M). Group II consists of FANCD2 and I, both of which are ubiquitylated by FA group I proteins and proposed to bind to DNA (Knipscheer et al., 2009; Wang, 2007). Group III consists of FANCD1 (BRCA2), J and N and are connected to breast cancer (Wang, 2007). Homozygous mutations in any of the *FANC* genes cause the FA disorder, while heterozygous mutations in any of the group III *FANC* genes predispose female carriers to breast cancer (Knipscheer et al., 2009; Wang, 2007). Some of the FA proteins have enzymatic activities: FANCM (group I) contains domains for helicase and endonuclease activities and has translocase activity; FANCJ (group III) is a DNA helicase interacting with BRCA1; and finally FANCD1 (BRCA2) (group III) is a regulator of RAD51.

Even though the FA pathway is replication dependent, an involvement of PCNA has not yet been demonstrated (McCabe et al., 2009; Wang, 2007). In paper 1, however, we report that one of the core FA proteins, FANCC (group I), contains the APIM motif. The functionality of APIM in FANCC, however, remains to be determined.

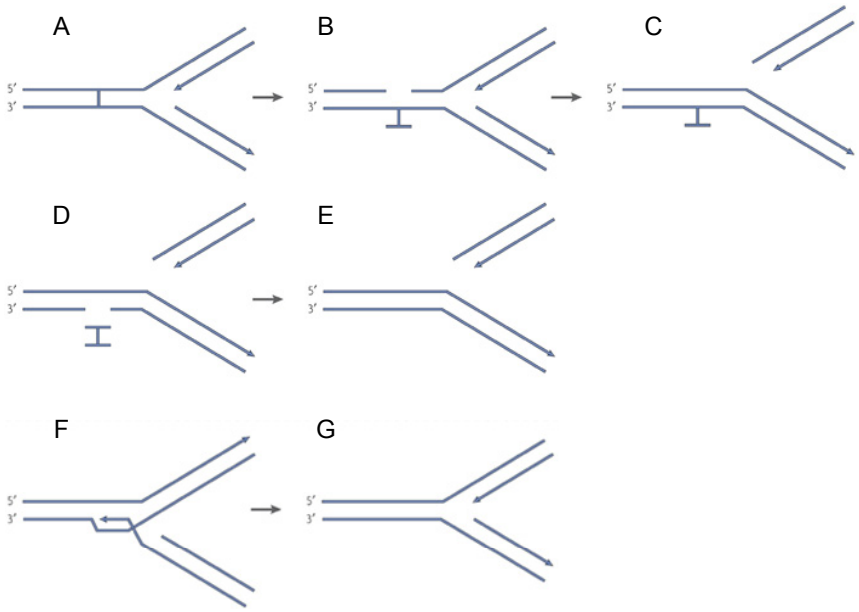

Figure 7. One of the models proposed to describe replication coupled repair of ICLs. A: Replication fork arrest at ICL recruits and activates the core FA proteins (group I). Group I FA ubiquitylates group II FA proteins. B: Unhooking of the crosslink by XPF-ERCC1 and MUS81-EME1. C: FA group III facilitates clearing of DNA and loading of TLS polymerases, creating a DSB. D-E: NER removes the remaining adducts and repairs the gap. F: FA group II recruits BRCA1-RAD51, promoting HR. G: Resolution of the recombination by FA helicases, and replication fork restart. Adapted from (Wang, 2007).

There are several theories for how repair of replication associated ICLs takes place. One model proposed by Wang is shown in figure 7 (Wang, 2007). The damage recognition is believed to be initiated by either collapsed replication fork or the FA proteins (McCabe et al., 2009). Recognition is tightly followed by incision near the ICL involving the NER heterodimer ERCC1/XPF and MUS81/EME1 unhooking the ICL

(7B). The remaining gap may be resynthesized by TLS forming a DSB (7C) (Nojima et al., 2005; Wang, 2007). The FA group III protein FANCJ helicase is believed to facilitate clearing of DNA and loading of the TLS polymerase (Wang, 2007). The remaining DSB is further repaired by the HR pathway shown to be essential for error-free repair of ICLs, where RAD51 plays an important role (7F) (Godthelp et al., 2002; Nojima et al., 2005). Finally, the FA helicases (FANCJ and M) are believed to resolve the recombination enabling restart of the replication fork (Wang, 2007).

To complicate the story further, the previously mentioned replication coupled MMR is also involved in ICL repair. Upon treatment with crosslinking anti-cancer drugs such as cisplatin, MMR is reported to signal cell cycle arrest due to the so-called futile cycle, eventually leading to strand break and cell death (Jiricny, 2006; Meyers et al., 2003). Thus, the MMR pathway induces cell death in response to cisplatin treatment, and deficient MMR is related to increased resistance to cisplatin (Jiricny, 2006; Meyers et al., 2003; Wang and Lippard, 2005). Interestingly, the NER complexes XPA - RPA and XPC - HR23B have been reported to cooperate with the MMR complex MutSβ in recognition of ICLs (Thoma et al., 2005; Zhao et al., 2009).

An HR-independent repair of ICL, probably also independent of replication, exists. This repair involves TLS together with the NER pathway and is unlike replication-dependent repair of ICL, error-prone (Wang et al., 2001). NER is believed to incise on both sides of the lesion, followed by TLS and another action of the NER pathway, resulting in removal of the ICL (McCabe et al., 2009; Wang et al., 2001). In this case, TLS is predominantly carried out by Pol ζ, at least in mouse and avian cells (Shen et al., 2006). Pol ζ is dependent on PTM modified (monoubiquitylated) PCNA and Rev1 for bypass of ICLs, in a process recently suggested to be independent of replication (Karras and Jentsch, 2010; Shen et al., 2006). A direct interaction between Pol ζ and PCNA has, however, not been demonstrated (Shen et al., 2006). Notably, Pol ζ contains the APIM motif described in paper 1. The PCNA form binding to the APIM containing proteins also has a PTM modification; however, the nature of this PTM is not yet known.

Clinical features of patients with hereditary defects in DNA repair pathways

The importance of the particular DNA repair pathways are demonstrated by the severe consequence for people with congenital DNA repair impairments, leading to predisposition to cancer or other diseases.

Perhaps the most pronounced disorder comes from defects in the NER pathway, with three rare human disorders, Xeroderma pigmentosum (XP), Cocayne syndrome (CS) and trichothiodystrophy (TTD). The skin cancer disease XP arises from mutations in one of the seven *XP* genes. The XP patients exhibit a >1000-fold increase in the incidence of sun-induced skin cancer, and 20% of the patients also develop neurological abnormalities dependent on which gene is defective (Cleaver et al., 2009; Hakem, 2008; Hoeijmakers, 2001). XP patients with defects in the GGR damage recognition proteins XPC and XPE have the lowest level of neurological damage, probably because RNA polymerase II is unaffected (Cleaver et al., 2009). CS is a rare human autosomal recessive inherited genetic disease. CS patients have growth retardation, progressive cognitive impairment and they die young. Similar to the XP patients they are also excessively sun sensitive, but do not seem to be predisposed to skin cancer. The CS disorder is caused by mutations in *CSA* or *CSB* genes, and CS cells therefore fail to recover gene transcription after DNA damage. Arrested RNA polymerase will induce apoptosis, thus damaged cells are lost rather than mutated. This explains why CS is not associated with increased cancer risk (Cleaver et al., 2009; Thoms et al., 2007). TTD is also a rare human autosomal recessive disorder caused by defective NER. In this case, the mutations are found in the *XPB* or *XPD* helicase genes. The TTD patients suffer from brittle hair and nails, dwarfism and ataxia, and half of the patients are sensitive to sunlight, however, as for CS patients, TTD patients do not seem to be predisposed to skin cancer (Cleaver et al., 2009; Thoms et al., 2007).

Sun sensitivity is also observed in patients with mutations in the TLS polymerase Pol η demonstrating its role in UV damage bypass (Pfeifer et al., 2005; Stary and Sarasin, 2002). These patients have normal NER function, and the disorder was therefore named

XPV prior to the identification of Pol η in 1999 (Masutani et al., 1999). The XPV patients are not as sensitive to UV light as the NER deficient XP patients; however, they are significantly predisposed to various types of skin cancers as a response to UV light from the sun (Inui et al., 2008).

Defects in the MMR pathway are related to development of hereditary non-polyposis colon cancer (HNPCC). Mutations in the *MSH2* or *MLH1* genes are most common (Hakem, 2008), but such mutations only constitute 3-4 % of all colorectal cancer incidents (Hsieh and Yamane, 2008). Defects in SSBR also lead to rare genetic disorders; Ataxia-oculomotor apraxia 1 (AOA1) and the even more seldom disorder; Spinocerebellar ataxia with axonal neuropathy 1 (SCAN1). None of the disorders result in genetic instability; instead they are characterized by ataxia and other neurological defects (Caldecott, 2008). The AOA1 disorder originates from mutations in the gene encoding aprataxin and SCAN1 from mutations in the gene encoding TDP1, both binding partners of the scaffolding proteins XRCC1 (Caldecott, 2008; Plo et al., 2003).

Defects in BRCA1 and BRCA2 proteins predispose women to breast and ovarian cancer. As described previously, these proteins are important for HR repair of the fatal DSBs. BRCA1 is furthermore important for cell cycle regulation by interacting with numerous cell cycle checkpoint and repair proteins through its BRCT domain. Moreover, BRCA1 is involved in DNA replication by regulating the ubiquitylation status of Topo II α (Huen et al., 2010). Pernicious homozygous mutations in any of the two *BRCA* genes result in enhanced chromosomal alterations termed gross chromosomal rearrangements which are the leading cause of cancerous mutations (Yu et al., 2000). For breast cancer, only 5 to 10% of the women carry mutation in *BRCA1* or *BRCA2*. However, a woman carrying *BRCA1* or *BRCA2* mutations in one allele, has 40 to 80% chance of developing breast cancer, making these mutations the strongest breast cancer predictors know (Fackenthal and Olopade, 2007).

Furthermore, homozygous mutations in any of the 13 *FANC* genes result in the genetic cancer predisposition syndrome FA. Genetically, the disease is characterized by chromosomal instability and hypersensitivity to ICLs (Wang, 2007). The phenotype is

characterized by bone marrow failure and high risk of myelodysplasia, acute nonlymphocytic leukemia and certain epithelial malignancies. In addition, the patients usually have particular features such as growth retardation, small head size, and café-au-lait spots; however, some patients acquire the characteristic features in adulthood (Bagby and Alter, 2006). As discussed for ICL repair, the FA pathway seems to work in tight collaboration with HR, however, the FA patients does not seem to be predisposed to breast or ovarian cancer. Heterozygous mutation in the group III *FANC* genes (*FANCJ* and *FANCN* in addition to *FANCD1*/*BRCA2*), however, seems to predispose women to breast cancer (Wang, 2007).

DNA repair and cancer therapy

The overall goal of cancer chemotherapeutic treatment is to impose a huge enough threat to the cells' DNA to make the cells give up and die. Our natural defense against these threats, DNA repair, will counteract these insults, and will ironically impair the cancer treatment. One approach for improving cancer treatment is therefore to modulate the DNA repair to increase the drug efficacy. Another approach is to take advantage of the cancer cells' defective DNA repair pathways, such as in the case of the BRCA1 and 2 deficient breast and ovarian cancers, which have reduced ability to repair DSBs. However, each cancer is genotypically different, thus personalized medicine has a potential for utilizing the particular genetic features for each of the cancer incidents.

Alkylating agents are widely used in cancer treatment (table 1). The direct repair protein MGMT is important for repair of O-alkylated lesions; however, the enzyme is inactivated and degraded after repair (Kaina et al., 2007). Thus, pseudo substrates for MGMT can be used in combination with temozolomide (which causes O^6meG). This will decrease the amount of active MGMT, and increase the effect of temozolomide. A class of pseudo substrates for MGMT is currently being tested in clinical trials in combination with temozolomide (Sharma et al., 2009).

At this date, the hottest DNA repair protein target for cancer therapy is PARP. Most is known about PARP1, which is involved in several DNA repair pathways, but most

importantly in BER/SSBR. Inhibition of PARP1 will impair SSBR, leading to the formation of DSBs. The use of PARP inhibitor has been suggested to work as a combination therapy for several types of cancers (Helleday et al., 2008); however, the indisputably best effects of PARP inhibitors is seen in BRCA1 and BRCA2 deficient cancer cells. Women with inherited heterozygous BRCA1 and BRCA2 defects have one functional allele; however, the cells that have developed into tumor cells have lost the functionality of both alleles (Yu et al., 2000). Consequently, the cancer cells in these patients display impaired DSBR by the HR pathway (Bryant et al., 2005; Farmer et al., 2005). By threating cells with PARP inhibitors, the SSBR is impaired, leading to accumulation of SSBs. SSBs left unrepaired form fatal DSBs. During DNA replication in normal repair proficient cells, these DSBs are repaired by HR. However, in the HR impaired BRCA1 and BRCA2 deficient cancer cells, more DSBs will remain unrepaired, leading to collapse of the replication fork and subsequent cell death (Bryant et al., 2005; Farmer et al., 2005). The use of PARP inhibitors is in phase III clinical trials and show promising results for these patients (Rouleau et al., 2010).

As our knowledge about the whole DNA repair machinery grows - which proteins are involved in repair of the various DNA lesions, how the proteins are modified and how they work together in complexes to efficiently repair the DNA - new possibilities for modulation of DNA repair emerge. Overexpression of an APIM containing fusion protein sensitized cells to various DNA damaging agents, including several anti-cancer drugs (paper 1 and 2). Thus, the APIM-peptide may be a potential DNA repair modulator for future cancer treatment. The intense basic research on DNA repair the last few decades will likely identify several new ways to modulate DNA repair, providing us with better and more customized cancer medicine in the future.

AIMS OF THE STUDY

The overall goal of this thesis was to study protein interactions in DNA repair using sophisticated *in vivo* cell biology based experiments. Use of confocal microscopy enables the study of fluorescently tagged proteins in living cells, in an environment close to reality. Our aim was to identify the regions within the particular DNA repair proteins responsible for complex formation with other DNA repair proteins and the "Maestro of the replication fork", PCNA. Furthermore, we wanted to investigate the biological significance of these interactions in terms of repair efficiency and cell survival.

In 2003, our group published that hABH2 colocalizes with PCNA in replication foci (Aas et al., 2003); however, unlike many other known proteins colocalizing with PCNA, it did not contain the PIP-box. The first aim of this thesis was therefore to reveal whether hABH2 and PCNA were directly interacting, and to find the region of hABH2 responsible for its complex formation with PCNA. The search for the PCNA interacting region in hABH2 turned into an identification of five conserved amino acids constituting a novel PCNA interacting motif, which we termed APIM. This motif was found in more than 200 proteins, many with distinct roles in DNA repair. Whether APIM was a functional motif in these proteins, however, was not known. Thus, the next aim was therefore to investigate the functionality of APIM in XPA, with an essential but not yet fully understood role in NER.

Numerous papers dealing with XRCC1 and its binding partners have been published. XRCC1 contains three conserved domains, reported to bind various repair proteins *in vitro*. However, which of these domains that is responsible for its localization to replication and to damaged areas, as well as its ability to recruit its binding partners, has been somewhat elusive. Furthermore, several SNPs within XRCC1 are associated with cancer. Therefore, the final and slightly different aim of this thesis was to study the ability of the various XRCC1 deletion mutants and SNP variants for intra nuclear migration, complex formation, protein recruitment, and repair capacity.

PAPER SUMMARY

Paper 1, Journal of Cell Biology 2009:

Identification of a novel, widespread, and functionally important PCNA-binding motif

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hABH2 colocalizes with PCNA in replication foci (Aas et al., 2003), but lacks the PCNA interacting peptide (PIP) box found in many proteins colocalizing with PCNA (Warbrick, 2000). Thus, the work for this paper started with dissecting hABH2, finding the smallest possible part responsible for its colocalization with PCNA, and examination of whether this sequence also mediated direct binding to PCNA.

By fluorescently tagged overexpressed proteins, confocal imaging, dot blot and pull-down assays we found that 5 conserved amino acids in the N-terminal of hABH2 were responsible for the colocalization between hABH2 and PCNA. Dot blot and subsequent *in vivo* confocal imaging showed that a conserved aromatic amino acid in position 2 (F, Y or W) of the 5 amino acid motif was crucial for its binding to PCNA. Positive fluorescence resonance energy transfer (FRET) indicates that the proteins are within 10 nm, and pull-down of crosslinked proteins indicates that the proteins are within 0.2 nm of one another (Matyus, 1992; Vasilescu et al., 2004). By using these two approaches, we found it most likely that the interaction between hABH2 and PCNA was direct. We termed this 5 amino acid motif AlkB homologue 2 PCNA interacting motif (APIM). Results from the fractionated pull-down assays suggested that only a part of the available hABH2 and PCNA - predominantly in the chromatin enriched fraction, interacted. This may indicate that PCNA, hABH2, or both need to be PTM modified to interact. This was supported by 2 dimensional gel electrophoresis analyses which

showed that APIM pulled down a modified form of PCNA; however, the nature of this modification is still enigmatic.

The functionality of APIM in hABH2 was illustrated by mutating F in position 4 in hABH2 to A, which totally disrupted colocalization with PCNA. Moreover, overexpression of APIM-YFP in S-phase cells threated with MMS lead to an elevated level of 3meA, and increased the sensitivity to MMS, measured by colony forming and MTT assays. However, APIM also sensitized cells to other DNA alkylating agents (Carmustine/BCNU, Mitomycin C and Temozolomide) introducing DNA damage not believed to be repaired by hABH2.

Using the APIM motif (K/R)-(F/Y/W)-(L/I/V/A)-(L/I/V/A)-(K/R) as a query, we obtained hundreds of hits in the Swiss-Prot/TreEMBL database. Of these, about 200 proteins had conserved APIM and were believed to be localized in the nucleus. Many of these proteins are involved in cellular stress-responses to DNA damage. Of the APIM containing proteins found, we verified functional APIM sequences in four proteins in addition to hABH2: transcription factor II S Like; transcription factor II-I; DNA topoisomerase II α; and RAD51B. All proteins colocalized and gave positive FRET with PCNA in replication foci. Importantly, mutation of the conserved amino acid (F to A) in APIM sequence in all these proteins either abolished colocalization or reduced FRET with PCNA, demonstrating the functionality of APIM in these proteins. In fact, the general transcription factor TFII-I has four APIM motifs. Mutation of either one of these motifs reduced FRET, and mutation of all four together totally abolished colocalization with PCNA. This suggests that several motifs can compete for stronger binding to PCNA.

To summarize, this paper presents the finding of a new PCNA interacting motif, called APIM, first discovered in hABH2. This motif binds directly to a modified form of PCNA in replication foci. A conserved APIM is found in more than 200 nuclear proteins, and a functional motif is demonstrated for 4 of these in addition to hABH2. Overexpression of this motif sensitizes cells to various DNA damaging agents introducing various types of lesions in DNA.

Paper 2, Manuscript submitted May 2010:

Proper functioning of the Xeroderma Pigmentosum group A protein is dependent on interaction with PCNA

Karin M. Gilljam, Rebekka Müller, and Marit Otterlei

The identified PCNA interacting motif, APIM (paper 1), was found in more than 200 proteins; however, the functionality of APIM in these proteins is not known. This paper investigated the functionality of APIM in the core NER pathway protein Xeroderma pigmentosum group A (XPA).

Experimentally, we made use of fluorescently tagged overexpressed proteins, confocal imaging, FRET, dot blot, pull-down, siRNA knock down, and MTT assay to examine the functionality of APIM in XPA. We found that XPA colocalized with PCNA in replication foci, and that XPA and PCNA were co-immunoprecipitated. Similar to what we found for the hABH2 - PCNA interaction (paper 1), the XPA - PCNA interaction seemed to be stronger in the chromatin enriched fraction than in the soluble fraction, probably requiring the PTM form of PCNA found in paper 1. Furthermore, FRET analysis indicated that XPA and PCNA were in close proximity. The APIM motif found in XPA is conserved within the DNA binding part of XPA, and these 5 conserved amino acids from XPA were sufficient for a close interaction with PCNA both *in vitro* (dot blot) and *in vivo* (confocal imaging including FRET measurements).

Unlike hABH2, transcription factor II S Like, transcription factor II-I, DNA topoisomerase II α, and RAD51B in paper 1, mutation of APIM in XPA did not reduce colocalization nor FRET with PCNA in HeLa cells. However, XPA has been reported to form dimers (Yang et al., 2002), and our FRET measurements supported this. Therefore, we used XP-A deficient fibroblast cells (GMO4429) for colocalization and FRET analysis. We found a strong reduction in FRET between XPA mutated in APIM and PCNA compared to the wild type constructs. However, the mutant XPA still colocalized

with PCNA, probably through its other binding partners. Notably, one of its binding partners, named XPA binding protein 2 (XAB2) also contains APIM (paper 1).

We found that overexpression of an APIM-peptide severely increased the sensitivity for damage from UV-B irradiation, which primarily is repaired by the NER pathway (Nouspikel, 2009). Importantly, by using siRNA knock down of XPA, we showed that the increased sensitivity against UV-B in cells overexpressing APIM compared to control cells was dependent on the presence of XPA. This strongly suggests that the UV-B sensitizing effect of APIM overexpression was due to reduced NER imputable to impaired binding between XPA and PCNA.

To summarize, this paper shows that XPA colocalizes and binds directly to PCNA through the newly discovered APIM motif, and that overexpression of APIM sensitized cells to UV-B only when XPA is present.

Paper 3, manuscript:

The NLS to BRCT1 region of XRCC1, harbouring the three most common single nucleotide variations, is essential for the scaffolding function of XRCC1.

Audun Hanssen-Bauer[†], Karin Solvang-Garten[†], Karin M. Gilljam, Kathrin Thorseth, David M. Wilson III, Mansour Akbari, and Marit Otterlei

[†] These authors contributed equally to this work

The scaffolding protein XRCC1 is composed of several conserved domains, all involved in interactions with different proteins. However, which of these domains is important for the scaffolding function, and the ability of XRCC1 to be recruited to sites of DNA damage or DNA replication, is somewhat elusive. Furthermore, SNP variants of XRCC1 have been associated with increased cancer incidents. Thus, in this paper we examined three different deletion mutants and three different SNP variants of XRCC1 and compared them with the full length, conservative protein in an $XRCC1^{-/-}$ background.

To investigate which part of XRCC1 was required for recruitment to sites of DNA damage, we used fluorescently tagged deletion mutants of XRCC1 (N-terminal, internal and C-terminal mutant). By introducing local damage by near UVA laser (405nm) during confocal imaging, we found that the BRCT1 region (present in both the internal and the C-terminal mutants) was essential for migration to sites of DNA damage. By co-expressing the XRCC1 deletion mutants with PCNA, PNK and Pol β, we found that the mutants carrying the suggested binding sites from *in vitro* experiments (Fan et al., 2004; Whitehouse et al., 2001), in addition to the BRCT1 domain, increased the recruitment of these proteins to micro-irradiated regions. Furthermore, we found that the BRCT1 region was absolutely required for colocalization of XRCC1 with sites of DNA replication, and that this colocalization was independent of the XRCC1-PCNA interaction sites.

Comet assay analysis, measuring single and double strand breaks as well as alkali labile sites, revealed that only the internal deletion mutant containing the region from NLS through BRCT1 had the capability to partly complement XRCC1 in XRCC1$^{-/-}$ cells after MMS challenge. This mutant neither binds Pol β nor LIG3, but contains the reported binding site for the 3-methyl-adenine DNA glycosylase (MPG) (Campalans et al., 2005), important for repair of methylated bases from MMS exposure. Thus, this mutant had the ability to colocalize with PCNA and MPG - two properties likely important for the complementation of XRCC1 for alkylation repair. The complementation observed despite the lack of Pol β and LIG3 binding sites may indicate that the repair of alkylated bases by MPG is most important during replication when other polymerases and DNA ligase I from the replication machinery are available. The internal mutant also possesses the binding site for OGG1, NEIL2 and NTH1 repairing oxidative base damage (Campalans et al., 2005; Marsin et al., 2003), however, this mutant could not complement for XRCC1 after H_2O_2 challenge.

The three common SNPs in XRCC1, Arg194Trp, Arg280His and Arg399Gln have been associated with increased incidence of specific cancers. All these SNPs are found within the important NLS - BRCT1 region of XRCC1. When comparing these SNP variants of XRCC1 with the conservative protein in our cell biology based experiments, we could not detect significant differences with regard to intra-nuclear localization or their ability to recruit Pol β or PNK to micro-irradiated regions. However, we found a slightly reduced ability to form and to maintain foci upon micro-irradiation as well as a slightly different repair profile after MMS and H_2O_2 treatment in two of these SNP variants compared with the conservative XRCC1. Small variations in repair profiles could have significant implications *in vivo*.

To summarize, the BRCT1 domain of XRCC1 is important for its accumulation to local DNA damage, and the mutants carrying the suggested binding sites for PCNA and PNK recruited these proteins to the micro-irradiated regions. The internal NLS - BRCT1 domain was further required for partial complementation of XRCC1 for alkylation damage repair. This part of XRCC1 carries three known SNPs showing slightly different accumulation upon micro-irradiation and *in vivo* repair efficiency.

DISCUSSION OF RESULTS AND PLANS FOR THE FUTURE

To increase the efficiency of DNA repair, proteins cluster together to be at the right place at the right time. Efficient DNA repair is vital during DNA replication, and the replication associated repair complexes - often mediated through binding to PCNA - has been our main focus.

Complex formation with PCNA increases the DNA repair efficiency and cell survival

Direct repair of alkylation damage by hABH2 has previously been reported to be localized to the replication fork (Aas et al., 2003), and to be required for efficient repair of 1meA during replication (Ringvoll et al., 2006). In paper 1, we showed that the interaction between hABH2 and PCNA is direct, mediated by a five amino acid motif which we termed APIM. Overexpression of this motif sensitized cells to damage from MMS, and increased the amount of 1meA in MMS threated HeLa cells arrested in S-phase. The sensitization by APIM is probably caused by blockage of the binding site for hABH2 on PCNA as illustrated in figure 8. This strongly indicates that hABH2 works more optimal while it is in complex with PCNA. We believe that hABH2 is bound to PCNA that is present close to the front of the replication fork during steady state replication of the genome. Alternatively, it may be in the vicinity of the replication fork and bind to PCNA with high affinity when the replication is stalled, similar to what is suggested for the TLS polymerases (Kannouche et al., 2004).

The core NER protein XPA has many binding partners, but the endonuclease XPG has been the only NER protein reported to bind to PCNA (Camenisch and Nageli, 2008; Gary et al., 1997). However, a recent publication investigating the assembly and disassembly of the NER proteins after UV-irradiation showed that XPA, not XPG, remained present in the final repair synthesis step, only accompanied by PCNA and RPA (Luijsterburg et al., 2010). They also suggested that XPA binds to the repair synthesis intermediate with high affinity. In accordance with this, we suggested that XPA binds directly to PCNA in paper 2. Importantly, our results showed that the

interaction is mediated by APIM and that proper function of XPA for UV damage repair is dependent on interaction with PCNA. Our data indicates that the interaction between XPA and PCNA takes place in replication foci, although NER is considered to be replication independent. Thus, interaction at the replication foci might be an artifact of overexpression due to direct interaction, enabling overexpressed PCNA to transport overexpressed XPA to these foci. Alternatively, XPA might in fact have an important role close to the replication fork. Bulky adducts and crosslinks block replication (Kaufmann, 2007; Unsal-Kacmaz et al., 2007; Wang and Lippard, 2005). Thus, keeping the NER core protein XPA in front of the replication fork could enable pre-replicative NER removal of the crosslink, allowing progression of the replication. The TLS polymerases may bypass the same bulky adducts, however, in an error-prone manner (Loeb and Monnat, 2008). NER, on the other hand has generally been considered to be error-free (Wood and Shivji, 1997; Youngs and Smith, 1973), although this has recently been challenged (Ogi et al., 2010). Ogi and colleagues suggest that not only Pol δ and Pol ε but also the "sloppy" TLS polymerase Pol κ may participate in the repair synthesis step in NER. Nevertheless, the presence of replication coupled repair by NER would be beneficial for the genome integrity. The confocal images show a clear colocalization between XPA and PCNA (paper 2), but compared to hABH2 (paper 1), more XPA seems to be in the nucleoplasm, indicating that only a fraction of the available XPA binds to PCNA. XPA may therefore have more than one role in the cell, which may mirror the contradictory published material concerning both the function as well as the cellular localization of XPA (Asahina et al., 1994; Bartels and Lambert, 2007; Lambert and Yang, 2000; Luijsterburg et al., 2010; Rademakers et al., 2003; Wu et al., 2007).

The BER/SSBR complexes appear to be comprehensive and intricate, and whether the BER/SSBR repair foci are pre-formed or formed sequentially upon DNA damage is part of an ongoing debate (Akbari et al., Unpublished; Akbari et al., 2010; Dianov et al., 2003; Fan and Wilson, 2005). Both XRCC1 and PCNA can be referred to as scaffolding proteins, since they have no enzymatic activity but instead mainly exert their effects through interaction with other proteins. PARP1 is reported to be important in BER, mainly through its recognition and binding to single strand breaks (Caldecott, 2008); however, recent work in our group suggests that PARP1 is important for recruitment of

long patch BER proteins (Akbari et al., Unpublished). PARP1 is reported to bind to the internal NLS-BRCT1 mutant of XRCC1 (Masson et al., 1998), the mutant required for complementation of XRCC1 in alkylation damage repair (paper 3). However, the C-terminal mutant, which also possesses the binding site for PARP1, did not have the capacity to complement for XRCC1. Thus, SSB recognition or recruitment of long patch repair proteins is not likely to be important in this case. It is more likely that the internal mutant's ability to bind to PCNA and MPG is its cardinal trait in complementation of XRCC1 in alkylation damage repair. It has recently been shown that the repair of alkylation damage from MMS, primarily executed by BER, is essential for fork progression during replication, at least in yeast (Vazquez et al., 2008). Moreover, MPG has been reported to be up-regulated in the S-phase and to interact with PCNA through an "inverted" PIP-box (Bouziane et al., 2000; Xia et al., 2005). This supports that the function of this protein is dependent on binding to PCNA, apparently recruited by XRCC1.

The potential binding of OGG1, NEIL2 and NTH1 to the same internal XRCC1 deletion mutant during exposure to oxidative damage from hydrogen peroxide did not support repair. This may indicate that repair of oxidative damage is not linked to replication to the same extent as the repair of alkylation damage, or that the main products of hydrogen peroxide treatment are SSBs. OGG1 has previously been reported to be constitutively expressed throughout the cell cycle (Dhenaut et al., 2000), whereas NEIL2 and NTH1 are reported to be up-regulated during S-phase (Hazra et al., 2002; Luna et al., 2000), and NTH1 to interact with PCNA (Oyama et al., 2004). A study has shown that knock down of both OGG1 and NTH1 individually, sensitized cells to hydrogen peroxide, demonstrating that this is a substrate recognized by these glycosylases (Yang et al., 2006). However, Yang and colleagues used ~100× more hydrogen peroxide compared to what we used in our comet assays. This dose would have wiped out our XRCC1 deficient cells, but perhaps a higher dose of hydrogen peroxide gives more oxidative damage.

PTMs on PCNA mediate the binding of APIM containing proteins, possibly inducing DNA repair, cell cycle arrest, and re-adjustments in transcription

We found that the PCNA form pulled down by APIM was modified (paper 1), and we are currently working on identification of this PTM. Compared to the main form of PCNA (pulled down by anti-PCNA antibodies), the form pulled down by APIM had a rare modification, making PCNA slightly more basic but not much larger. Thus, it is not likely that this modification is an ubiquitylation which is reported to signal the switch from a replicative to a TLS polymerase upon arrest of the replication fork (Lee and Myung, 2008). It is more likely that this modification is an acetylation, a PCNA modification reported to be enriched in the chromatin bound fraction and up-regulated during S-phase (Naryzhny and Lee, 2004). Thus, this modification fits well with our data; however, we have not been able to identify any acetylations on PCNA by Western blots. In terms of charge and size, the APIM specific modification also resembles the reported cancer specific methyl esterification on PCNA (Hoelz et al., 2006); however, APIM containing proteins also interact with PCNA in noncancerous cells. We believe that APIM specific PTM modification of PCNA may be induced by DNA damage, signaling a switch in PCNA's binding preference from PIP-box containing proteins, to APIM containing proteins as illustrated in figure 8. This will explain why APIM expression sensitized cells to DNA damaging agents, whereas unthreated cells seemed to be unaffected by APIM (paper 1 and 2).

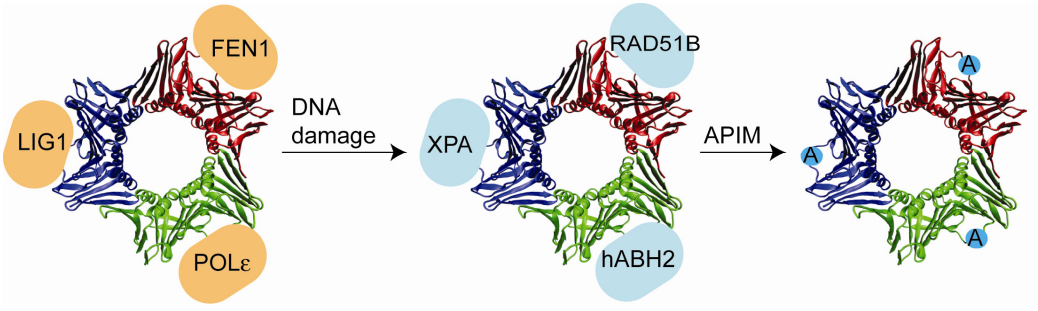

Figure 8. Suggested change in PCNA's affinity towards PIP-box containing proteins (orange) to affinity towards APIM containing proteins (blue) as a result of APIM specific PTMs on PCNA after DNA damage. Expression of the APIM peptide (small blue dots) blocks the binding of APIM containing proteins to PCNA.

Cells overexpressing APIM are hyper-sensitive to crosslinking agents such as Carmustine/BCNU and Mitomycin C (paper 1) as well as cisplatin (unpublished data), forming intrastrand crosslinks and ICLs. The cellular response to these crosslinks involves several processes including cell signaling and activation of various DNA repair pathways such as NER, HR, FA and TLS (Wang and Lippard, 2005; Wang, 2007). Impaired function of XPA may contribute to the increased sensitization to crosslinks; however, we did not observe any sensitization to cisplatin when we knocked down XPA by siRNA. It is not likely that diminished hAHB2 affects the sensitization to these agents; however, reduced function of RAD51B may reduce the efficiency of HR and thereby the error-free ICL repair. Moreover, the reason for APIM's sensitization to crosslinking agents is probably imputable to reduced function of several of the APIM containing proteins listed in http://tare.medisin.ntnu.no/pcna/index.php, published in paper 1. Among these are the HR proteins pro-BRE and hSMC5, and the FA protein FANCC mentioned previously. Preliminary studies regarding the core FA protein FANCC show that FANCC colocalizes with PCNA, possibly through a functional APIM motif (Müller et al., in prep). Also, the MAP kinase pathway contains three APIM containing proteins; MAPKAP Kinase 2 and 5 (MK2 and MK5) and mitogen-activated protein kinase 15. MK2 is known to be activated upon cell stress including DNA damage (Kyriakis and Avruch, 2001), and a recent study has suggested a role for MK2 in cell cycle arrest in response to DNA damage (Reinhardt and Yaffe, 2009). An ongoing study at our lab suggests that the MAP-kinase pathway is partly impaired by overexpression of APIM, possibly contributing to the sensitization to crosslinking agents (Müller et al. in prep).

The transcription machinery controls and adjusts the protein production according to the cells' demand. After cell stress such as DNA damage, transcription of stress-related genes is stimulated while transcription of growth-related genes is repressed (Lopez-Maury et al., 2008). In paper 1, our strongest evidence for the functionality of APIM as a PCNA interacting motif was the transcription factor TFII-I with four conserved functional APIM motifs. TFII-I is a growth factor induced transcription factor which has been shown to participate in transcription of Cyclin D1, which is important for cell cycle entry into S-phase as a response to growth signal (Desgranges and Roy, 2006).

Vice versa, the same group suggested that TFII-I is ubiquitylated and degraded following genotoxic stress, resulting in repression Cyclin D1 transcription and thereby cell cycle arrest. Thus, a presence of TFII-I in front of the replication fork would efficiently sense the DNA damage and signal for cell cycle arrest. Furthermore, two RNA polymerase II subunits contain APIM, whereas the largest subunit (RPB1) has shown to colocalize with PCNA in replication foci (Halvei, 2009, unpublished Master thesis). This supports a close interaction between DNA replication and transcription, which has already been suggested (Gilchrist et al., 2008; Malyavantham et al., 2008). A close interaction between these two processes, which previously was referred to as separate (Wei et al., 1998), enables communication of replication arrest upon encountering of DNA damage. This may lead to transcription repression of growth-related genes, while transcription of stress-related genes may be stimulated. Our data from paper 1, suggests that APIM could be important in this linkage between DNA replication and transcription, possibly signaled by APIM-specific PTMs on PCNA.

In vivo versus *in vitro* approach for the study of protein complexes

Traditionally, protein interactions have been studied *in vitro*, inside a tube. Our approach; however, has been to identify protein-protein interactions and protein localization in live cells *in vivo*. Many of our results could not be obtained from purified proteins and *in vitro* experiments. Examples are our endless unsuccessful attempts to show specific binding between purified full length hABH2 and PCNA *in vitro*, before we realized that the PCNA form binding to APIM is PTM modified. Thus, specific high affinity interaction is likely dependent upon this modification. XRCC1 is similar to PCNA, a scaffold protein which is PTM modified. The exact nature and extent of PTM modifications on XRCC1 and PCNA are not fully understood. However, proper protein modifications as well as correct intra-cell and intra-nuclear localization are vital in order to study and identify new interaction partners.

In cases where the alternative PTMs are known and can be mimicked, *in vitro* assays can be useful. If we can mimic the specific PTM required for APIM's binding to PCNA, we can execute *in vitro* assays to quantitatively elucidate the stimulatory effect of

hABH2's binding to PCNA. However, whether the binding between hABH2 and PCNA increases the activity of hABH2 in a reaction tube where the enzyme and substrate are in close proximity is uncertain. Another urgent issue that needs to be addressed is to elucidate where on PCNA the APIM motif binds. If we manage to acquire specific *in vitro* binding between APIM and PCNA, they can be crystallized together, or alternatively be analyzed by Small angle X-ray scattering (SAXS) experiments. The PIP-box has been reported to bind into a hydrophobic pocket of PCNA buried under the interdomain connecting loop (Bruning and Shamoo, 2004; Gulbis et al., 1996), and whether the APIM-peptide binds to or overlap with this loop is of interest since this could tell us whether PIP and APIM collaborate and mutually exclude each other.

APIM versus PIP

For 11 years (Warbrick, 1998), the PIP-box was the only known PCNA interacting motif, and in many papers we find the sentence *"…PIP box, was found in … and most other PCNA-binding partners*" (Moldovan et al., 2007). In fact, in the database search performed for paper 1, we found 226 nuclear proteins with conserved APIM and "only" 198 with conserved PIP-box, indicating that at least as many proteins contain the APIM motif. The functionality of PIP and APIM in most of these proteins, however, remains to be determined. The majority of the PIP-containing proteins are directly involved in DNA replication or in processes tightly bound to the replication such as the MMR pathway. On the other hand, APIM containing proteins appear to be involved in processes more important after cell stress. In support for this, overexpression of a PIP-box peptide (from p21) completely blocked DNA replication (Mattock et al., 2001), while overexpression of APIM had little or no effect on the cell growth without addition of cytotoxic agents (paper 1 and 2).

APIM Therapeutics AS

The APIM motif is now being patented, and a company APIM Therapeutics AS, has been established. Exposing various cancer cells to an APIM-derived peptide has shown that the toxic effect of APIM alone is a matter of concentration. Also, the toxicity varies significantly between different cancer cells. Certain cancer cell lines, such as lymphoma

and myeloma cells, are particularly sensitive to the peptide without additional drugs, whereas other cancer cell lines such as osteosarcoma and breast cancer cells appear to be unaffected by APIM expression alone, but display an increased sensitivity when exposed to APIM in combination with cancer chemotherapy. Moreover, preliminary studies using a Xenograft prostate cancer mouse model have shown that the mice receiving the APIM peptide together with cisplatin displayed delayed tumor growth. Importantly, the APIM motif is not toxic to the mice as judged by body weight loss at receivable doses; thus, the APIM-peptide has a potential as a DNA repair modulator in future cancer treatment.

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Paper I

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Identification of a novel, widespread, and functionally important PCNA-binding motif

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Numerous proteins, many essential for the DNA replication machinery, interact with proliferating cell nuclear antigen (PCNA) through the PCNA-interacting peptide (PIP) sequence called the PIP box. We have previously shown that the oxidative demethylase human AlkB homologue 2 (hABH2) colocalizes with PCNA in replication foci. In this study, we show that hABH2 interacts with a posttranslationally modified PCNA via a novel PCNA-interacting motif, which we term AlkB homologue 2 PCNA-interacting motif (APIM). We identify APIM in >200 other proteins involved in DNA maintenance, transcription, and cell cycle regulation, and verify a functional APIM in five of these. Expression of an APIM peptide increases the cellular sensitivity to several cytostatic agents not accounted for by perturbing only the hABH2–PCNA interaction. Thus, APIM is likely to mediate PCNA binding in many proteins involved in DNA repair and cell cycle control during genotoxic stress.

Introduction

Proliferating cell nuclear antigen (PCNA) is a member of the conserved sliding clamp family of proteins. It is essential for chromosomal DNA replication and important for several DNA transactions, such as DNA repair, epigenetic modification, chromatin assembly and remodeling, sister chromatid cohesion, and cell cycle control (Moldovan et al., 2007). Numerous proteins involved in these processes are localized in so-called replication factories, and many of these proteins interact with PCNA through the conserved sequence called the PCNA-interacting peptide (PIP) box (QxxL/I/MxxHF/DF/Y; Warbrick, 2000). However, several PCNA-binding proteins do not contain a PIP box (Fan et al., 2004; Moldovan et al., 2007). Furthermore, posttranslational modifications (PTMs) of PCNA have been reported to regulate the affinity to its binding partners, as illustrated by polymerase switch (Lehmann et al., 2007).

Human cells are exposed to alkylating compounds produced endogenously from environmental sources and drugs used in cancer treatment (Drabløs et al., 2004). Proteins involved in DNA repair and cell cycle control are interesting targets to increase the efficacy of chemotherapy (Helleday et al., 2008). The DNA damage introduced, such as alkylation adducts and interstrand cross-links, may lead to miscoding, replication arrest, double-strand breaks, and/or cell death. The simpler lesions, such as methylated bases, are repaired by base excision repair (BER), oxidative demethylation, or methyl transfer, depending on the type of damage (Sedgwick et al., 2007). The BER enzyme 3-methyladenine DNA glycosylase (AAG/MPG; removes 3meA) and the oxidative demethylase human AlkB homologue 2 (hABH2; repairs 1meA and 3meC) are both localized in proximity of replication foci (Aas et al., 2003; Xia et al., 2005). Although MPG contains an "inverted" PIP box sequence for interaction with PCNA, no PIP box is found in hABH2.

In this study, we demonstrate that hABH2 interacts with PCNA through a novel PCNA-interacting motif, AlkB homologue 2 PCNA-interacting motif (APIM), and that APIM is a functional PCNA-binding motif important for several proteins involved in DNA maintenance and cell cycle regulation after DNA damage.

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Abbreviations used in this paper: APIM, AlkB homologue 2 PCNA-interacting motif; BER, base excision repair; FRET, fluorescence resonance energy transfer; hABH2, human AlkB homologue 2; HcRed, *Hereactis crispa* RFP; IP, immunoprecipitation; LC, liquid chromatography; MMC, mitomycin C; MMS, methyl methanesulfonate; MS, mass spectrometry; PCNA, proliferating cell nuclear antigen; PCV, packed cell volume; pI, isoelectric point; PIP, PCNA-interacting peptide; PTM, posttranslational modification; TMZ, temozolomide; Topo, topoisomerase; WB, Western blot; WT, wild type.

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Results and discussion

The 10 N-terminal amino acids in hABH2 are essential for colocalization with PCNA

To identify the region in hABH2 responsible for localization in replication foci during S phase (Aas et al., 2003), we coexpressed PCNA tagged with a blue variant of GFP (CFP-PCNA) and various hABH2 deletion mutants fused with a yellow GFP variant (YFP) because GFP-tagged PCNA is known to form foci representing sites of replication (Leonhardt et al., 2000). First, we verified that hABH2-YFP colocalized with endogenous PCNA similar to coexpressed, tagged PCNA (Fig. 1 A, rows 1 and 2). Next, we found that deletion of the 10 N-terminal amino acids in hABH2 totally abolished the colocalization with PCNA. Remarkably, these 10 amino acids fused to YFP were sufficient for colocalization with PCNA (Fig. 1 A, rows 3 and 4). Notably, coexpression of CFP-PCNA increased the localization of full-length hABH2 (hABH2$_{1–261}$-YFP) but not hABH2$_{11–261}$-YFP in nuclear foci, suggesting a direct interaction between PCNA and hABH2 mediated by the 10 N-terminal amino acids of hABH2.

To investigate the potential hABH2–PCNA interaction in more detail, soluble and chromatin-enriched protein extracts were prepared from cells expressing hABH2-YFP, hABH2$_{11–261}$-YFP, or YFP and subjected to coimmunoprecipitation (co-IP) using anti-YFP antibodies (α-YFP). Notably, low levels of PCNA were pulled down from the soluble cell fraction, whereas PCNA was readily pulled down from the chromatin-enriched fraction. Moreover, removal of the 10 N-terminal residues in hABH2 markedly decreased the amount of PCNA pulled down (Fig. 1 B). The hABH2–PCNA interaction was confirmed by reciprocal experiments using extracts from cells expressing YFP-PCNA (Fig. 1 C) and also by targeting endogenous PCNA (Fig. 1 D). In both cases, more hABH2 was pulled down from the chromatin-enriched fractions than from the soluble fractions (Fig. 1, C and D), even though both proteins were present in the soluble fraction (Fig. 1 D, input). Collectively, these results support the idea that hABH2 interacts with PCNA and that the binding sequence is contained within hABH2's 10 N-terminal amino acids. The apparent preferential association of hABH2 and PCNA in the chromatin-enriched fraction may indicate that a subfraction of either of the proteins exists in a PTM form, promoting the interaction. Alternatively, the presence of other proteins may mediate the observed interaction. A bridging effect caused by DNA interaction was considered less likely because the chromatin-enriched fraction was subjected to extensive DNase and RNase treatment before co-IP.

hABH2 directly interacts with PCNA through a novel PCNA-binding motif

Sequence alignment of ABH2s from several species shows that the seven N-terminal amino acids are highly conserved (Fig. 2 A) and have the apparent consensus Met-Asp-Lys/Arg-Phe-(Leu/Val/Ile)$_2$-Lys/Arg. The flanking amino acids (8–10) are not conserved. Dot blot assays against mutant versions of this sequence indicated that the most important determinant for binding to PCNA was an aromatic residue at position 4 because Tyr could substitute for Phe at this position, whereas Ala abolished the interaction (Fig. 2 B and not depicted). We verified the sequence specificity for the PCNA interaction in vivo by expressing the conserved amino acids 1–7 of hABH2, and variants in which Phe4 was substituted by Tyr, Trp, or Ala, in fusion with YFP and tested their subnuclear localization. Expressed fusion proteins containing an aromatic amino acid in position 4 colocalized with PCNA when expressed alone (Fig. 2 C, rows 1 and 2) and when coexpressed with CFP-PCNA (Fig. 2 C, rows 3–5). Analogous to what was found in dot blot assays, the F4A mutation severely reduced the colocalization with PCNA (Fig. 2 C, row 6). By measuring fluorescence resonance energy transfer (FRET), we found that both full-length hABH2-YFP and hABH2$_{1–10}$-YFP as well as hABH2$_{1–7}$F4W-YFP are in very close proximity with CFP-PCNA because fluorescent tags must be <100 Å apart to give positive FRET (Mátyus, 1992).

To further investigate the proximity between hABH2 and PCNA, we performed in vivo cross-linking in cells stably expressing hABH2$_{1–7}$-YFP-Flag and hABH2$_{1–7}$F4A-YFP-Flag using formaldehyde. Formaldehyde induces heat-reversible cross-links of proteins that are within ∼2 Å of one another (Vasilescu et al., 2004). Extracts from these cells were used for IP with α-Flag. After elution with Flag peptide, cross-links in half of the samples were reversed. In Fig. 2 E (lanes 3 and 11), bands containing both PCNA and Flag are identified at molecular masses of ∼70–75 kD (1: PCNA cross-linked to hABH2$_{1–7}$-YFP-Flag), 100–130 kD (2: PCNA dimer or trimer cross-linked to hABH2$_{1–7}$-YFP-Flag), and 160–190 kD (3: PCNA trimer cross-linked to two or three hABH2$_{1–7}$-YFP-Flag). Bands 1 and 2 are much stronger in the IP from cells expressing hABH2$_{1–7}$ wild type (WT) than from cells expressing the hABH2$_{1–7}$F4A mutant, and band 3 is not detected in the IP from cells expressing the hABH2$_{1–7}$F4A mutant. Notably, after reversal of the cross-links (lanes 4 and 12), only PCNA and Flag bands of 35 kD were identified, suggesting that the larger bands detected in lanes 3 and 11 were cross-linked with hABH2$_{1–7}$-YFP-Flag and PCNA. Together with the FRET, these data strongly support a direct interaction between hABH2$_{1–7}$ and PCNA.

Our data from co-IP experiments (Fig. 1, B–D) indicated that more complexes of hABH2 and PCNA were pulled down from chromatin-enriched fractions, suggesting potential involvement of PTMs. Therefore, we analyzed the isoform distribution of PCNA cross-linked to hABH2$_{1–7}$-YFP-Flag by 2D Western blot (WB) analysis and compared it with the total repertoire of PCNA isoforms present in the same cell extract (Fig. 2 F). We included purified RAD51 as an internal standard because its isoelectric point (pI; 5.4) is close to the pI of unmodified PCNA (4.6). Our results indicate that the PCNA variants cross-linked to hABH2$_{1–7}$-YFP-Flag (top membrane) are shifted toward a more acidic pI without significantly changing the vertical migration. Multiple isoforms of PCNA with pI between 4 and 5 have previously been identified, although the exact nature of most of these modifications is not known (Naryzhny, 2008). Most PCNA present in a cell (lower membrane), and the low levels (Fig. 2 E, lane 7) of PCNA cross-linked to hABH2$_{1–7}$F4A-YFP-Flag (mutant; mid membrane), have a higher pI than the PCNA pulled down by

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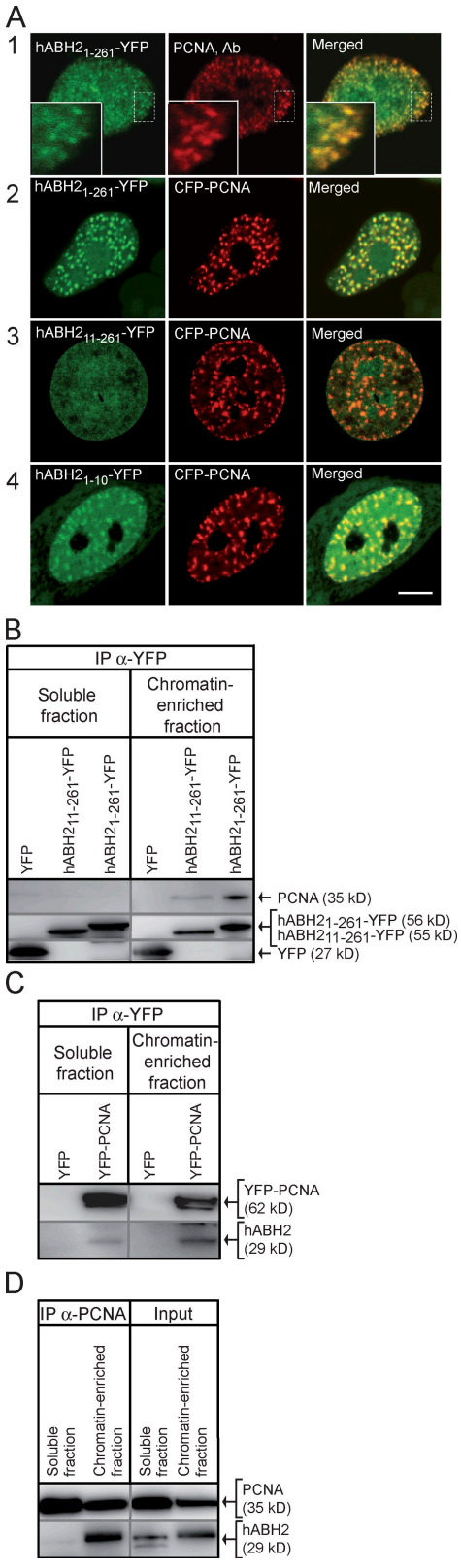

Figure 1. **The 10 N-terminal amino acids of hABH2 are important for interaction with PCNA.** (A) Confocal fluorescence images of full-length hABH2-YFP with endogenous PCNA (row 1) and hABH2 constructs co-expressed with CFP-PCNA in live cycling HeLa cells. Insets show a higher magnification view of boxed regions. Bar, 5 µm. (B) Co-IP of PCNA from HeLa cells stably expressing hABH2-YFP constructs using α-YFP beads. (C) Co-IP of hABH2 from cells stably expressing YFP-PCNA using α-YFP beads. (D) Co-IP of hABH2 from cells only expressing endogenous proteins using α-PCNA beads. Input is 3.3% of cell extract used for IP. Black lines, separate membranes; gray lines, same membranes.

hABH2$_{1–7}$-YFP-Flag. PTMs on PCNA may explain why our attempts to analyze the PCNA–hABH2 interactions using purified recombinant full-length proteins in in vitro experiments gave inconclusive data.

Collectively, these results reveal a novel PCNA-binding site within the conserved seven N-terminal amino acids of hABH2. Based on the alignment of the different ABH2s, the dot blot assay, and the in vivo imaging results, APIM was defined as [KR]-[FYW]-[LIVA]-[LIVA]-[KR].

Overexpression of APIM decapeptide fused to YFP reduces repair of 1meA and sensitizes cells to DNA alkylation damage

hABH2 is known to repair 1meA and 3meC generated by the S_N2-alkylating agent methyl methanesulfonate (MMS) (Aas et al., 2003; Ringvoll et al., 2006). To examine whether expression of APIM interfered with the function of hABH2 by perturbation of the PCNA binding, we exposed cells expressing hABH2$_{1–10}$-YFP or only YFP to MMS and analyzed removal of 1meA in DNA by liquid chromatography (LC)/mass spectrometry (MS)/MS. Cells were arrested at the G1/S border and treated with MMS for 1 h. For arrested cells, a 13% significant increase of 1meA was seen in APIM-YFP–expressing compared with YFP-expressing cells (Fig. 3 A). This is likely the result of reduced removal of 1meA by endogenous hABH2 during incubation with MMS. These results indicate that the hABH2–PCNA interaction is required for efficient removal of 1meA in cells arrested at the G1/ S transition.

Next, we exposed cell lines expressing hABH2$_{1–10}$-YFP, hABH2$_{1–7}$F4A-YFP, and YFP to MMS and measured their colony-forming capacity. We found a fivefold decrease in colony-forming units in cells overexpressing functional hABH2$_{1–10}$-YFP compared with the cells expressing mutated APIM and only YFP (Fig. 3 B). These results strongly suggest that binding of APIM to PCNA increases MMS cytotoxicity. We subsequently exposed hABH2$_{1–10}$-YFP– and YFP-expressing cells to MMS as well as three other alkylating agents, BCNU (carmustine), temozolomide (TMZ), and mitomycin C (MMC), and measured cell growth for 4 d (MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]). Unlike MMS, the other alkylating agents are believed to introduce damage not repaired by hABH2 but by several different repair pathways, including direct methyl transfer by O^6-methylguanine-DNA methyltransferase, nucleotide excision repair, BER, mismatch repair, and homologous recombination (Sedgwick et al., 2007). Over expression of hABH2$_{1–10}$-YFP had little effect on the growth rate in untreated cells, whereas it strongly sensitized cells to all the alkylating agents (Fig. 3 C). These results suggested that the hypersensitivity to genotoxic agents was caused by inhibiting the function not only of hABH2 but also of other proteins involved in genome maintenance.

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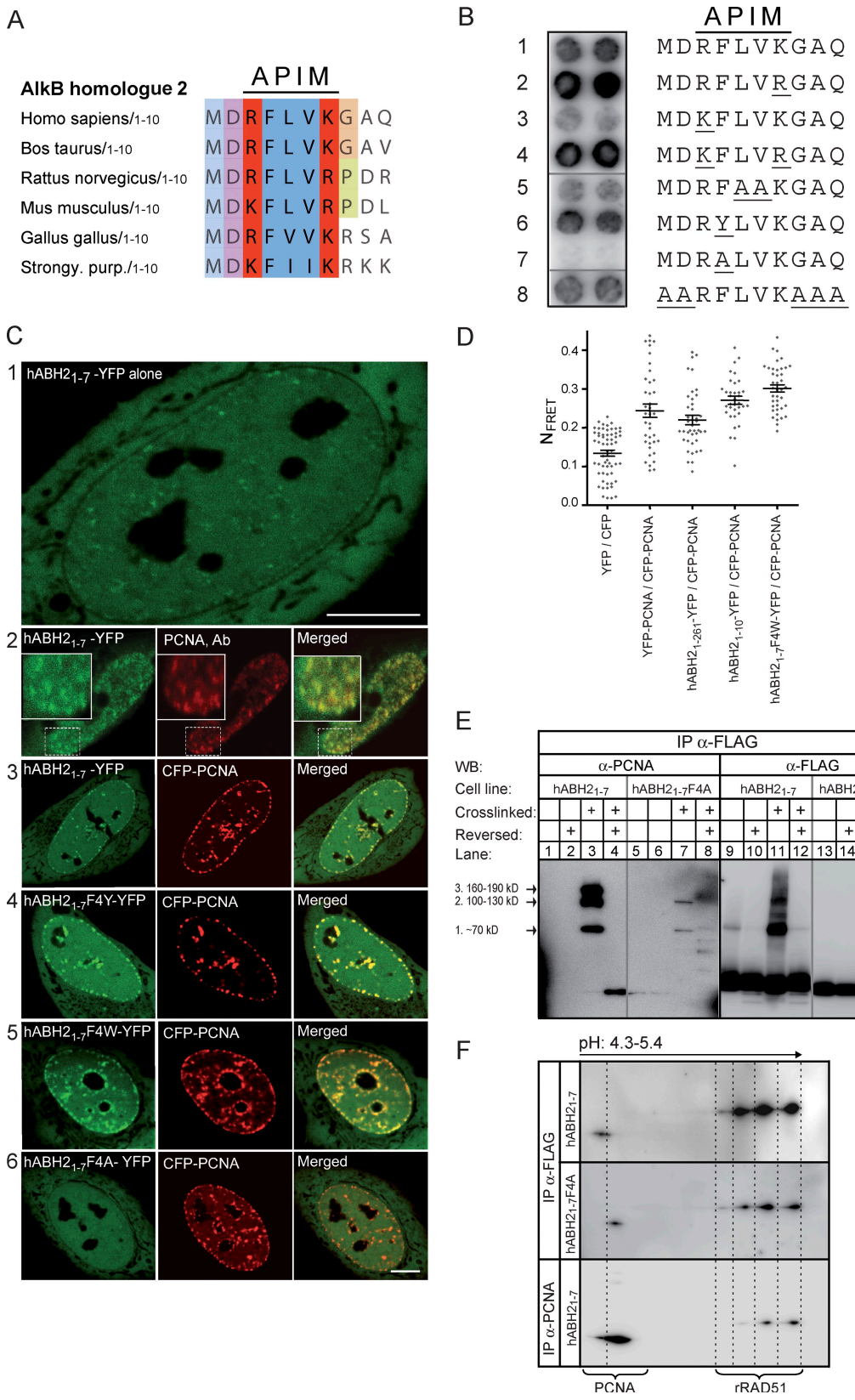

Figure 2. **Close interaction between the N terminus of hABH2 and a modified form of PCNA.** (A) Sequence alignment of the 10 N-terminal amino acids of ABH2 homologues from different species (colors as given by Clustal X). (B) PCNA binding to hABH2$_{1-10}$ peptide variants (dot blot). Row 1 shows the hABH2$_{1-10}$ peptide, and rows 2–8 show peptides where different amino acids are substituted (underlined in the right panel; data from one membrane). (C) Confocal images of HeLa cells. Row 1 shows hABH2$_{1-7}$-YFP expressed alone (live cells), row 2 shows hABH2$_{1-7}$-YFP with endogenous PCNA, and rows 3–6 show various hABH2$_{1-7}$-YFP F4 variants coexpressed with CFP-PCNA (live cells). Insets show a magnified view of the boxed areas. Bars, 5 µm. (D) N$_{FRET}$ measurements. YFP/CFP (vectors only) and YFP-PCNA/CFP-PCNA were used as background and positive controls, respectively. Data shown

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APIM is found in many proteins involved in genome maintenance and cell cycle control

Using the APIM motif as the query, we obtained 636 hits in the Swiss-Prot/TrEMBL database. After discarding nonnuclear proteins and proteins in which APIM is not conserved, this was reduced to 226 hits (Table I; see http://tare.medisin.ntnu.no/pcna/index.php for complete query results and experimental procedures). Nine of these proteins also contained the PIP box consensus (Table I).

Among the proteins found in the query, we examined the APIMs more closely in four human proteins in addition to hABH2. We named the first protein examined TFIIS-like (TFIIS-L) because it contains the conserved N-terminal domain I found in elongation factor TFIIS (Cramer, 2004). The function of this protein is unknown. However, like hABH2, TFIIS-L contains an APIM within its seven N-terminal amino acids. We next examined the multifunctional transcription factor TFII-I, which contains four APIMs. TFII-I is a transcription factor critical for cell cycle control and proliferation and has also recently been suggested to have a role in DNA repair (Desgranges and Roy, 2006). Finally, we examined APIM in DNA topoisomerase (Topo) II α, which is involved in postreplicative DNA decatenation and DNA segregation (Agostinho et al., 2004), and the RAD51 paralogue RAD51B, which is involved in homologous recombination, centrosome function, and chromosome segregation (Date et al., 2006). The APIM sequences in all these proteins are conserved throughout evolution (Fig. 3 D). Among these proteins, only Topo II α has been reported to localize to nuclear S phase foci (Lou et al., 2005) and to contain a putative PIP box (QttLaFkp; amino acids 1,277–1,284; Niimi et al., 2001). We cloned the proteins as YFP fusions and found that all were nuclear proteins accumulating in various numbers of visible foci (Fig. 3 D), many of which represent replication foci (see following paragraph). Endogenous TFII-I was also present in foci colocalizing with endogenous PCNA (unpublished data).

APIM is a functional PCNA-interacting motif

Substitution of Phe4 to Ala in APIM impaired binding between hABH2-derived peptides and PCNA (Fig. 2); thus, we wanted to examine whether the corresponding mutation had a similar effect on the full-length hABH2, TFIIS-L, TFII-I (in one and four APIMs), Topo II α, and RAD51B. Mutation of APIM in all these proteins, except Topo II α, strongly reduced colocalization with PCNA when coexpressed with WT proteins (Fig. 4 A, rows 2–7), suggesting that impaired APIM reduced the PCNA interaction. However, coexpression of WT hABH2-CFP and WT hABH2-YFP resulted in foci containing both fusion proteins (Fig. 4 A, row 1). Mutations of APIM in TFIIS-L, or in either one of the four APIMs of TFII-I, did not cause visible reduction in colocalization with PCNA when cotransfected with PCNA alone (unpublished data), but a reduction in FRET could be detected in these cases (Fig. 4 B, green). Thus, higher FRET between PCNA and WT proteins, and the fact that WT proteins outcompete the mutant proteins for binding to PCNA when coexpressed (Fig. 4 A, rows 3 and 4), suggested that the affinity of the mutant proteins for PCNA was reduced. Only a minor reduction in colocalization with PCNA was observed for the mutant Topo II α when coexpressed with WT Topo II α. However, a reduction in FRET was also detected in this case (Fig. 4 B). Because Topo II α is a homodimer (Nettikadan et al., 1998), binding to PCNA could be mediated through its nonmutated endogenous or coexpressed WT partner. Altogether, these results strongly suggest that APIM is a functional PCNA-binding motif in all these proteins.

The RAD51B S phase spots were on average less bright than the spots for the other APIM-containing proteins, and clear colocalization with PCNA was seen in only ∼20% of the S phase cells in comparison with 95–100% for hABH2, TFIIS-L, TFII-I, and Topo II α. This indicates that the PCNA–RAD51B interaction is less prominent and might require specific cell conditions.

In summary, the work presented in this study strongly indicates that APIM is a functional, widespread PCNA-interacting motif found in many proteins involved in genome maintenance. Among other interesting APIM-containing proteins are the poly(ADP-ribose) family (PARP-1, -2, and -4), the FANCC protein, the REV3L subunit of translesion polymerase ζ, several E3 ubiquitin protein ligases, subunits of the general transcription factors II and III, members of the MAPK pathway, many serine/threonine protein kinases, and three subunits of RNA polymerase II and III (Table I). Interestingly, recent data indicate a partial overlap between regions of replication and transcription (Malyavantham et al., 2008); thus, APIM could possibly be involved in linking transcription and cell cycle regulation to PCNA/replicative processes after genotoxic stress.

The scaffold protein PCNA interacts with numerous proteins in a well-orchestrated fashion, thus constituting a foundation for many vital cellular processes. Interactions with PCNA are likely to be regulated at several levels; e.g., by PTMs as well as through several PCNA-interacting motifs (Moldovan et al., 2007). Interestingly, PCNA-binding peptides containing the PIP box fused to GFP are reported to block colony formation when expressed in untreated freely cycling HeLa and U2OS cells (Warbrick, 2006). Cells expressing APIM-YFP had normal capacity for colony formation in untreated cells, but these cells showed increased sensitivity to alkylating agents. We suggest that impaired PCNA binding of several APIM-containing proteins, in addition to hABH2, contributes to the

are the result of three individual experiments (mean ± SEM; n = 35–50). (E) Cross-linked and reverse cross-linked IPs (α-Flag) from cells stably expressing hABH2$_{1–7}$YFP-Flag and hABH2$_{1–7}$F4A-YFP-Flag. The eluted fractions were analyzed for the presence of PCNA and Flag fusion proteins by WB. (F) 2D gel electrophoresis followed by WB analysis of PCNA immunoprecipitated from cross-linked hABH2$_{1–7}$-YFP-Flag (top membrane; α-Flag) and hABH2$_{1–7}$F4A-YFP-Flag (mid membrane; α-Flag). Total PCNA was immunoprecipitated with α-PCNA beads (lower membrane). Purified recombinant RAD51 (rRAD51) was added as an internal standard. Dotted lines illustrate the vertical alignment of the membranes. (B and E) Gray lines indicate that intervening lanes have been spliced out.

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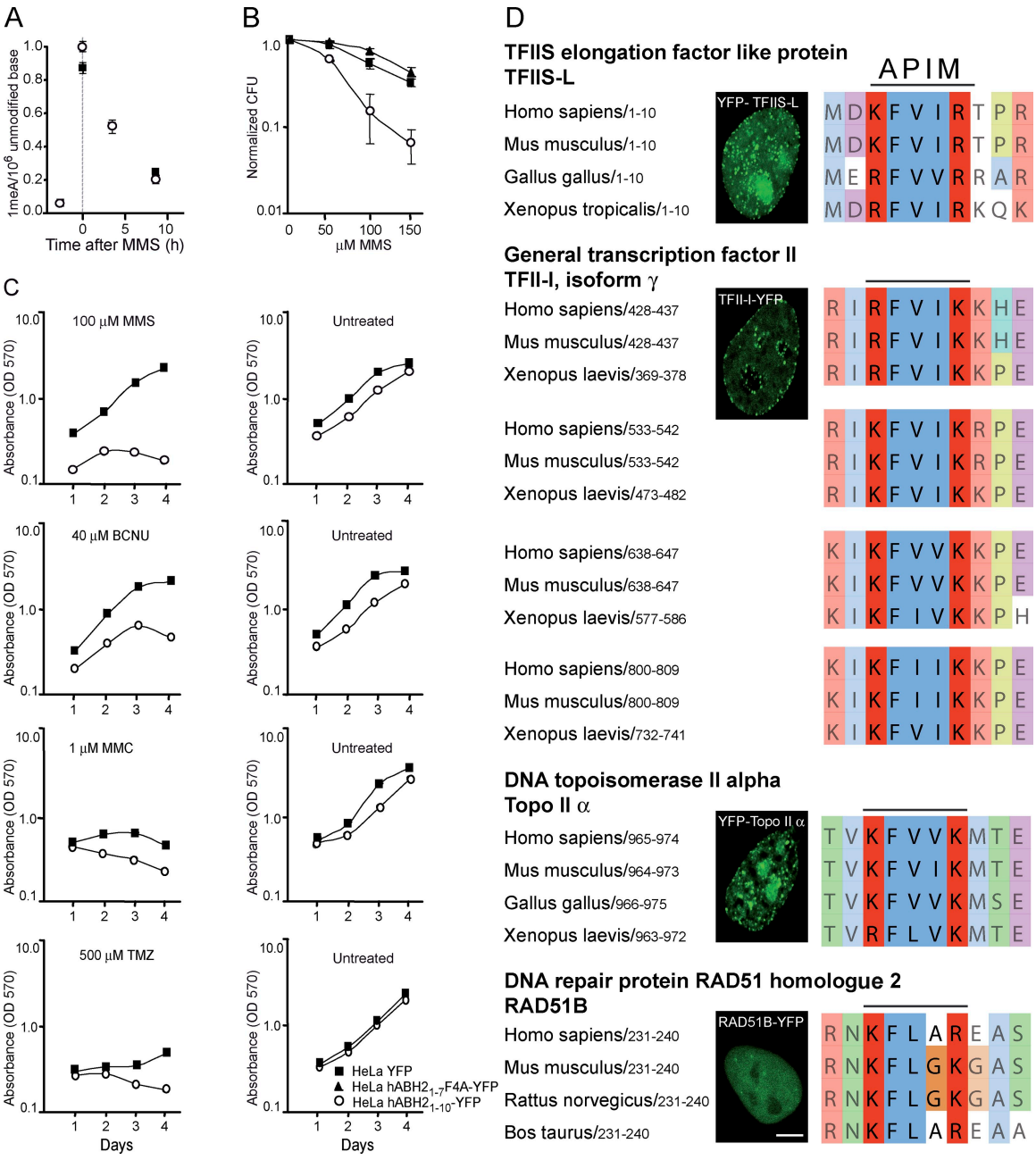

Figure 3. **Expression of APIM decapeptide sensitizes cells to alkylating agents, and several foci-forming proteins contain conserved APIM.** (A) 1meA in DNA isolated from YFP (closed squares)- and hABH2$_{1–10}$-YFP (open circles)–expressing cells after exposure to 1.2 mM MMS for 1 h before release from the G1/S border (mean ± SEM; n = 4–5). (B) Clonogenic assay comparing the MMS sensitivity between cells expressing hABH2$_{1–10}$-YFP (open circles), hABH2$_{1–7}$F4A-YFP (closed triangles), or only YFP (closed squares; mean ± SD; n = 2–4). CFU, colony-forming unit. (C) Cell growth of HeLa cells stably expressing YFP (closed squares) and hABH2$_{1–10}$-YFP (open circles) measured by MTT assay after continuous exposure to MMS, BCNU, MMC, and TMZ. The growth rates of unexposed cells are shown in the right lane. (D) Conservation of APIM in TFIIS-L, TFII-I, Topo II α, and RAD51B. These proteins are shown as YFP fusion proteins. Bar, 5 μm.

hypersensitivity to cytostatic drugs seen in APIM-expressing cells and that coordinated binding of APIM-containing proteins to PCNA might be an important response mechanism subsequent to DNA damage.

Materials and methods

Expression constructs

Cloning of the fluorescently tagged expression constructs CFP-PCNA, *Hereactis crispa* RFP (HcRed)–PCNA, and hABH2$_{1–261}$-YFP/-CFP has been described previously (Aas et al., 2003; Otterlei et al., 2006). Using phABH2$_{1–261}$-YFP as a template, phABH2$_{1–10}$-YFP and phABH2$_{11–261}$-YFP were generated by PCR and cloned into pYFP-N1 (Clontech Laboratories, Inc.) using NdeI–AgeI and AgeI–EcoRI, respectively. The PCR product from EST (image clone 5176979 [BC035374] Resource Center/Primary Database) was cloned into pYFP- and pCFP-C1 (HindIII–Acc651) to make pYFP- and pCFP–TFIIS-L. pTFII-I–YFP and –CFP were generated by PCR amplification of TFII-I from pI3CX–TFII-I (provided by R.G. Roeder, The Rockefeller University, New York, NY) and cloning into pYFP- and pCFP-N1 (SacI–ApaI). pYFP– and pCFP–Topo II α were made by switching the EGFP tag (EcoRI blunt–NheI) with YFP and CFP tag (XhoI blunt–NheI) from pEGFP–Topo II α (pT104-1; provided by

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Table I. **Selected proteins containing APIM**

Type/group of proteins	Proteins	Source
Proteins containing PIP box and APIM	**DNA ligase I**, MDN1 Midasin, ubiquitin thioesterase FAF-X, protein 18 homologue (hVPS18), cytokine signaling 6 (SOCS-6), Topo II β, IκB-related protein, UHRF2, PARP4	Moldovan et al., 2007
DNA polymerase	**Pol ζ catalytic subunit** (hREV3L)	Moldovan et al., 2007
DNA ligases	**DNA ligase I**, DNA ligase IV	Moldovan et al., 2007
Topo	**Topo II** α and β	This study; Niimi et al., 2001; Lou et al., 2005
DNA repair proteins	**hABH2**, **PARP-1**, -2, and -4, **RAD51B**, **FANCC**, XPA	This study; Simbulan-Rosenthal et al., 1999; Jacquemont and Taniguchi, 2007
DNA repair–associated/ interacting proteins	XPA-binding protein 2, BRCA1/BRCA2-containing complex subunit 45 (prot-BRE), x-ray radiation resistance-associated protein 1	NA
Sister chromatid cohesion	***N*-acetyltransferase ESCO1/EFO1**, **hSMC5**	Potts et al., 2006; Moldovan et al., 2007
Chromatin remodelling and DNA-binding proteins	Chromodomain helicase DNA-binding proteins 3–5, p325 subunit of remodeling and spacing factor chromatin–remodelling complex, **telomeric repeat–binding protein 2 (TRF2)**	Opresko et al., 2004
E3 ubiquitin ligases	**UHFR1**, UHFR2, UBR1, UBR2, ring finger proteins 3, 17, and 151, probable E3 ubiquitin protein ligase MYCBP2	Bronner et al., 2007
Transcription factors	**TFIIS-L**, **TFII-I**, TFIIE-α, sterol regulatory element-binding transcription factor 2 (SREBF2), TFIIIC subunit α, TFIID 100 kD subunit (TAF5), TFIIIC 102 kD subunit (TF3C γ), transcription factor–like protein MRG15 and X (mortality factor 4–like proteins 1 and 2), E2 transcription factor 7	This study
Cell cycle regulators	Cell division cycle-associated 2, Bcl2-interacting mediator of cell death, testis spermatocyte apoptosis-related gene 2 protein	NA
Protein kinases	Serine/threonine (S/T) protein kinases SRPK1 and -2, 33 and MST4, leucine-rich repeat S/T protein kinase 1, STK23 (S/T protein kinase 23), S/T protein kinase PLK3, microtubuli-associated S/T protein kinase, microtubuli-associated S/T protein kinase 1, MAPKAP kinase 2 (MK2) and 5 (MK5), mitogen-activated protein kinase 15 (MSK-15)	NA
Methyltransferase	H3 lysine 4–specific MLL3, H3-K9 methyltransferase 5, putative rRNA methyltransferase 3	NA
Cancer-associated antigens	Melanoma-associated antigen E1 (MAGE E1), MAGE B18, MAGE-G1, natural killer tumor recognition protein (NK-TR), Myc-binding protein–associated protein, Myb-binding protein 1A, hepatoma-derived growth factor–related protein 2 isoform1, serologically defined colon cancer antigen 1	NA
RNA polymerase and ribosome-associated proteins	RNA polymerase II, largest subunit (RPB1), RNA polymerase III subunit 5 (RPC5), RNA polymerase II 140 kD (RPB2), UTP14A U3 small nucleolar RNA-associated protein 14 homologue A, 60S ribosomal protein L18, 60S ribosomal protein L35, TAF5-like RNA polymerase II p300 (PAF65-beta), mediator of RNA polymerase II transcription subunit 12 homologue	NA

NA, not applicable. Bold indicates proteins localized in replication foci under normal conditions or after DNA damage. The full lists of hits for the APIM and PIP motifs are available at http://tare.medisin.ntnu.no/pcna/index.php.

W.T. Beck, University of Illinois, Chicago, IL). RAD51B was amplified by PCR from pET15b-RAD51B (provided by S. Yokoyama, RIKEN Genomic Sciences Center, Kanagawa, Japan) and cloned into pYFP- and pCFP-N1 using XhoI and SacII. The hABH2$_{1-7}$-YFP constructs, including the F4 mutants, were made by annealing oligos with XhoI–EcoRI overhang followed by cloning into pYFP-N1 mutated in the ATG codon. The Flag constructs were generated by PCR amplification of the 3× Flag tag from p3×Flag–CMW-14 (Sigma-Aldrich) followed by cloning into pCFP-N1 in the BsrGI–XbaI site. All point mutations were made by site-directed mutagenesis (QuickChange II; Agilent Technologies) according to the manufacturer's instructions. Restriction enzymes and calf intestinal alkaline phosphatase were obtained from New England Biolabs, Inc., and the oligonucleotides were obtained from MedProbe Eurogentech. All constructs were verified by sequencing.

Confocal imaging

HeLa cells were examined 16–48 h after transient transfection (by Fugene 6 [Roche] or Lipofectamine 2000 [Invitrogen] according to the manufacturer's recommendations) of CFP, YFP, and HcRed fusion constructs. Fluorescent images were aquired using a laser-scanning microscope (LSM 510 Meta; Carl Zeiss, Inc.) equipped with a Plan Apochromat 63× 1.4 NA oil immersion objective. The images were acquired in the growth medium of the cell with the stage heated to 37°C using LSM 510 software (Carl Zeiss, Inc.). For the two-color images, CFP was excited at λ = 458 nm and detected at λ = 470–500 nm, and YFP was excited at λ = 514 nm and detected at λ = 530–600 nm using consecutive scans. When three-color images were acquired, YFP was excited at λ = 488 nm and detected at λ = 530–600 nm, HcRed was excited at λ = 543 nm and detected at λ > 560 nm, and the CFP settings were kept as for the

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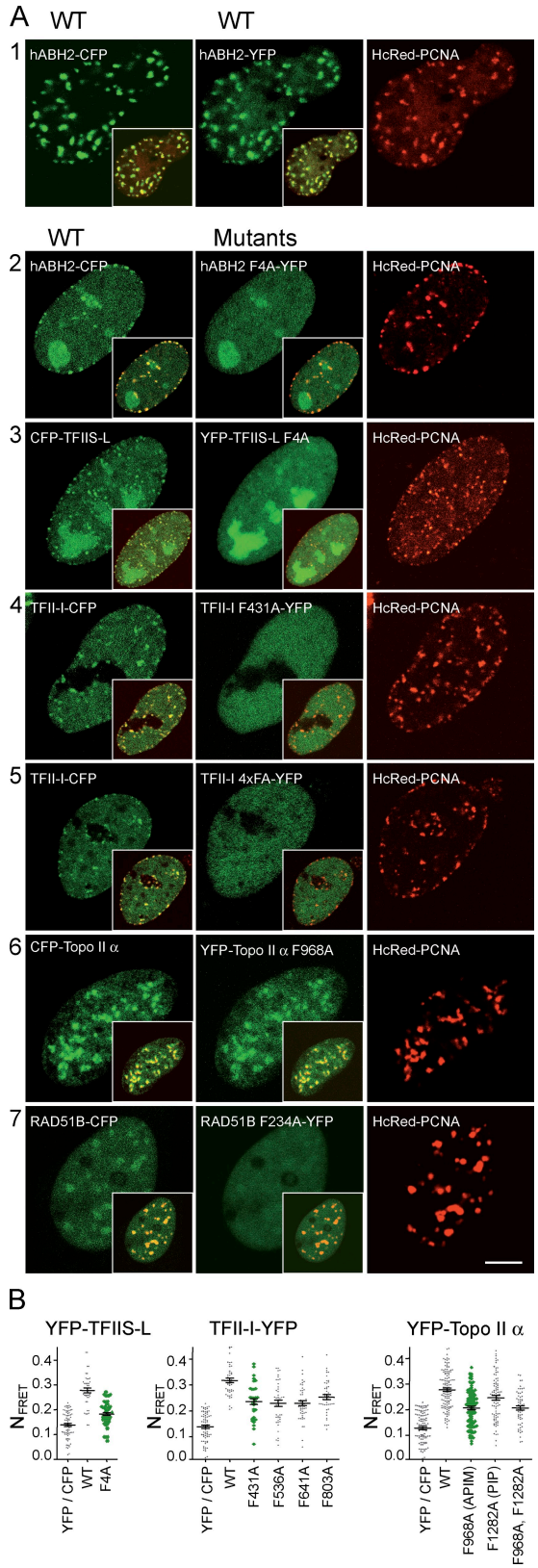

Figure 4. **Point mutations in APIM result in disrupted colocalization and reduced FRET.** (A) Row 1 shows confocal images of cotransfected WT hABH2-CFP, WT hABH2-YFP, and HcRed-PCNA. Rows 2–7 show confocal images of the WT proteins with CFP tag (left; green) cotransfected with YFP-tagged proteins mutated in APIM (middle; green), and HcRed-PCNA (right; red) in live cycling HeLa cells. Insets show merged images with PCNA. Bar, 5 µm. (B) N_{FRET} calculated for constructs in which single APIM mutation does not disrupt colocalization. WT and mutant proteins (YFP fusions of TFIIS-L, TFII-I, and Topo II α) are coexpressed with CFP-PCNA. YFP/CFP (vectors only) were used as background. Data are the results of two (TFIIS-L and TFII-I) to four (Topo II α) independent experiments. Error bars indicate mean ± SEM (n = 36–119).

two-color images. The thickness of the slice was 1 µm. No image processing, except contrast and intensity adjustments, were performed.

Immunofluorescence

HeLa cells were fixed in freshly made 2% paraformaldehyde on ice for 10 min before cold (−20°C) methanol was added, and the cells were incubated at −20°C for 20 min. All dilutions and washes were performed in 2% FCS in PBS. The cells were washed (three times for 5 min) before addition of 1 µg/ml α-PCNA (PC10; Abcam) and incubation for 1 h at 37°C. Finally, the cells were incubated for 1 h at 37°C with the secondary antibody Alexa Fluor 546 goat anti–mouse (diluted 1:2,000; Invitrogen). After washing, the cells were analyzed in a laser-scanning microscope (LSM 510 Meta; described in the previous paragraph), with excitation at 546 nm and detection >560 nm for goat anti–mouse and 488 nm excitation and detection between 505 and 550 nm for YFP, using consecutive scans.

FRET measurements

FRET occurs if the tags (YFP and CFP) are <100 Å (10 nm) apart (Mátyus, 1992). We detected FRET using the sensitized emission method, measuring acceptor (YFP) emission upon donor (CFP) excitation. FRET was scored when the intensity of emitted light from YFP after excitation of the CFP fluorochrome was stronger than the light emitted by CFP- or YFP-tagged proteins alone after excitation with the YFP and CFP lasers, respectively (bleed through), given by the equation $FRET = I_2 - I_1 (I_{D2}/I_{D1}) - I_3 (I_{A2}/I_{A3})$, in which I indicates mean intensities. YFP and CFP (vectors only) were used to measure background FRET because of dimerization of the tags, and YFP-PCNA and CFP-PCNA (because PCNA is a homotrimer) were used as positive control. FRET > 0 was normalized for expression levels using the equation $N_{FRET} = FRET/(I_1 \times I_3)^{1/2}$ (Mátyus, 1992; Xia and Liu, 2001; Otterlei et al., 2006). N_{FRET} was calculated from mean intensities within a region of interest containing >25 pixels in which all pixels had intensities <250, and the mean intensities were between 100 and 200. Channels 1 (CFP) and 3 (YFP) were measured as described for imaging, and channel 2 (FRET) was excited with λ = 458 nm and detected at λ = 530–600 nm. I_{D1-D3} and I_{A1-A3} were determined for cells transfected with CFP and YFP constructs only with the same settings and fluorescence intensities as cotransfected cells (I_1 and I_3).

Culture of cell lines and preparation of cell extracts

HeLa (cervical cancer) cells stably expressing the constructs of interest were prepared by transfection (Fugene 6) followed by cell sorting or cloning by dilution, and prolonged culturing in 400 µg/ml selective (using genticine; G418; Invitrogen) high glucose, 4.5 g/liter DME (BioWhittaker) supplemented with 10% FCS, 250 µg/ml amphotericin B (Sigma-Aldrich), 100 µg/ml gentamycin (Invitrogen), and 1 mM glutamine (BioWhittaker). The cells were cultured at 37°C in a 5% carbon dioxide–humidified atmosphere. Fractionated cell extracts from HeLa were prepared by resuspending the cell pellets in 1× packed cell volume (PCV) in buffer I (10 mM Tris-HCl, pH 8.0, and 50 mM KCl) and 1× PCV in buffer II (10 mM Tris-HCl, 100 mM KCl, 20% glycerol, 0.5% Nonidet P-40, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 1× complete protease inhibitor [Roche], and phosphatase inhibitor cocktail [PIC I and II; Sigma-Aldrich]). Cells were incubated under constant shaking for 30 min at 4°C, centrifuged at 2,000 rpm, and the supernatant (soluble fraction) was harvested. The pellet (containing nuclei) was resuspended in 1× PCV of buffer III (10 mM Tris-HCl, pH 8.0, and 100 mM KCl), 1× PCV buffer II, and sonicated. The sonicated nuclear pellet was incubated with 2 µl DNase/RNase cocktail I (200 U/µl Omnicleave Endonuclease; Epicentre Technologies), 1 µl DNase (10 U/µl; Roche), 1 µl bensonase (250 U/µl; EMD), 1 µl micrococcal nuclease (100–300 U/mg; Sigma-Aldrich), and 10 µl RNase (2 mg/ml; Sigma-Aldrich) per 30 mg cell extract at 37°C for 1 h. This fraction, denoted chromatin-enriched fraction, was dialyzed against buffer II and III followed by clearance by centrifugation before IP.

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Formaldehyde cross-linking of proteins in intact cells
Cells were harvested and washed twice with cold PBS. 5–6 × 10^6 cells were resuspended in 10 ml PBS containing 0.25% formaldehyde and incubated at 37°C for 20 min. Cross-linking was stopped by adding glycine (final concentration 0.125 M). Cells were collected by centrifugation and washed once in PBS, resuspended in 8× PCV in buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5% NP-40, 1 mM DTT, and complete protease inhibitor) containing 5 µl Omnicleave, and sonicated. DNase/RNase cocktail I was added, and the homogenate was incubated at room temperature for 1 h and dialyzed at 4°C overnight in buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 1 mM DTT, and complete protease inhibitor).

co-IP
An in-house affinity-purified rabbit polyclonal antibody raised against GFP protein, which also recognizes YFP and CFP proteins (called α-YFP), and monoclonal α-PCNA antibody (PC10; Santa Cruz Biotechnology, Inc.) were covalently linked to protein A paramagnetic beads (Invitrogen) according to instructions provided by New England Biolabs, Inc. 1,500 µg total cell–protein of the fractions was incubated with an additional 2 µl Omnicleave during IP with 10 µl antibody-coupled beads under constant rotation at 4°C over night (IP). The beads were washed four times with 200 µl 10 mM Tris-HCl and 50 mM KCl, pH 8, with a 5-min incubation on ice in between. The beads were resuspended in NuPAGE (Invitrogen) loading buffer and 1 mM DTT, heated, and the IP elutions were separated on 4–12% Bis-Tris-HCl (NuPAGE) gels. 50 µg cell extract was used for input.

IP of cross-linked protein extracts
Cross-linked Flag fusion proteins were immunoprecipitated using anti-Flag M2 affinity gel (herein referred to as α-Flag; Sigma-Aldrich) according to the manufacturer's protocol. The resin was prepared by washing once with 0.1 M glycine and 0.5 M NaCl, pH 3.0, and three times with TBS buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 10 mM Na butyrate, and 20 mM NaF). 2.5 mg and 5 mg (hABH2$_{1–7}$-YFP and hABH2$_{1–7}$F4A-YFP, respectively) of cross-linked extracts were incubated with 20 µl or 40 µl resin, respectively (packed gel volume), for 2 h at 4°C under constant rotation. The resin was washed three times with 500 µl of TBS buffer, and the cross-linked Flag fusion proteins were eluted by incubating the resin with 100 µl 3× Flag peptide in TBS buffer (final concentration of 450 ng/µl) for 30 min at 4°C under constant rotation. The cross-linking was reversed by a 30-min incubation at 95°C. For further WB analyses, the IP elution fractions were heated in 1× LDS sample buffer (NuPAGE) and 0.1 M DTT (65°C for 10 min) before loading 4–12% Bis-Tris-HCl (NuPAGE) gels.

2D gel electrophoresis
Immunoprecipitates of cross-linked extracts of hABH2$_{1–7}$YFP–3× Flag (5 mg) and hABH2$_{1–7}$F4A-YFP-3× Flag (10 mg) pulled down with α-Flag resin (40 µl and 80 µl, respectively) and by magnetic α-PCNA–coupled beads (50 µl beads; 2 mg extract) was subjected to 2D Western analysis. The resin was washed three times with 500 µl of TBS buffer. The resin was washed once in 20 mM Tris HCl, pH 7.4, 50 mM NaCl, 10 mM Na butyrate, and 20 mM NaF, and the cross-linked Flag fusion proteins were eluted by incubating the resin with 100 µl 3×Flag peptide in this buffer (final concentration of 450 ng/µl) for 30 min at 4°C under constant rotation. First dimension: after IP and elution, the cross-links were reversed (see previous paragraph), the α-PCNA beads were washed three times with 10 mM Tris-HCl and 50 mM KCl (1 ml), and resuspended in 340 µl destreak with 1% IPG buffer, pH 4–7 (GE Healthcare). After incubation overnight in a shaker at 4°C, the elutions were collected in separate vials without α-PCNA beads. 20 ng recombinant RAD51 protein (molecular mass, 37 kD; pI, 5.4; provided by I. Hickson Weatherall, University of Oxford, Oxford, England, UK) was added to each sample to serve as an internal standard. The samples were used to rehydrate immobiline DryStrips (18 cm; pH 4–7; GE Healthcare) overnight. The isoelectric focusing was performed according to the manufacturer's instructions in the IPGphor II unit (GE Healthcare). After isoelectric focusing, strips were cut after pH 5.5, and the pieces from pH 4–5.5 were incubated in equilibrium buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) containing 1% DTT for 15 min followed by a 15-min incubation in the same buffer containing 2.5% iodoacetamide instead of DTT. Second dimension: the strips were loaded onto NuPAGE Novex 4–12% gels (Invitrogen).

WB
After gel electrophoresis, the polyvinylidene fluoride membranes (Immobilon; Millipore) were blocked in 5% low fat dry milk in PBST (PBS with 0.1% Tween 20). The primary antibodies, α-PCNA (PC10), α-hABH2 (monoclonal; Sigma-Aldrich), and α-Flag (monoclonal; Sigma-Aldrich), as well as the secondary antibodies, polyclonal rabbit anti–mouse IgG/HRP and polyclonal swine anti–rabbit IgG/HRP (Dako), were diluted in 1% dry milk in PBST. The membranes were treated with chemiluminescence reagent (SuperSignal West Femto Maximum; Thermo Fisher Scientific), and the proteins were visualized in Image Station (2000R; Kodak).

Dot blot analysis of predicted PCNA-binding peptides
An amino PEG500-UC540 sheet (acid hardened with improved stability) containing dots of 28 nmol peptide (stained with Ponceau to visualize the spots) was prepared at the peptide synthesis laboratory at The Biotechnology Center (University of Oslo, Oslo, Norway). The membrane was probed with 1 µg/ml PCNA for 2 h followed by probing with α-PCNA (PC10) and developed as described for WB. Data extracted from one representative dot blot is shown.

Sequence analysis
Details are provided at http://tare.medisin.ntnu.no/pcna/index.php.

MTT assay
HeLa cells stably expressing hABH2$_{1–10}$-YFP and YFP were seeded into 96-well plates (4,000 cells/well) and incubated for 3 h. Various doses of MMS (Acros Organics), BCNU (1,3-Bis(2-chloroethyl)-1-nitrosurea; Sigma-Aldrich), TMZ (4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo [4.3.0] nona-2,7,9-triene-9-carboxamide; Sigma-Aldrich), and MMC (6-amino-1,1a,2,8,8a,8b-hexahydro-8-(hydroxymethyl)-8a-methoxy-5-methyl-azirino[2′,3′:3,4] pyrrolo[1,2-a]indole-4,7-dione carbamate; Sigma-Aldrich) were added to the wells. The cells were exposed continuously until harvest. MTT was added to the cells, the OD was measured at 570 nm, the mean from at least six wells was used to calculate cell survival, and the SD was smaller than the size of the dots. Data presented show growth from one representative experiment and has been reproduced at least two times.

Clonogenic assay
750 cells were seeded out in 10-cm cell culture dishes in 10 ml growth media and grown for 10 d. The cells were fixed in 6% glutaraldehyde in PBS for 15 min at room temperature, washed once in PBS, and stained with crystal violet, and colony-forming units were counted. Only colonies consisting of at least 50 cells were included. Data presented are mean ± SD from two (hABH2$_{1–7}$F4A-YFP) and four (hABH2$_{1–10}$-YFP and YFP) independent experiments.

Quantitation of 1meA in DNA
HeLa cells stably expressing hABH2$_{1–10}$-YFP and YFP were synchronized by the double thymidine block and analyzed by flow cytometry to verify the cell cycle phase. The DNA analysis of the cells was performed after methanol fixation (70%), RNase treatment (100 µg/ml in PBS at 37°C for 30 min), and propidium iodide staining (50 µg/ml in PBS at 37°C for 30 min) on an FACS flow cytometer (Canto; BD).

During G1/S arrest, the cells were treated with 1,200 µM MMS for 1 h, released, and harvested at defined time points. The cell pellets were washed with ice-cold PBS, spun down, snap frozen in liquid N_2, and stored at −80°C before use. DNA was isolated using DNeasy Blood and Tissue kit (QIAGEN). DNA samples were degraded enzymatically to deoxynucleosides and analyzed by LC/MS/MS using an HPLC system (Prominence; Shimadzu) interfaced with a triple-quadrupole mass spectrometer (API5000; Applied Biosystems), essentially as described previously (Ringvoll et al., 2006). 1meA and unmodified deoxynucleosides were monitored in multiple-reaction monitoring mode using the mass transitions 266.2→150.1 (1meA), 252.2→136.1 (deoxyadenosine), 243.2→127.1 (thymidine), 268.2→152.1 (deoxyguanosine), and 228.2→112.1 (deoxycytidine). Quantization was accomplished by comparison with pure deoxynucleoside standards.

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158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.

166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.
168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

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178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS

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

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201. Knut Jørgen Arntzen: PREGNANCY AND CYTOKINES
202. Henrik Døllner: INFLAMMATORY MEDIATORS IN PERINATAL INFECTIONS
203. Asta Bye: LOW FAT, LOW LACTOSE DIET USED AS PROPHYLACTIC TREATMENT OF ACUTE INTESTINAL REACTIONS DURING PELVIC RADIOTHERAPY. A PROSPECTIVE RANDOMISED STUDY.
204. Sylvester Moyo: STUDIES ON STREPTOCOCCUS AGALACTIAE (GROUP B STREPTOCOCCUS) SURFACE-ANCHORED MARKERS WITH EMPHASIS ON STRAINS AND HUMAN SERA FROM ZIMBABWE.
205. Knut Hagen: HEAD-HUNT: THE EPIDEMIOLOGY OF HEADACHE IN NORD-TRØNDELAG
206. Li Lixin: ON THE REGULATION AND ROLE OF UNCOUPLING PROTEIN-2 IN INSULIN PRODUCING ß-CELLS
207. Anne Hildur Henriksen: SYMPTOMS OF ALLERGY AND ASTHMA VERSUS MARKERS OF LOWER AIRWAY INFLAMMATION AMONG ADOLESCENTS
208. Egil Andreas Fors: NON-MALIGNANT PAIN IN RELATION TO PSYCHOLOGICAL AND ENVIRONTENTAL FACTORS. EXPERIENTAL AND CLINICAL STUDES OF PAIN WITH FOCUS ON FIBROMYALGIA
209. Pål Klepstad: MORPHINE FOR CANCER PAIN
210. Ingunn Bakke: MECHANISMS AND CONSEQUENCES OF PEROXISOME PROLIFERATOR-INDUCED HYPERFUNCTION OF THE RAT GASTRIN PRODUCING CELL
211. Ingrid Susann Gribbestad: MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF BREAST CANCER
212. Rønnaug Astri Ødegård: PREECLAMPSIA – MATERNAL RISK FACTORS AND FETAL GROWTH
213. Johan Haux: STUDIES ON CYTOTOXICITY INDUCED BY HUMAN NATURAL KILLER CELLS AND DIGITOXIN
214. Turid Suzanne Berg-Nielsen: PARENTING PRACTICES AND MENTALLY DISORDERED ADOLESCENTS
215. Astrid Rydning: BLOOD FLOW AS A PROTECTIVE FACTOR FOR THE STOMACH MUCOSA. AN EXPERIMENTAL STUDY ON THE ROLE OF MAST CELLS AND SENSORY AFFERENT NEURONS

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216. Jan Pål Loennechen: HEART FAILURE AFTER MYOCARDIAL INFARCTION. Regional Differences, Myocyte Function, Gene Expression, and Response to Cariporide, Losartan, and Exercise Training.
217. Elisabeth Qvigstad: EFFECTS OF FATTY ACIDS AND OVER-STIMULATION ON INSULIN SECRETION IN MAN
218. Arne Åsberg: EPIDEMIOLOGICAL STUDIES IN HEREDITARY HEMOCHROMATOSIS: PREVALENCE, MORBIDITY AND BENEFIT OF SCREENING.
219. Johan Fredrik Skomsvoll: REPRODUCTIVE OUTCOME IN WOMEN WITH RHEUMATIC DISEASE. A population registry based study of the effects of inflammatory rheumatic disease and connective tissue disease on reproductive outcome in Norwegian women in 1967-1995.
220. Siv Mørkved: URINARY INCONTINENCE DURING PREGNANCY AND AFTER DELIVERY: EFFECT OF PELVIC FLOOR MUSCLE TRAINING IN PREVENTION AND TREATMENT
221. Marit S. Jordhøy: THE IMPACT OF COMPREHENSIVE PALLIATIVE CARE
222. Tom Christian Martinsen: HYPERGASTRINEMIA AND HYPOACIDITY IN RODENTS – CAUSES AND CONSEQUENCES

223. Solveig Tingulstad: CENTRALIZATION OF PRIMARY SURGERY FOR OVARAIN CANCER. FEASIBILITY AND IMPACT ON SURVIVAL
224. Haytham Eloqayli: METABOLIC CHANGES IN THE BRAIN CAUSED BY EPILEPTIC SEIZURES
225. Torunn Bruland: STUDIES OF EARLY RETROVIRUS-HOST INTERACTIONS – VIRAL DETERMINANTS FOR PATHOGENESIS AND THE INFLUENCE OF SEX ON THE SUSCEPTIBILITY TO FRIEND MURINE LEUKAEMIA VIRUS INFECTION
226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
227. Vibeke Nossum: THE EFFECT OF VASCULAR BUBBLES ON ENDOTHELIAL FUNCTION
228. Sigurd Fasting: ROUTINE BASED RECORDING OF ADVERSE EVENTS DURING ANAESTHESIA – APPLICATION IN QUALITY IMPROVEMENT AND SAFETY
229. Solfrid Romundstad: EPIDEMIOLOGICAL STUDIES OF MICROALBUMINURIA. THE NORD-TRØNDELAG HEALTH STUDY 1995-97 (HUNT 2)
230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

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235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
240. Arne Kristian Myhre: NORMAL VARIATION IN ANOGENITAL ANATOMY AND MICROBIOLOGY IN NON-ABUSED PRESCHOOL CHILDREN
241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
242. Beate Sitter: TISSUE CHARACTERIZATION BY HIGH RESOLUTION MAGIC ANGLE SPINNING MR SPECTROSCOPY
243. Per Arne Aas: MACROMOLECULAR MAINTENANCE IN HUMAN CELLS – REPAIR OF URACIL IN DNA AND METHYLATIONS IN DNA AND RNA
244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

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248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS

251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

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

280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA
286. Per Magnus Haram: GENETIC VS. AQUIRED FITNESS: METABOLIC, VASCULAR AND CARDIOMYOCYTE ADAPTATIONS
287. Agneta Johansson: GENERAL RISK FACTORS FOR GAMBLING PROBLEMS AND THE PREVALENCE OF PATHOLOGICAL GAMBLING IN NORWAY
288. Svein Artur Jensen: THE PREVALENCE OF SYMPTOMATIC ARTERIAL DISEASE OF THE LOWER LIMB
289. Charlotte Björk Ingul: QUANITIFICATION OF REGIONAL MYOCARDIAL FUNCTION BY STRAIN RATE AND STRAIN FOR EVALUATION OF CORONARY ARTERY DISEASE. AUTOMATED VERSUS MANUAL ANALYSIS DURING ACUTE MYOCARDIAL INFARCTION AND DOBUTAMINE STRESS ECHOCARDIOGRAPHY
290. Jakob Nakling: RESULTS AND CONSEQUENCES OF ROUTINE ULTRASOUND SCREENING IN PREGNANCY – A GEOGRAPHIC BASED POPULATION STUDY
291. Anne Engum: DEPRESSION AND ANXIETY – THEIR RELATIONS TO THYROID DYSFUNCTION AND DIABETES IN A LARGE EPIDEMIOLOGICAL STUDY
292. Ottar Bjerkeset: ANXIETY AND DEPRESSION IN THE GENERAL POPULATION: RISK FACTORS, INTERVENTION AND OUTCOME – THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
293. Jon Olav Drogset: RESULTS AFTER SURGICAL TREATMENT OF ANTERIOR CRUCIATE LIGAMENT INJURIES – A CLINICAL STUDY
294. Lars Fosse: MECHANICAL BEHAVIOUR OF COMPACTED MORSELLISED BONE – AN EXPERIMENTAL IN VITRO STUDY
295. Gunilla Klensmeden Fosse: MENTAL HEALTH OF PSYCHIATRIC OUTPATIENTS BULLIED IN CHILDHOOD
296. Paul Jarle Mork: MUSCLE ACTIVITY IN WORK AND LEISURE AND ITS ASSOCIATION TO MUSCULOSKELETAL PAIN
297. Björn Stenström: LESSONS FROM RODENTS: I: MECHANISMS OF OBESITY SURGERY – ROLE OF STOMACH. II: CARCINOGENIC EFFECTS OF *HELICOBACTER PYLORI* AND SNUS IN THE STOMACH

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298. Haakon R. Skogseth: INVASIVE PROPERTIES OF CANCER – A TREATMENT TARGET ? IN VITRO STUDIES IN HUMAN PROSTATE CANCER CELL LINES
299. Janniche Hammer: GLUTAMATE METABOLISM AND CYCLING IN MESIAL TEMPORAL LOBE EPILEPSY
300. May Britt Drugli: YOUNG CHILDREN TREATED BECAUSE OF ODD/CD: CONDUCT PROBLEMS AND SOCIAL COMPETENCIES IN DAY-CARE AND SCHOOL SETTINGS
301. Arne Skjold: MAGNETIC RESONANCE KINETICS OF MANGANESE DIPYRIDOXYL DIPHOSPHATE (MnDPDP) IN HUMAN MYOCARDIUM. STUDIES IN HEALTHY VOLUNTEERS AND IN PATIENTS WITH RECENT MYOCARDIAL INFARCTION
302. Siri Malm: LEFT VENTRICULAR SYSTOLIC FUNCTION AND MYOCARDIAL PERFUSION ASSESSED BY CONTRAST ECHOCARDIOGRAPHY
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304. Lasse Løvstakken: SIGNAL PROCESSING IN DIAGNOSTIC ULTRASOUND: ALGORITHMS FOR REAL-TIME ESTIMATION AND VISUALIZATION OF BLOOD FLOW VELOCITY
305. Elisabeth Olstad: GLUTAMATE AND GABA: MAJOR PLAYERS IN NEURONAL METABOLISM

306. Lilian Leistad: THE ROLE OF CYTOKINES AND PHOSPHOLIPASE A_2s IN ARTICULAR CARTILAGE CHONDROCYTES IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS
307. Arne Vaaler: EFFECTS OF PSYCHIATRIC INTENSIVE CARE UNIT IN AN ACUTE PSYCIATHRIC WARD
308. Mathias Toft: GENETIC STUDIES OF LRRK2 AND PINK1 IN PARKINSON'S DISEASE
309. Ingrid Løvold Mostad: IMPACT OF DIETARY FAT QUANTITY AND QUALITY IN TYPE 2 DIABETES WITH EMPHASIS ON MARINE N-3 FATTY ACIDS
310. Torill Eidhammer Sjøbakk: MR DETERMINED BRAIN METABOLIC PATTERN IN PATIENTS WITH BRAIN METASTASES AND ADOLESCENTS WITH LOW BIRTH WEIGHT
311. Vidar Beisvåg: PHYSIOLOGICAL GENOMICS OF HEART FAILURE: FROM TECHNOLOGY TO PHYSIOLOGY
312. Olav Magnus Søndenå Fredheim: HEALTH RELATED QUALITY OF LIFE ASSESSMENT AND ASPECTS OF THE CLINICAL PHARMACOLOGY OF METHADONE IN PATIENTS WITH CHRONIC NON-MALIGNANT PAIN
313. Anne Brantberg: FETAL AND PERINATAL IMPLICATIONS OF ANOMALIES IN THE GASTROINTESTINAL TRACT AND THE ABDOMINAL WALL
314. Erik Solligård: GUT LUMINAL MICRODIALYSIS
315. Elin Tollefsen: RESPIRATORY SYMPTOMS IN A COMPREHENSIVE POPULATION BASED STUDY AMONG ADOLESCENTS 13-19 YEARS. YOUNG-HUNT 1995-97 AND 2000-01; THE NORD-TRØNDELAG HEALTH STUDIES (HUNT)
316. Anne-Tove Brenne: GROWTH REGULATION OF MYELOMA CELLS
317. Heidi Knobel: FATIGUE IN CANCER TREATMENT – ASSESSMENT, COURSE AND ETIOLOGY
318. Torbjørn Dahl: CAROTID ARTERY STENOSIS. DIAGNOSTIC AND THERAPEUTIC ASPECTS
319. Inge-Andre Rasmussen jr.: FUNCTIONAL AND DIFFUSION TENSOR MAGNETIC RESONANCE IMAGING IN NEUROSURGICAL PATIENTS
320. Grete Helen Bratberg: PUBERTAL TIMING – ANTECEDENT TO RISK OR RESILIENCE ? EPIDEMIOLOGICAL STUDIES ON GROWTH, MATURATION AND HEALTH RISK BEHAVIOURS; THE YOUNG HUNT STUDY, NORD-TRØNDELAG, NORWAY
321. Sveinung Sørhaug: THE PULMONARY NEUROENDOCRINE SYSTEM. PHYSIOLOGICAL, PATHOLOGICAL AND TUMOURIGENIC ASPECTS
322. Olav Sande Eftedal: ULTRASONIC DETECTION OF DECOMPRESSION INDUCED VASCULAR MICROBUBBLES
323. Rune Bang Leistad: PAIN, AUTONOMIC ACTIVATION AND MUSCULAR ACTIVITY RELATED TO EXPERIMENTALLY-INDUCED COGNITIVE STRESS IN HEADACHE PATIENTS
324. Svein Brekke: TECHNIQUES FOR ENHANCEMENT OF TEMPORAL RESOLUTION IN THREE-DIMENSIONAL ECHOCARDIOGRAPHY
325. Kristian Bernhard Nilsen: AUTONOMIC ACTIVATION AND MUSCLE ACTIVITY IN RELATION TO MUSCULOSKELETAL PAIN
326. Anne Irene Hagen: HEREDITARY BREAST CANCER IN NORWAY. DETECTION AND PROGNOSIS OF BREAST CANCER IN FAMILIES WITH *BRCA1*GENE MUTATION
327. Ingebjørg S. Juel : INTESTINAL INJURY AND RECOVERY AFTER ISCHEMIA. AN EXPERIMENTAL STUDY ON RESTITUTION OF THE SURFACE EPITHELIUM, INTESTINAL PERMEABILITY, AND RELEASE OF BIOMARKERS FROM THE MUCOSA
328. Runa Heimstad: POST-TERM PREGNANCY
329. Jan Egil Afset: ROLE OF ENTEROPATHOGENIC *ESCHERICHIA COLI* IN CHILDHOOD DIARRHOEA IN NORWAY
330. Bent Håvard Hellum: *IN VITRO* INTERACTIONS BETWEEN MEDICINAL DRUGS AND HERBS ON CYTOCHROME P-450 METABOLISM AND P-GLYCOPROTEIN TRANSPORT
331. Morten André Høydal: CARDIAC DYSFUNCTION AND MAXIMAL OXYGEN UPTAKE MYOCARDIAL ADAPTATION TO ENDURANCE TRAINING

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332. Andreas Møllerløkken: REDUCTION OF VASCULAR BUBBLES: METHODS TO PREVENT THE ADVERSE EFFECTS OF DECOMPRESSION
333. Anne Hege Aamodt: COMORBIDITY OF HEADACHE AND MIGRAINE IN THE NORD-TRØNDELAG HEALTH STUDY 1995-97
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