Interplay of the Ubiquitin Proteasome System with Nucleotide Excision Repair

Samenspel tussen het ubiquitin proteasome systeem en nucleotide excisie herstel

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Chapter 1

General introduction and scope of the thesis

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Nothing lasts forever, which is undoubtedly true for the macromolecules involved in the make-up of our cells. Various sources of endogenous and exogenous damage only speed up this process. Radical formation, mostly caused by oxygen-dependent energy consumption, is a major source of endogenous damage attacking our cells when energy is converted. Sources of exogenous damage are air pollution such as exhaust-gasses and cigarette smoke, certain types of electromagnetic waves including UV light, X- and gamma-rays, numerous chemical compounds etc. Three major classes of macromolecules susceptible to damage can be identified: lipids, proteins and nucleic acids. All three have different and common mechanisms that deal with the damaged molecules.

Lipids that are maintained by protein complexes in cell membranes form the barrier between the cell and its surroundings. Their occasional damage is restored by a constant turnover, which will not be further discussed here [1,2].

Proteins are executing almost all processes in the cell. They also function as building blocks for the skeleton of the cell. The characteristics of proteins, including their stability therefore vary widely depending on their function. Proteins with a specific task are frequently degraded after their action has been completed. Cellular functions critically depend on the lifespan of specific proteins, suggesting a tightly regulated and protein-specific protein degradation (proteolysis) machinery. So all proteins are turned over at their own variable pace including those proteins that are damaged via endogenous or exogenous sources. Moreover, proteins need to be folded to function properly in a process that often fails [3]. The misfolded proteins are either re-folded or degraded [4]. Protein turnover is accomplished by degrading used/old/damaged proteins and the synthesis (if necessary) of new ones.

Specific and aspecific degradation of proteins is performed by proteases. A cellular compartment specialized in waste processing called the lysosome contains enzymes including proteases that degrade all contents. Engulfment at the surface of the lysosome by invagination or septation results in the uptake of cytoplasm followed by the degradation of its components in a process referred to as micro-autophagy. Macro-autophagy involves the formation of cytosolic double-membrane vesicles that sequester parts of the cytoplasm, including even entire cell organelles. Fusion of the completed vesicle, an auto-phagosome, with the lysosome results in the delivery of its content into the lumen of the degradation compartment. Autophagy is the primary/major intracellular pathway for the degradation and recycling of long-lived proteins and organelles [5]. Short-lived proteins need special care inducing a quicker targeted degradation performed by the ubiquitin proteasome system (UPS). In this system target proteins are modified by the covalent attachment of a small protein/polypeptide called ubiquitin. Ubiquitylation occurs via the successive action of three enzymes, the activator, a conjugator and a ligase. The latter two determine the specificity of the target. Certain conjugated ubiquitins are ubiquitylated themselves, giving rise to the well-known poly-ubiquitin trees. Poly-ubiquitylated proteins are brought to the 26S proteasome that hydrolyses the target protein ultimately into amino acids. Although hydrolysis would in principle be an energy generating process,

26S-mediated proteolysis appeared an energy-consuming process [6]. The UPS will be discussed in more detail in **chapter 3**.

Poly-nucleic acids such as DNA and RNA encoded by the DNA form the third class of macromolecules susceptible to damage. Newly transcribed copies of a gene can replace damaged RNA molecules. However DNA faces the unique problem that it is the blueprint of life. Proper functioning of cells and organisms rely on a faithful copying of the genetic information encoded by the DNA. This vast amount of information deposited in DNA ultimately directs all processes in the cell. Expression of most of the genes encoded on the genome is strictly regulated. Regulation of gene expression depends for an important part on the compaction status of the gene locus. The majority of the genome in all eukaryotic cells is highly compacted in chromatin. The DNA is wrapped around nucleosomes consisting of the core histones 2A (H2A), 2B (H2B), 3 (H3) and 4 (H4) and a linker histone1. Compaction of chromatin is regulated by modification of histone tails by acetylation, methylation, phosphorylation, ribosylation and ubiquitylation. A combination of histone tail modification and chromatin-binding proteins determine the accessibility of the DNA. The DNA including its protein packing/surroundings is referred to as the chromatin.

DNA, like most macromolecules, is sensitive for certain types of endogenous and exogenous damage in spite of its rather stable double helix structure. Moreover, the DNA-transacting processes, transcription, replication and recombination are in itself a source of DNA damage due to the complexity of the chromatin. Cellular functioning and reliable transmission of genomic information require that the integrity of the genome is maintained. Several DNA repair and genome maintenance processes take care that this integrity is indeed maintained as much as possible. Due to the difference in damage type, localization and the phase of the cell cycle different repair pathways/genome maintenance mechanisms exist [7]. These different repair pathways and their role in genome maintenance are discussed in **chapter 2**.

If the damage load becomes too high, the cell commits suicide e.g. apoptosis or enters a permanent quiescence state e.g. senescence. Both scenarios prevent that unwanted mutations cause uncontrolled cell growth as a process that potentially could lead to cancer. In most cancers cell cycle control or genome maintenance genes are mutated. As a matter of fact, the p53 gene leading to apoptosis is mutated in most tumors, emphasizing the importance of this mechanism [8,9] and implying that apoptosis is a tumor-preventing process. Apoptosis (and senescence) is further an essential mechanisms for the architecture of the body they play an important role in development. The flip side however of a too robust induction of these processes by e.g. DNA damage is that it potentially depletes the germ line (i.e. cell renewal capacity). Apoptosis or senescence in the germ line itself has an even more profound effect of depletion. Cell depletion ultimately leads to a lower capacity or even complete organ failure as steps in the aging process. In fact in segmental premature aging models and most likely in natural aging as well, the body turns down the metabolism/energy consumption by hormonal control in anticipation of potential or accumulated damage (van der Pluim et al., submitted). It can be concluded that the prevention of cancer, induced by DNA damage has an unwanted side effect namely aging of the body. The lifespan of an organism will thus critically depend on the balance of tolerating lesions (at the expense of mutagenesis) and cell destruction (or senescence). The body will inevitably age in the prevention of cancer [10].

In addition to the potential failure in genome maintenance, possible loss of several cellular control mechanisms may disrupt normal cellular homeostasis leading to apoptosis as well, (also proteasomal) stress leads to apoptosis causing aging.

As mentioned above, a tight regulation of the various genome maintenance mechanisms is vital. Several ways to regulate this have evolved. A common theme in these is the usage of posttranslational modifications such as phosphorylation and ubiquitylation. The latter is, as a posttranslational modification, next to “a license to kill” also involved in many other non-proteasomal-related processes. Examples of both degradation- and non-degradation-related ubiquitylation events in the response to DNA damage are discussed in **chapter 4**.

In the experimental chapters 5 and 6 the regulatory mechanism of one genome maintenance sub-pathway (the global genome nucleotide excision repair pathway) is studied. In **chapter 5** the dynamic behavior of the damage recognition protein, XPC, in living cells is presented. The complex partner of XPC, the Homologue of Rad23B (HR23B) has a dual function in the UPS and DNA repair. The repair function of this protein is outlined in **chapter 6**. A novel DNA damage-induced histone ubiquitylation is described in **chapter 7**. **Chapter 8** concerns the presence of HR23B in cellular inclusions related to many neurodegenerative diseases. In **chapter 9** the findings of the experimental chapters 5-8 are discussed and some future prospects are presented.

Chapter 2

Genome maintenance

Genome maintenance

Preservation of the genetic code is not only important for the progeny but also for proper cellular functioning. However, replication errors, illegitimate recombination and direct interference with the DNA structure continuously challenge the genetic information. Several genome mechanisms have evolved to deal with the various genetic insults that arise in the cellular live cycle [7]. The characteristics of these lesions, the phase of the cell cycle and the cell type will determine how a cell responds to DNA lesions. The different DNA damage response (DDR) mechanisms, including DNA repair and signaling pathways will be briefly outlined in this chapter. Two of these pathways (i.e. nucleotide excision repair and translesion synthesis) will be discussed in more detail because in these the connection between genome maintenance and the ubiquitin proteasome system is the most evident.

2.1 DNA repair mechanisms

2.1.1 *Homologous Recombination and Non-Homologous End Joining*

DNA double strand breaks can be resolved by two distinct processes, homologous recombination (HR) and non-homologous end joining (NHEJ). In NHEJ two broken ends are relinked. However, processing of DNA breaks may result in the loss of a few base pairs. NHEJ is therefore a less accurate repair mechanism and prone to mutagenesis [11].

In HR the information on the sister chromatid is used as a template for the repair of the broken chromatid. HR is only active in S and G2 phases of the cell cycle since the presence of a homologous chromatid is essential [12,13].

2.1.2 *Base Excision Repair*

DNA damage involving only one strand and generally mildly affects base pairing, such as to the sugar or the base moieties of the nucleotides can be repaired by base excision repair (BER). For a large part BER lesions do arise spontaneously (deamination or hydrolysis) or are produced by cellular metabolism (for example reactive oxygen species ROS). Small alterations in the helix usually don't have dramatic effects on progression of transcription and replication. However, they may decrease fidelity of replication resulting in a mutation if not repaired. BER is therefore an important and vital mechanism to protect against spontaneous mutagenesis. The reaction is initiated by a group of lesion specific glycosidases with overlapping affinity for BER-type of lesions. Recognition results in the removal of the affected base followed by the nuclease mediated strand incision leaving an abasic site. The subsequent one or two nucleotide gap is filled in by DNA polymerase β. Only one or a few bases will be excised and inserted as a result of such repair.

2.1.3 *Nucleotide Excision Repair*

Lesions that cause serious helix distortion and only affect one strand are generally repaired by Nucleotide Excision Repair, a pathway that is discussed in more detail later in this chapter.

2.2 Replication-associated genome surveillance

2.2.1 *Mismatch repair (MMR*)

The so-called mismatch repair system restores mistakes in replication due to the incorporation of a wrong nucleotide or caused by slipping of the DNA polymerase. The mismatch is recognized and removed by the subsequent action of several different proteins (or complexes). The replication machinery then re-synthesizes a new strand using the original template [14].

2.2.2 *Translesion DNA synthesis (TLS)*

Replication of damaged DNA faces the unique problem that lesions that inhibit or stall replication are detrimental for cellular duplication, however tolerating replication over lesions enhances mutagenesis. Two classes of systems are operational in eukaryotic cells for damage avoidance and/or damage tolerance: the DNA template switch in combination with a recombination step (DNA damage avoidance mechanism) and translesion DNA synthesis (TLS) as an alternative DNA damage tolerance mechanism. The alternative polymerases of TLS can bypass lesions on which the normal high fidelity polymerases would jam. However, these polymerases operate on undamaged DNA with extreme low fidelity. This low degree of fidelity is partly explained by the absence of the exonucleolytic proofreading as performed by high fidelity polymerases. Moreover, these polymerases have a more open active site that can accommodate (helix-distorting) lesions better than polymerases that replicate with high fidelity possibly also explaining their low degree of fidelity [15]. Interestingly, the mechanism(s) for the switch from “normal” replication polymerases to “special” TLS polymerases is as yet not completely understood. This switch must be strictly regulated in view of the extremely low fidelity of the TLS polymerases. Accidental replication of undamaged DNA by a TLS polymerase instead of a replication polymerase would presumably increase the mutation rate substantially. A spatial separation of the different polymerases is highly unlikely since most TLS polymerases are localized in replication foci [16-18]. Modifications (e.g. ubiquitylation and SUMOylation) of the proliferating nuclear antigen (PCNA) ring determine in yeast which polymerase is sustained on the DNA [19-21]. The mechanism behind this switch is discussed in more detail in chapter 4.

2.3 DNA damage-induced signaling

Genotoxic stress does not only trigger DNA damage repair pathways, it also induces a cell cycle checkpoint (CCC) signaling pathway collectively referred to as the DNA damage response (DDR). In parallel a MAPKinase signaling pathway is induced, which will not be discussed here. Signaling is important for the activation of certain repair genes and induces cell cycle arrest. Cell cycle blocks either occur in G1/S, intra S, or G2/M thereby providing more time for DNA repair to occur or leading to apoptosis when too many lesions are encountered. Important initiators in mammalian DDR are the phosphatidylinositol 3-kinase-like kinases (PIKKs): ataxia telangiectasia mutated (ATM), ATM- and Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). The PIKK family members are activated upon (DNA) stress and consequently induce a cascade of kinases by phosphorylating downstream targets, including p53 and the effector kinases CHK1 and CHK2.

Classically, ATM is regarded as the primary regulator induced by DSBs whereas ATR is mostly triggered by replication blocks, hypoxia and UV light [22-24].

Upon stalling of the DNA polymerase extensive regions of ssDNA are formed. Replication protein A (RPA), essential for DDR, has affinity for exposed ssDNA and thus covers these regions [25]. ATR interacting protein (ATRIP) binds ssDNA-RPA structures [26]. Similarly, the alternative clamp loader, Rad17-RFC_{2-5} [27,28], binds to the same structure independent of ATR as well [29]. Rad17 recruits upon binding the rad9-Hus1-Rad1 (9-1-1) complex that displays structural similarities to the PCNA clamp [30,31]. The chromatin bound 9-1-1 complex then mediates the phosphorylation and activation of substrates by ATR including Rad17 and Chk1 [32-34]. The latter stabilizes stalled replication forks, inhibits the firing of late origin replication sites and arrests cells in G2/M [35].

ATM exists as a homodimer under unchallenging conditions, DNA damage induces auto-phosphorylation and the dimer becomes dissociated and subsequently targets other substrates, including Chk2 [36]. The initiation of the cascade occurs at the damaged sites, however the activated Chk2 quickly diffuses through the nucleus allowing activation of downstream targets pan-nuclear [37]. This trans-acting signaling warrants cell cycle arrest when anywhere in the nucleus a genomic insult is detected. At least some of these phosphorylation steps occur at the damaged site since the phosphorylation of an H2B-Chk2 chimera was restricted to the vicinity of the damaged site only [37]. The Mre11/rad50/Nbs1 (MRN) complex is necessary for the activation of ATM [38,39]. Although the MRN complex binds and tethers DSBs it is not clear whether it functions up- or downstream (or both) of ATM activation [40-42].

2.4 Nucleotide Excision Repair (NER)

Distortion of the DNA helix by DNA lesions has dramatic effects on both transcription and replication, in most cases even a complete stalling of the polymerase elongation. Unresolved stalling of RNA polymerase II (RNAPII) ends in apoptosis emphasizing the importance of the clearing of these lesions [43]. Well-known causes for helix-distorting lesions are compounds in cigarette smoke, DNA intercalating agents, many chemicals used in chemotherapy, and UV light. Especially UV-C light gives rise to well-characterized photoproducts such as the cyclobutane pyrimidine dimers (CPDs) and pyrimidine- (6,4)-pyrimidone photoproducts (6-4PPs) [44,45].

NER repairs a wide variety of helix-distorting lesions, including 6-4PPs, CPDs. NER is a multistep process encompassing: lesion recognition, helix unwinding and lesion verification, incision, gap-filling/synthesis and ligation (fig1).

Repair efficiency of NER is dependent on the type of lesion (extent of helix distortion) and its location (compactness of the chromatin and or transcriptionally active sites). Two distinct ways of lesion recognition can be distinguished in NER that give rise to the two sub pathways, the transcription-coupled repair (TC-NER) sub pathway and the global genome NER (GG-NER) sub pathway. In TC-NER a stalled RNAPII is thought to lead to the binding of the transcription factor IIH (TFIIH) complex after which the lesion is repaired. For successful repair the Cockayne syndrome (CS) protein B and CSA proteins are essential, but their exact function in repair is unknown. TC-NER is restricted to the transcribed strand of active genes, lesions anywhere else in the genome, including the non-transcribed strand are targeted by GG-NER. In GG-NER lesions are recognized by the

concerted action of two complexes: the XPC complex and the damaged DNA binding (DDB) complex. Lesion recognition in GGR is discussed in more detail later in this chapter.

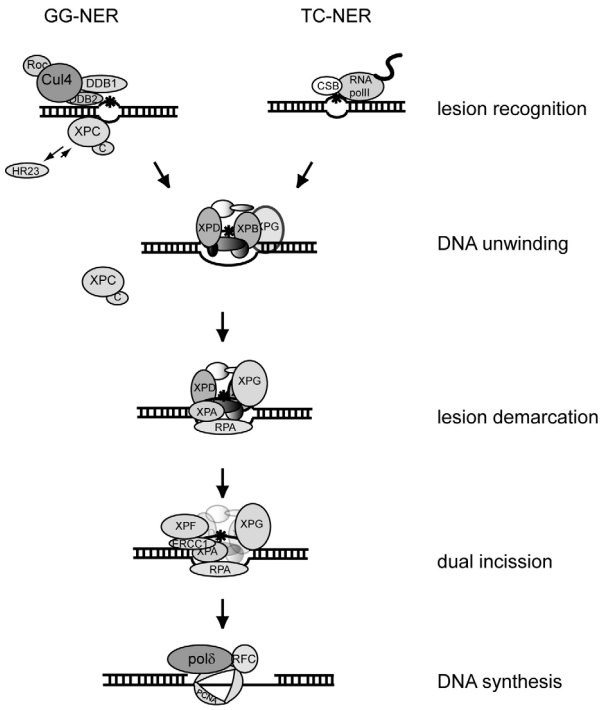

Fig1 Basic NER scheme.
In the transcribed part of the genome, the TFIIH complex is recruit by the stalled RNA polymerase with the help of the CSB and CSA protein. In the non-transcribed part of the genome lesions are recognized by the XPC complex assisted by the UV-DDB complex subsequently other NER factors are recruited. The helix is opened, in an ATP-dependent manner by the helicases in the TFIIH complex. The pre-incision complex and the excision of the lesion-containing strand is oriented by XPA and RPA. After excision, performed by both ERCC1/XPF and XPG, the remaining gap is filled in by the polymerase, PCNA and RFC. As a last step the nick is sealed by a ligase.

Upon lesion recognition, XPC recruits TFIIH [46,47], a ten-component complex [48,49] that contains the two helicases, XPB and XPD responsible for unwinding the 3'-5' and 5'-3' direction of the damaged helix respectively [50,51]. Besides NER, TFIIH is also essential for transcription initiation by RNAPII [52], and is involved in RNA polymerase I-driven transcription [53,54]. TFIIH functions in transcription and in NER by locally unwinding (melting) of the helix at promoters or lesions respectively. TFIIH melts a 25-nucleotide region on both sides located next to a genuine NER lesion. Only the helicase activity of XPB is essential for transcription, whereas XPD has probably a more structural function in this process [55]. However, for NER both helicase activities are essential [56]. TFIIH might be involved in verification of the lesion, in addition to its role in helix opening. Recruitment of TFIIH to non-NER lesions by the recognition factories does not result in further assembly of the subsequent NER factors. How this possible lesion verification by TFIIH is accomplished is as yet not known.

Following unwinding both XPA and RPA are incorporated into the growing NER complex. XPA is essential for NER but its exact role is still a matter of debate. It has been proposed that XPA is either involved in recognition or verification of the lesion since it has increased affinity for damaged

DNA in vitro. XPA is probably essential for the recruitment of other repair factors thereby stabilizing the complex as well. In addition, multiple protein interactions of XPA with other NER factors have been found, such as with TFIIH, ERCC1 and RPA [57-59]. The latter likely covers the undamaged 25 nucleotide single stranded DNA. The protection of the undamaged strand by RPA directs the orientation of the two structure-specific endonucleases responsible for the excision of the damaged strand [60]. The 3' endonuclease XPG binds subsequent to the opening of the helix in a process that is independent of XPA [61,62]. The 5' cut is performed by the heteromeric ERCC1/XPF complex [63]. A 24-32-bp patch including the lesion is excised by the action of the endonucleases [64]. After excision the replication machinery formed by PCNA, RFC and the DNA polymerase δ or ε fill in the gap thereby using the undamaged daughter strand as a template. As a final step a ligase seals the nick.

Three rare autosomal recessive human disorders are associated with mutations in genes involved in NER. Xeroderma pigmentosum (XP) is the most frequent of the three. Seven complementation groups (XP-A till XP-G) corresponding to different NER genes have been identified. Patients typically suffer from UV sensitivity and high (skin) cancer incidence. Mutations in the CSA or CSB genes, involved in TCR, give rise to Cockayne syndrome (CS). Patients display symptoms associated with premature aging and mental retardation. A third NER deficient disease is trichothiodytrophy (TTD), clinical features are similar to CS with additional scaling skin and brittle hair and nails [65].

2.5 Global Genome NER

In order to initiate a multi protein complex, such as NER, proper selection of the initiation site (i.e. DNA lesions) is a prerequisite. Within global genome NER (GG-NER) two complexes, with increased affinity for damaged DNA, have been identified. Namely the XPC complex and the damage DNA binding (DDB) complex. XPC binds to Cen2 and one of the two Homologs of the yeast Rad23 protein HR23A and HR23B [66-69]. Both Cen2 and the two Rad23 proteins are abundantly present. These proteins have other functions in addition to DNA repair whereas no extra functions have been found for XPC. Cen2 is localized at the centrosome and is essential for centriole duplication hence its name. Both Rad23 homologs are receptors of the UPS (for more details see chapter 3).

2.5.1 *GG-NER in humans and in mice*

Patients carrying (null) mutations in both XPC alleles typically have a high risk for (skin) cancers [7,70]. Human XP-C fibroblasts are moderately sensitive to UV in comparison with XP-A cells that lack both subpathways of NER (both TC-NER and GG-NER) [70,71]. Moreover, in a quantitative repair assay (unscheduled DNA synthesis), XP-C cells exhibit ~15-30% of wild type repair capacity while XP-A cells have <5% of wild type repair capacity. This suggests that the severe reduction of GG-NER only partly contributes to UV-hypersensitivity. The very toxic damages in the transcribed part of the genome that block transcription are efficiently removed in XP-C cells explaining the lower survival as compared to the (repair in) other NER mutants. UV-induced mutations accumulate in XP-C cells since they lack repair in the non-transcribed part of the genome and in the non-transcribed strand. Replication over persisting lesions, including the low fidelity trans lesion-polymerases will introduce

these mutations. Certain types of mutations affect the critical balance of processes that regulate the cell cycle, which possibly leads to uncontrolled cell growth. Mouse models lacking XPC (knock-out; K.O. mouse models) have a higher tumor incidence upon DNA damage compared to wild type litter mates [72,73] whereas heterozygous animals display also an increased risk for developing skin cancer (although much less compared to K.O. littermates). The latency for developing skin cancer after UV-irradiation is reduced in XPC heterozygous animals in comparison with their wild type littermates [74]. Moreover, K.O. and heterozygous XPC mice suffer from a higher spontaneous mutation frequency while XPA K.O. mice did not, clearly emphasizing the importance of an efficient GG-NER recognition process [75]. However, no difference in spontaneous tumorigenesis was found between XPC K.O. mice and their wild type littermates [74]. Possibly explained by the presence of a functional TC-NER in XPC mutant mice. In XPA K.O. cells accumulated DNA damage most likely triggers apoptosis via the arrest of RNA polII driven transcription [43]. Indeed, high levels of UV-induced apoptosis are observed in XPA K.O. cells but not in XPC K.O. cells [76,77]. This suggests that mutations in XPA K.O. mice are cleared via apoptosis while in XPC K.O. mice they accumulate in the untranscribed part of the genome [75]. Both the increase in spontaneous mutagenesis and the decrease in the latency for developing UV-induced skin cancer in K.O. and heterozygous animals imply an allelic insufficiency, further suggesting that the amount of XPC is a critical determinant of NER capacity.

In the human population certain types of cancer, lung and skin, display genetic aberrations in the xpc locus [78] but it is as yet not clear whether these are the cause or the consequence of the tumor. Moreover, certain single nucleotide polymorphism (SNP) in the xpc locus are associated with higher cancer risks [79,80]. Recently, alterations in the HR23B mRNA were reported associated with elevated cancer risks [81,82].

XP-E patients carry mutations in the DDB2 or p48 gene [83,84]. DDB2 forms with DDB1 or p127 the most significant components of the DDB complex (see below for other complex partners). Only mutations within the DDB2 gene have thus far in the human population been associated with XP-E possibly explained by the fact that all other components of the UV-DDB complex have additional, probably vital functions, besides NER. XP-E patient cells are only mildly UV sensitive and display even higher repair capacities than XP-C cells [71]. XP-E cells characteristically exhibit 50% of unscheduled DNA synthesis. Typically, CPD removal is almost completely absent and 6,4PP removal is delayed in the cells of XP-E patients, but only at low UV dose [85].

2.5.2 *Transcriptional response*

Cells that lack a functional p53 have a slower CPD removal [86]. This reduced repair is not caused by a direct participation of p53 in NER as no accumulation within local UV-damaged sites was observed [87] nor can it be found in chromatin immuno-precipitations of repair sites. This suggests that p53 influences GGR in a more indirect manner. p53-deficient cells lack the normal transcriptional increase in XPC and DDB2. XPC mRNA initially decreases (1-6 hour post UV) and then increases up to 24 hours post treatment [88,89]. Similar to XPC there is a transcriptional increase of DDB2 as well. Up-regulation starts after 8 hours and is maintained up to 24 hours [86].

In rodents the p53-inducible element is mutated in the promoter of DDB2. The UV-induced p53 response elevates DDB2 levels in humans that facilitate efficient repair [86,90]. Rodents lack this

response and are therefore hampered in the efficient removal in those lesions for which DDB2 is involved [91,92]. Despite the lack of UV-inducibility, rodents are not completely deprived of DDB2. Knockout models are more cancer prone compared to wild type littermates indicating that even the low levels of DDB2 in mice are important for a successful repair [93,94]. Enhancing the level of DDB2 in mice did indeed reduce the carcinogenic effects although similar repair kinetics as found in e.g. in human cells were not achieved [95].

In contrast to the drop in the mRNA levels, no initial drop in XPC protein levels could be detected. However, protein levels do rise 8-24 hours post UV [89] at least in part as the consequence of increased transcription. Endogenous XPC is a relatively stable protein since no drop could be demonstrated within 24 hr after inhibition of *de novo* synthesis (personal communication R. Nishi). The stability of XPC might explain the absence of a decrease in protein levels. A significant increase in fluorescence was observed 8 hours after UV in cells expressing XPC-GPF under control of a CMV promotor, indicating that induction of XPC is more than only a transcriptional response [96].

2.5.3 *Affinity for damaged DNA*

Both the XPC- and the UV-DDB complexes are involved in the recognition of helix-distorting lesions. XP-C cells lack both CPD and 6,4PP removal whereas XP-E cells are mainly deficient in CPD repair and have a slower 6,4PP removal. This suggests that UV-DDB assists XPC binding *in vivo*. *In vitro* XPC recognizes 6,4PP relatively well, while almost no affinity for CPDs is detected [97,98]. Chemical helix-distorting modifications to the DNA, like cholesterol or N-acetoxy-2-acetylaminofluorene (NA-AAF) are also substrates for the XPC complex. XPC exhibits an exceptionally broad lesion recognition potential, even ordinary helix bubbles are good substrates [98,99]. The XPC complex has an increasing affinity for those lesions that display unfavorable thermodynamic changes in the helix, it might be that XPC specifically probes such lesions [100]. Single molecule studies using scanning force microscopy (SFM) revealed that the XPC complex induces or stabilizes a bend angle on cholesterol substrates [101]. This implies that the XPC-damaged DNA complex is kept in an open state. Recently, Buterin et al showed that XPC binds to the undamaged strand opposite to the helix-disturbing lesion [102]. This observation suggests the following sequence of events: the XPC complex senses energetically unfavorable distortions, immobilizes on the opposing strand (i.e. the actual only common structure in all the different NER-inducing lesions) and thereby stabilizes the lesion. Subsequently, the TFIIH complex binds directly to XPC. The partial open status of the DNA frame is beneficial since it might be a better initiation point for the helicases (similar to the function of TFIIH in transcription initiation) whereas there might also be less steric hindrance of the XPC complex.

UV-DDB complex has a much higher affinity for UV-induced lesions in comparison to XPC/hHR23B complex [103]. UV-DDB binds to both CPD and 6,4PP, albeit with a higher affinity for the latter. In fact, UV-DDB is stimulating XPC binding on UV lesions [85]. Recently it was shown that UV-DDB also binds to AP sites and mismatches [104]. In contrast to XPC/hHR23B, UV-DDB affinity decreased when the mismatch (or bubble size) increased. XPC binds with a similar affinity to 3 or 5 bp bubbles while UV-DDB affinity clearly drops for substrates with more than 3 bp mismatches. Unfortunately, these studies are not conducted at the same time and under the same conditions. NER repairs a remarkable wide variety of helix-distorting lesions that don't share any structural relationship,

rather than disrupting Watson&Crick base pairing. Since NER acts on a wide variety of structurally unrelated lesions a highly specific recognition complex with explicit affinity for certain types of lesions might therefore not be beneficial. UV-DDB might serve as an (essential) enhancer for only a subset of lesions including those induced by UV.

2.5.4 *Post-translational modifications*

DDB1 and DDB2 reside in a larger complex containing cullin4a, Roc1 and all the components of the COP9 signalosome (CSN) [105]. Cullin4a and Roc1 display E3 ubiquitin ligase activity and indeed ubiquitylate DDB2 both *in vitro* and in living cells [106-108]. DDB2 ubiquitylation in living cells results in its rapid degradation by the 26S proteasome [109,110].

Cullins form a family of proteins (cul1, 2,3, 4a, 4b and 5 and the related cul7, PARC and APC2) that interacts with ROC1 (RING of Cullin) via their conserved carboxy terminus. The actual E3 ligase activity is displayed by ROC1, which also recruits the E2 to the complex. Cullins bind via the conserved amino-terminus to an adapter protein (or –complex). The adaptor protein (–complex) facilitates the binding to a specific substrate as opposed to most ligases that bind directly to their substrate. Many other targets, besides DDB2, have been identified. Cullin4a knock-out mice are embryonic lethal and die between blastocyst stage and embryonic day 7.5 emphasizing its importance and the likelihood of other target(s) [111].

The CSN displays deubiquitylation (DUB) activity that counteracts the E3 ligase activity of the cullin4a and roc1. CSN is essential for development and is involved in the regulation of the cullins [112].

Upon UV the UV-DDB complex binds to chromatin and induces dissociation of CSN from the UV-DDB/cullin4a/roc1 complex. Simultaneously, Cullin4a becomes NEDDylated, which activates its E3 ligase activity [105]. The activation in combination with the absence of the counteracting DUB activity of the CSN facilitates the (poly)ubiquitylation and subsequent degradation by the proteasome of DDB2. Next to an effect on DDB2, UV-damage also triggers the degradation of the replication-licensing factor CTD in a cullin4A- and DDB1-dependant fashion [113,114]. This reaction does not require DDB2 and shows that the ubiquitylation/deubiquitylation regulatory unit connected to DDB1 has multiple functions in the DDR [115]. Yeast cells lack DDB2 but have a DDB1 ortholog. Studies in fission yeast revealed that DDB1 mutant cells are mildly UV-sensitive and have a slow S phase progression [116].

Recently, the UV-DDB dependent ubiquitylation of XPC was reported as well [117,118]. UV-induced DNA damage triggered XPC (poly)ubiquitylation in all except XP-E cells. Ubiquitylated species were both bound as well as unbound to the chromatin. Surprisingly, polyubiquitylated XPC is not degraded but the polyubiquitin modification disappeared in time, possibly by an as yet unidentified DUB activity. So upon UV-irradiation the E3 ligase activity associated with UV-DDB ubiquitylates both DDB2 and XPC. Consequently, DDB2 is degraded while XPC is not. Ubiquitylated XPC had a higher affinity for both damaged- as undamaged-DNA compared to unmodified XPC [118]. Since polyubiquitylated XPC species could be detected in the DNA-unbound fraction it might be that the E3 ligase activity displayed by the UV-DDB complex is only necessary for an initial round of NER (i.e. of those lesions that require a functional UV-DDB activity). Subsequent recognitions e.g. binding to NER

lesions could be performed without the enhancing activity of the UV-DDB complex. The degradation of DDB2 ensures that the amount of polyubiquitylated XPC stays limited, which is necessary since the modified species enhances unspecific DNA binding of XPC as well, a process that likely hampers other DNA transacting processes. After most of the 6,4PP lesions are removed, XPC polyubiquitylation is reversed and DDB2 levels are restored via the p53-dependent transcriptional response.

2.5.5 *XPC complex partners*

The binding domain of cen2 in XPC was mapped to residue 847-867. Even more precisely, three hydrophobic residues (W848, L851 and L855) are essential for cen2 binding. A mutated XPC incapable of binding displayed reduced 6,4PP removal compared to wild XPC. Moreover, Cen2 stimulated XPC binding to damaged DNA *in vitro* [119].

Rad23 is essential for XPC stability *in vivo*. Cells, which lack HR23A and HR23B, display a dramatic reduction of XPC levels. Rad23 seems to protect XPC from 26S-proteasome-mediated degradation, since ectopically expressed XPC becomes stabilized after blocking the 26S-proteasome [96]. A similar mechanism has been shown in yeast as well [120]. *In vitro* both HR23A and HR23B have a stimulatory role on the affinity of XPC for damaged DNA [68,121]. Moreover in yeast Rad4 (XPC) overexpression only partly rescued RAD23 K.O. UV-sensitivity [122]. This clearly indicates that there are other functions of the Rad23 proteins in addition to the regulation of XPC levels. In chapter 6 we show that Rad23 proteins are indeed essential for the binding of XPC on damaged DNA. In the absence of both Rad23 proteins, both endogenous as well as over expressed XPC fail to accumulate on local DNA damage. Moreover, no immobilization (binding to DNA) of XPC-GFP can be detected in cells missing both HR23A and HR23B. Interestingly, HR23B (and HR23A) itself does not accumulate at local damaged areas and is not immobilized after UV irradiation in living cells. Indeed the amount of HR23B in complex with XPC reduces dramatically after UV irradiation both *in vitro* (using immunoprecipatation) as well as in living cells (using fluorescent energy transfer, FRET).

2.6 Nucleotide excision repair and chromatin

Within NER, the basal mechanism of repairing helix-disturbing lesions is rather well understood as determined *in vitro* on naked DNA templates. However, little is known yet about the contribution of the chromatin structure during and after DNA repair. Already in the seventies it has been proposed that chromatin remodeling is essential for efficient NER [123], and indeed reconstituted nucleosomes have a clear inhibitory effect on NER rates *in vitro* [124,125]. Opening of the chromatin has been proposed as a first step to allow efficient binding of the recognition complex, whereas presumably the original state of the chromatin has to be restored when repair is completed. These subsequent steps have been defined as recognition, repair and restoration [126,127].

2.6.1 *chromatin*

About 80% of the eukaryotic DNA is wrapped around nucleosomes. These consist out of two copies of H2A, H2B, H3 and H4. Initially DNA is wrapped around a $(H3/H4)_2$ tetramer, the association on either side of the H2A/H2B dimer results in the final nucleosome arranging 147 bp [128]. The various DNA-

histone interactions compensate for the energy cost of this bending and this results in a relatively stabile structure [128]. Besides the “core” octamer also the linker histone H1 plays a role in compacting chromatin. This linker histone is, however, not as stably anchored in the nucleosome as the core histones, as was shown by the exchange of transiently bound H1 revealed by photobleaching studies on GFP-H1 [129]. Although core histones form a more stable complex, each of the components display a differential kinetic assembly with the complex [130,131].
In higher eukaryotes different isoforms of the histones exist as is illustrated by the notion that besides the major H2A several minor variants including H2AX, H2AZ, macroH2A and H2A-Bbd are incorporated in nulceosomes, likely providing regulation of chromatin-associated processes. Further differential plasticity to the nucleosome is provided by as series of covalent modifications on histone tails, predominantly amino-terminal. These are: acetylation, phosphorylation, methylation, ribosylation and ubiquitylation (at the carboxy-terminus). Histone modifications have a major impact on the structural status of the chromatin, either compacting or opening. A combination of these modifications gives rise to the so-called histone code [132], thereby extending the information lying in the genetic DNA code (i.e. the epigenetic code) [133]. A vast amount of literature exists concerning the importance of both histone variants and histone modifications. For example, recently it has been shown that a specific histone modification (H3K36me) masks possible transcription initiation sites in the coding region [134]. Similarly, H2A.Z is preferentially incorporated in promoter regions thereby functioning in transcription initiation for at least a subset of genes. Both observations show and emphasize the importance of the nucleosome composition and structure [135]. Besides the histone modifications and histone variants, ATP-depending enzymes, the so-called chromatin remodeling enzymes, can modulate chromatin at the nucleosomal level. Roughly four classes of such enzymes exist (SWI/SNF, ISWI, CHD and INO80) that all contain a SNF2-like DEAD/H(SF2) ATPase subunit in their complex [128]. Chromatin remodeling induced by and in combination with the histone code and proteins that bind to the chromatin greatly influences all DNA-transacting processes including transcription, replication and repair.

2.6.2 *Chromatin remodeling and repair*

For long it is known that processing of DNA damage induces remodeling of chromatin. Within the first hours post irradiation a chromatin reshuffling takes place since in this period repair sites are enriched in endonuclease sensitive fragments [123,136]. Moreover, chromatin of newly repaired DNA resides relatively more in high salt soluble fractions usually associated with “active” chromatin [137].
There are indications that chromatin remodeling influences NER positively. For example, the repair rate in hyperacetylated chromatin areas is increased by using deacetylase inhibitors [138]. Histone acetylation is typically associated with activation of transcription e.g. with more open chromatin [139]. Moreover, the TATA-binding Protein-free TAF-containing (TFTC) complex, involved in transcription initiation [140], acetylates H3 after UV-induced DNA damage [141]. Chromatin-remodeling complexes added to *in vitro* repair reaction have a stimulatory role on damaged chromatin templates [142-144]. Though chromatin remodeling was found to be clearly linked with UV-damage repair, the enzymatic activities required and at which stage remodeling takes place (or which process activated by DDR) are unknown. The CSB protein, involved in TCR is a DNA-dependent ATPase of the SWI/SNF family and

the first DNA repair protein known to remodel the chromatin structure [145] by actively wrapping DNA around its surface, thereby influencing the DNA topology [146]. Although CSB immobilizes and accumulates at (TCR) repair sites [147] it is not clear whether the chromatin remodeling activity itself is necessary for TCR.

In contrast to the limited information on the possible influence of chromatin remodeling on NER, a large body of evidence exists implying a role of chromatin remodeling at DSB sites. For example the INO80 complex is implicated in DSB repair and binds specifically to yH2AX [148,149]. The histone variant H2AX is phosphorylated upon ionizing radiation [150]. It is believed that this histone modification is an important event in the repair of DSBs [151]. Indeed ino80 mutants in yeast are sensitive to damaging agents known to cause DSB [152]. Moreover, ino80 mutants are UV sensitive as well [152]. Although INO80 mutants grow less well in comparison to wt strains and therefore UV sensitivity could be a reflection of a more general problem this might imply that INO80 is involved in UV induced chromatin remodeling. A mammalian homologue of the yeast INO80 complex has been identified [153]. Next to the chromatin remodeling proteins other (non-histone) proteins also have an influence on the structure of the chromatin. For example, the nucleosome binding protein HMGN1 (high-mobility-group N1) is known to affect the stability of the higher-order chromatin structure [154]. Recently, it was shown that HMGN1 K.O mice are hypersensitive to UV irradiation [155]. In HMGN1 K.O. MEFs removal of UV-induced DNA damage in transcribed genes is reduced in comparison to damage removal in cells from wild type littermates. A transcribed gene in HMGN1 K.O. cells was digested with reduced efficiency by micrococcal nuclease activity whereas no effects on the total genome were observed [155]. This suggests that HMGN1 affects accessibility to transcribed genes. The UV sensitivity might therefore be explained by a slower rate of TC-NER. However, more research is necessary to see if there is a role of HMGN1 in either GG- or TC-NER. Currently, it is not clear if there is indeed an enrichment of HMGN1 on damaged DNA and if this is restricted to the transcribed genes or not.

2.6.3 *Post NER chromatin remodeling and histone alterations*

The nucleasomal reshuffling after UV irradiation, as mentioned above, shows that chromatin remodeling takes place in the DNA damage response. Analogous to transcription initiation and replication start also for DNA repair chromatin remodeling has been proposed to proceed binding of repair factors. However, no clear chromatin-remodeling prior or during the recognition step in GG-NER in living cells has been identified to date. It might even be possible that all the observed DNA-damage associated chromatin remodeling takes place after recognition or even repair. Interestingly, both the nucleases sensitivity as the enrichment of UV-irradiated chromatin in high salt, “active chromatin” fractions are much less in XPC deficient cells as compared to wild type cells [137,156]. Moreover, in wt cells DNA is still slightly more sensitive to staphylococcal nuclease and Dnase I treatment after the majority of the lesions are repaired [136] with also an overrepresentation of repair sites in short endonuclease fragments [157]. This suggests that the observed damage-dependent chromatin modifications are a consequence of repair rather than a prerequisite.

The accumulation of the chromatin assembly factors (CAF) and CAF dependent chromatin remodeling during repair synthesis also suggests that this type of chromatin rearrangement also takes

place after excision of the lesion [158,159]. Surprisingly, this chromatin rearrangement covers more than a single repair site and is independent of DNA synthesis/gap filling [160]. The distribution beyond the repair site suggests that these factors do not necessarily have to function in restoring the original state of the chromatin. Post repair induced chromatin remodeling might be crucial for other reasons, for example in repressing gene expression of damaged areas or inducing cell cycle arrests.

There is more evidence for a repair-dependent chromatin alteration. Recently, it was shown that H2AX is also phosphoryted after UV. This UV-induced γH2AX is dependent on ATR and interestingly absent in NER-deficient XP-A cells [161]. Contrary to the expectation, this ATP-dependent and UV-induced H2AX phosphorylation also occurs in non-dividing cells, ruling out that stalled replication forks (suggested to trigger ATR) induce this event.

We showed an increase in ubiquitylated H2A in the vicinity of UV-induced lesions (chapter 7). This UV-induced monoubiquitylation of H2A was dependent on functional NER e.g. it was dependent on excision. Moreover UV-induced H2A monoubiquitylation did not occur in cells mutated in ATR while it did in ATM-deficient cells. This suggests that the event is dependent on DNA damage-induced signaling. Although we observed a colocalization of this ubiquitylation event with γH2AX on local UV irradiated areas, the ubiquitylation appeared independent of the phosphorylation of H2AX. Using live cell imaging techniques we determined that roughly 50-200 ubiquitin molecules are conjugated to H2A per repair site. This strongly implies that the UV-induced uH2A signal spreads to adjacent nucleosomes (10-30 Kb). A remarkable spreading of γH2AX over a 50 Kb region around a single DSB was found [162]. Whether a similar spreading of γH2AX on UV-induced lesions occurs is however not known yet. Both post excision-, DNA damage induced-histone modifications alter the status of the histone code surrounding the damage. The notion that these modifications reside much longer at the damaged site as repair factors makes a possible function in restoring the original histone code highly unlikely. Moreover, both modifications and the CAF-induced rearrangement extend much further than the actual repair site. Apparently, the histone code is altered far beyond a repair site in a repair-dependent fashion. The notion that both UV-induced histone modifications are absent in ATR-deficient cells suggests that these have a function in DDR. Studies in yeast suggest a role for histone ubiquitylation in G1/S checkpoint signaling after UV-induced DNA damage [163]. Notably, in the yeast nucleosome H2B is the primary ubiquitination target instead of H2A in mammalian cells. Yeast strains expressing the mutant histone $H2B^{K123R}$ that cannot be ubiquitylated are viable and not overtly sensitive to UV light [164]. However, UV-induced phosphorylation of Rad53 (orthologue of Chk2) was abrogated in the $H2B^{K123R}$ strain resulting in a failure to block DNA replication after DNA damage induction [163].

H2AX knock out mice suffer from genome instability but are viable and repair DSBs in S phase [165,166]. Since it is generally believed that DSBs are extremely toxic, this implies that γH2AX is not essential for the initial repair of DSB but has other functions in genome maintenance e.g. in DNA damage-induced signaling and or as a facilitator of repair. Thus, it is tempting to speculate that γH2AX and uH2A may be part of the same postrepair epigenetic histone code involved in DNA damage signaling.

Strikingly both UV-induced H2AX phosphorylation and H2A ubiquitylation are regulated by ATR. It is still unclear how ATR-dependent signaling is initiated e.g. how do cells know when to activate the

DDR? Upon replication stalling ATRIP binds ssDNA-bound RPA and thereby initiates the ATR-dependent DDR. It has been proposed that all ATR-mediated signaling is triggered by replication stress only [167]. However, many findings dispute this. For example, H2AX phosphorylation (and H2A ubiquitylation) upon UV-induced damage occurs in non-replicative cells [161] (and personal observations). Moreover, wild type yeast can activate Mec1 (ATR) in G1 or G2 while NER-deficient yeast cells cannot, nor can they activate down stream effectors rad9 and rad53 [168,169]. Instead NER-deficient cells undergo a replication dependent S-phase arrest upon UV [170]. It is therefore likely that the processing of the lesion as well can trigger UV-induced signaling. The NER pathway is a likely candidate to fulfill such a processing, thereby functioning as the damage detector as well. Controversially, a third hypothesis states that ATR itself can sense DNA damage and that DDR is independent of NER in G1 cells [171].

The postrepair-induced histone modifications uH2A and yH2AX are likely to play a role in the DDR. The dependency of ATR suggests that they do not function in the initiation of DDR. The phenotype of the (yeast) mutants implies that they are essential in DDR. A possible function of these modifications might be to enhance or facilitate the response. Especially the notion that there is a dose-effect in DDR partly determining the outcome of events (arrest or apoptosis) suggests that such a histone modification might function as a threshold to determine their outcome and efficiency.

Chapter 3

The Ubiquitin Proteasome System

The Ubiquitin Proteasome System (UPS)

3.1 Ubiquitin and ubiquitin-like proteins

Ubiquitin is a highly conserved 76 amino acids polypeptide that is utilized as a protein modifier via conjugation to target proteins. Like other protein modifications such as phosphorylation and acetylation, ubiquitylation plays a crucial role in many cell processes by changing the structural and catalytic function of the targeted polypeptide. Originally it was believed that ubiquitin was present in all kingdoms explaining its name. Only after the sequencing of bacterial and archea genomes it became clear that ubiquitin is restricted to eukaryotes in which it is indeed ubiquitously present. The previously observed presence of ubiquitin in bacteria is most likely an artifact due to the usage of yeast extracts to grow bacteria [172].

In addition a large family of ubiquitin-like proteins exist. These polypeptides share structural similarities (e.g. the ubiquitin-fold) but usually a low sequence homology [173]. Two groups can be distinguished: (i) those who are free moieties and that can be conjugated themselves and (ii) those that contain an ubiquitin-like domain. Among the first class are for example the Small Ubiquitin-like Modifiers (SUMO1-3) and NEDD8. Most of these, including ubiquitin itself, need posttranslational proteolytic processing to reveal the (carboxy-)terminal di-glycine essential for the covalent attachment to target proteins (for details see below and [174]). Polypeptides that share the ubiquitin fold, but are without the terminal di-glycine, cannot be conjugated to target proteins hence they cannot be considered as protein modifications. The second group consists of proteins that contain an ubiquitin fold domain with high- (ubiquitin like domain) or with low sequence homology (Ubx domain). These domains often function as subcellular localization targeting elements [175].

Ubiquitin and ubiquitin-like proteins share a similar mechanism for their attachment to target proteins. The carboxy-terminal glycine (G) of ubiquitin is covalently conjugated to an amino group in the target protein. In most cases the ε-amino group of internal lysine residues within the target protein is attacked but amino-terminal conjugations have been described as well [176]. Conjugation takes place in a three-step mechanism involving the ubiquitin activating enzyme or E1, an ubiquitin conjugating enzyme or E2 and an ubiquitin ligase or E3 (Fig. 2). Within a cell the balance between ubiquitylated and deubiquitylated proteins is maintained by a combination of ubiquitin ligases and conjugases and enzymes that release the ubiquitin moieties, the deubiquitylation enzymes (DUB).

Several ubiquitin molecules can be attached to different target sites in one target protein, giving rise to multiple-monoubiquitylation [177,178]. However, the exact function of this multi-monoubiquitylation is substrate specific [179].

Ubiquitin itself can also be conjugated to conjugated-ubiquitin since ubiquitin contains several lysines, the outcome is a so-called poly-ubiquitin tree [180]. The distinct lysines in ubiquitin result in diverse poly-ubiquitin trees. The different poly-ubiquitin trees have different consequences. The best well known target is the lys 48 tree, which is the ticket to 26S-mediated degradation [180]. In yeast lys 29 trees are involved in degradation as well [181]. Lys6 polyubiquitin trees are rare, these were suggested to inhibit degradation [182]. Poly-ubiquitin trees through lysine 63 have a non-proteasomal role, examples of this have been found to function in endocytosis, ribosomal function and in DNA repair [20,183,184]. Particularly, a link of this modification with DNA repair is apparent since

yeast cells that only express Ub^{K63R} (Ublys63arg), grow and degrade normally but are slightly UV- and MMS-sensitive [185]. How uniform the polyubiquitin chains are is not known, *in vitro* so-called mixed polyubiquitin chains have been frequently detected, it might very well be that these play an important role *in vivo* as well (for review see[186]).

Several variations on ubiquitylation are possible with completely different outcomes. It has been described that the same residue in a single target protein can be mono-, poly-ubiquitylated or even modified by an ubiquitin-like peptide all with a different outcome; a prime example is the modification status of PCNA (chapter 4).

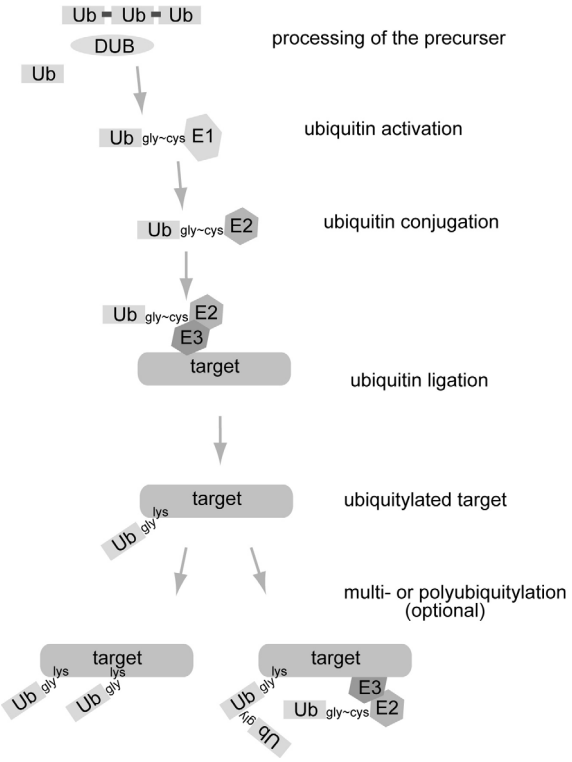

Fig. 2 Basic ubiquitylation scheme.
After processing of the ubiquitin precursor single ubiquitin moieties can be activated by the E1 enzyme. The E1 transfers the ubiquitin to an E2 enzyme. Finally ubiquitin is covalently bound to a target protein by the actions of both an E2 and an E3 enzyme. After initial monoubiquitylation a target can be further polyubiquitylated or multiubiquitylated.

3.2 Enzymes involved in ubiquitylation and deubiquitylation

As a first step the carboxy-terminus of ubiquitin is activated by the action of the E1 enzyme. Activation occurs in a two-step, ATP- and Mg-dependent reaction that results in E1~thiolester~ubiquitin intermediate. In all eukaryotes only one functional E1 is known. Upon activation ubiquitin is transferred

to a conserved cysteine in one of the distinct E2 enzymes. Again a thiolester intermediate is formed. Typically, E2 conjugation enzymes share a conserved globular domain. In contrast to the E1 many E2 conjugation enzymes have been identified in higher eukoryotes. The E2 enzyme conjugates the ubiquitin to the final substrate either directly or indirectly. In the later case ubiquitin is first transferred to a HECT (Homologous to E6AP Carboxy Terminus) domain, present within a subclass of E3 ligases and then transferred to the final substrate. Or, more commonly, the E2 enzyme conjugates the substrate with the help of an E3 ligase. In this case the ligase has an adaptor or bridging function. Most of the E3 ligases in this group contain a RING (Really Interesting New Gene) domain [174]. E3 ligases bind both the E2 and the substrate and thus determine in combination with the E2 the specificity. A specific preceding modification such as phosphorylation, glycosolation etc. of either the target or the ligase is often needed for efficient E3 substrate binding. Many RING domain E3 ligases reside in larger complexes, for example the SCF (Skp1-Cdc53/Cul-1-F-box protein) complexes [112] for which substrate- and E2-binding specificity are divided over the distinct complex partners. Although the exact number of E3 ligases is not known, in part due to the fact that other domains might also display E3 activity, the number in mammals has been estimated to be around 300. The U-box (Ufd2 homolgy) containing proteins bind oligoubiquitylated (one or two ubiquitins) substrates and assist the further polyubiquitylation of these substrates. Due to this property it has been proposed that they belong to a new class: the E4 enzymes [187]. Although currently this classification is part of a semantic debate in which it is questioned whether this enzyme represents a genuine class or a sub-E3 class.

Specific proteases exist that remove conjugated ubiquitin molecules from targets. Also in the processing of newly translated ubiquitin DUBs are involved. DUBs are divided in five different subclasses dependent on the active domain. Using BLAST searches on genome databases approximately a hundred of potential DUBs have been identified in the human genome [188]. Not surprisingly, DUBs play an essential role in many processes, including DNA repair, transcription regulation and protein degradation as they keep the balance of ubiquitylated versus non-ubiquitylated proteins.

The formation of polyubiquitin chains is still not fully understood. Several different variations on the basic formation of these polyubiquitin trees have been proposed [189]. Elongation factors such as the E4 enzyme might play a role, also the counteracting activity of the DUBs and the protection of shielding factors have to be considered. In addition a switch of either or both E2 and/or E3 might be a possibility.

3.3 Ubiquitin interacting motives

Recognition factors are involved in transducing and modulating ubiquitylation events into the downstream consequences. Several ubiquitin-binding domains (UBD) have been identified, among which are the UBA (ubiquitin association domain), CUE (coupling of ubiquitin conjugation to endoplasmic reticulum degradation), UIM (ubiquitin interacting domain), UEV (ubiquitin E2 variant), GLUE (GRAM-like ubiquitin-binding in Eap45), GAT (GGA (Golgi-associated gamma-adaptin homologous) and Tom1 (target of Myb 1)), PAZ (Polyubiquitin Associated Zinc finger) and the NZF (Npl4 zinc finger) [190,191]. The affinity of these domains for the different types of ubiquitylation (mono

versus the different polyubiquitin trees) is in general very low, typically a Kd in the order of 10-500 µM has been observed [191] probably explained by the reversible and transient nature of these interactions. The affinity of a UBD for a certain type of ubiquitylation can vary from protein to protein and may be defined by the surrounding amino acid context as is described for the UBA domain [192]. Moreover, many proteins contain one or multiple UBD domains. Together this multitude of enzymatic activities involved in the ubiquitylation equilibrium creates an enormous amount of possibilities to modulate protein function exceeding far beyond the surplus on phosphorylation regulated pathways. The combination of different posttranslational modifications even further increases the regulatory potential.

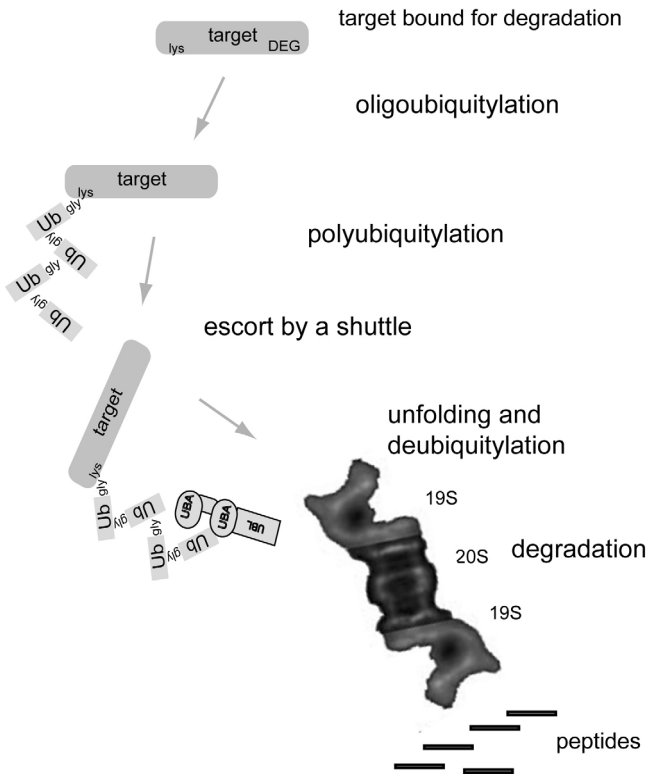

Fig. 3 Recruitment of polyubiquitylated proteins to the 26S proteasome.
Polyubiquitylated targets of the proteasome are escorted to the 19S cap of the 26S proteasome by shuttles such as the RAD23 proteins. At the 19S part of the proteasome deubiquitylation and unfolding takes place. Finally proteins are degraded by the protealytic activity of the inner rings of the 20S particle of the proteaome.

3.4 Protein degradation

26S-mediated protein degradation is the major proteolytic pathway for intracellular proteins in eukaryotes. It also plays an important role in MHC class I antigen presentation and the breakdown of misfolded proteins [193].

In general a protein that is recognized for degradation is marked with a lys48-linked polyubiquitin tree. Recognition can be established in several ways as is discussed in the review of Glickman and Ciechanover in 2002 [193].

Ubiquitylated proteins, targeted for degradation are initially oligoubiquitylated subsequently the AAA (ATP-ases associated with a wide variety of cellular activities) ATPase CDC48 or p97 binds the target. CDC48/p97 seems to be a critical switch that determines if a protein is further polyubiquitylated and subsequently degraded or stabilized and/or deubiquitylated [194].

Following polyubiquitylation the protein is targeted to the 26S proteasome for final degradation. Several shuttling proteins have been described, such as rpn10 (S5a), Rad23, Ddi1 and Dsk2, that facilitate targeting of substrates to the 26S proteasome [195,196]. For example, Rad23 binds both to the E4, Ufd2 and the proteasome. Furthermore it also recognizes polyubiquitylated proteins via its UBA domains [197-200]. The binding of Rad23 to Ufd2 and the affinity of the UBA domains for poly-ubiquitin chains suggest that Rad23 first binds polyubiquitin chains and subsequently binds to the proteasome with the UBL domain.

The 26S proteasome is composed of two subcomplexes, the 20S core particle and the 19S regulatory particle (fig 3). The core particle contains two α outer- and two β inner-rings that display the actual catalytic activity. The core particle is capped by 19S particles on one or on both ends. In higher eukaryotes the 19S particle can be replaced by the 11S particle, which is implicated in antigen presentation. The 19S particle is composed of a lid and a base. Recognition of polyubiquitylated proteins is performed by the 19S particle that binds to shuttles like Rpn10 (S5a), Rad23, Ddi1 and Dsk2. Also the deubiquitylation of substrates prior to degradation takes place at the 19S cap. The different AAA ATPase subunits in the base unfold the substrate whereupon it can enter the catalytic chamber of the 20S proteasome and subsequently be degraded [172].

3.5 Folding and the UPS

The correct folding of proteins in a unique three-dimensional structure is essential for their functioning. Such correct folding is not in all cases spontaneous, for a large number of proteins folding is aided by chaperones including the heat-shock proteins (Hsp) 40 and 70, Hsc70 and chaperonin. They fold both during *de novo* synthesis as well as under stress conditions such as heat-shock. Guided folding is essential to counteract misfolding [201]. Partially folded and misfolded states of proteins often tend to aggregate. A certain level of aggregation is tolerated in cells. However, often these aggregates are not properly or efficiently cleared, this may lead to formation of structured aggregates. The association of many neurodegenerative diseases such as Alzheimer and Huntington with cellular aggregates underscores the biological relevance of aggregate clearance/protein folding. It has to be said that the exact mechanism behind the origin of these diseases remains a mystery. The observed structures may be only secondary features not causing neuronal death that ultimately leads to the pathology of

these patients. Under normal circumstances misfolded proteins are either re-folded or degraded by the UPS system.

3.6 Rad23

The yeast Rad23 and its orthologs have a distinct function in DNA repair and in the UPS [202]. Rad23 proteins share a highly conserved domain structure but a poor sequence homology [203]. The amino-terminal UBL domain binds to the rpn1 and rpn2 subunits of the 19S base [197,200]. In addition to the direct interaction with the proteasome, the human orthologs of Rad23 also interact with another shuttle protein namely S5a [204]. The latter interaction however could not be confirmed in yeast [197]. Rad23 proteins contain two UBA domains, one at the carboxy-terminus and one in the middle of the protein that have affinity for lys48 polyubiquitin trees [205-207]. The carboxy terminal UBA domain harbors an intrinsic stabilization signal at the very carboxy-terminal part that prevents 26S-mediated degradation [208]. All these characteristics (substrate and proteasome binding) make the Rad23 proteins ideal shuttle-proteins to deliver substrates (marked by lys48 polyubiquitin chains) to the 26S proteasome [196,209]. They, most likely, protect polyubiquitylated proteins from premature deubiquitylation and deliver targets at the 19S cap. Other identified shuttles of the proteasome also contain the UBL/UBA domain structure. A potential redundancy between these shuttles might explain the relatively mild phenotype of rad23 mutants in *S.cereviseae*. Yeast cells mutated in both dsk2 and rad23 are temperature sensitive possibly due to a spindle pole body duplication problem [210]. On the contrary, in *S.pombe* Rhp23 (rad23 ortholog) single mutants are defective in NER, affected in protein degradation and have a delay in the G2 phase of the cell cycle [211].

Rad23 in mammals

In mammals two orthologs of the rad23 gene have been identified (HR23A and HR23B) [66]. In some species e.g. mouse and human even a third genomic copy can be found. This latter copy is considered to be a pseudo gene since no functional RNA has been identified yet. Interestingly, despite homology in the coding sequence the genomic organization in this third copy is not conserved between mice and human, suggesting that these triplications were evolutionary separated events.
K.O. mice of both HR23A and HR23B have been created [96,212]. The mHR23B-/- mice display a severe phenotype. The number of newborns is extremely low in comparison to the expected Mendelian birth ratio. Animals that do get born are smaller, have facial dismorphologies and the males are sterile [212]. The number of K.O. embryos reduces gradually during uterine development starting from day 12.5-13.5. Embryos at E13.5 are already smaller and anemic. In sharp contrast, HR23A-/- mice appear normal and do not display an apparent phenotype [96]. The double-crossing of HR23A-/- and HR23B-/- is lethal beyond day E8.5. HR23B K.O. with only one HR23A allele (HR23A+/-HR23B-/-) are embryonically lethal as well. Furthermore at day 13.5 embryos with this genotype are more anemic in comparison to HR23B knock out embryos.

Removal of only one rad23 gene (i.e. the single HR23A or HR23B K.O. mice), does not result in a NER-deficient phenotype. However, cells derived from day 8.5 double knock-out (DKO) embryo's do display a NER deficiency [96]. This suggests that for NER HR23A and HR23B are

interchangeable. The severe phenotype associated with HR23B, strongly suggests that HR23B has additional functions besides NER, different from that of HR23A. At least some of these functions can be partly rescued by HR23A. One explanation for the difference in phenotype and the failure of HR23A to rescue the lack of HR23B could be a difference in expression. In fibroblasts HR23B is ten times more abundant in comparison to HR23A [213]. Recently we observed that in cultured cells knocking-out one of the Rad23 orthologs resulted in a concomitant increase of the other (non published observations), suggesting a partly compensating response. However, other explanations such as a different substrate specificity of the different homologs should not be ruled out. It will be difficult to identify the diverse profiles of substrate recognition of the different HR23 proteins by using classical approaches (IP, GST-pulldown or yeast two hybrid screens) since the affinity of UBA domains for ubiquitin or poly-ubiquitin trees in general is not very high or only transient. As a matter of fact the need for proteasomal shuttles has only been revealed for a few substrates. In human cells it has been shown that HR23A and HR23B are involved in p53 degradation [214,215]. Conflicting evidence has been reported towards this issue; the first study report states that knock down of HR23A and HR23B results in an increase of p53 while the other claims an acceleration in p53 degradation, illustrating the difficulty to determine the effect of either HR23A and B on degradation of specific substrates.

Another shuttle of the 26S proteasome namely Dsk2 is duplicated in the mammalian system as well [216]. In addition to a variety of enzymes involved in ubiquitylation and deubiquitylation, also a group of distinct shuttling proteins contribute to the whole spectrum of the complex regulation of specificity and subsequent fate of the ubiquitylated polypeptides. The need of many distinct shuttles is unclear, explanations are differences in substrate specificity or expression pattern of these shuttles. Different binding partners could also clarify the need of many shuttles. For example, HR23B has been found to interact with peptide:*N*-glycanase (PNGase) a protein involved in the cleavage of oligosaccharides from glycoproteins [217,218]. PNGase functions in the endoplasmic reticulum associated degradation (ERAD) pathway that is involved in for instance the proteolysis of unfolded/misfolded proteins [219]. Both Rad23 and Dsk2 in yeast cells function indeed in the ERAD pathway [220]. Rad23 in complex with PNG1 regulates turnover of glycoproteins suggesting that substrate specificity of the proteasomal shuttles is at least partly determined by the complex partners [221].

3.6.2 *Rad23 in cell cycle regulation*

There is an evident proliferation problem associated with the level of Rad23 protein. Moreover the amount of Rad23 protein fluctuates throughout the cell cycle. Fission yeast cells mutated in rhp23 have a delay in G2 and wild type strains display low levels of Rhp23 protein in S phase [211]. In addition hHR23A levels have been shown to vary throughout the cell cycle (low during S phase as well) [222]. Although, cells deficient in HR23A do not display any proliferation problem, HR23B-/- cells (mouse embryonic fibroblasts) do (unpublished results). The level of HR23B drops in cells during S phase as well, and the protein appears absent from condensed chromosomes [223] (and personal observation), moreover the protein redistributes from a more nuclear localization to a more cytoplasmic localization during different stages of the cell cycle. The total number of dividing cells, as

measured by BrdU incorporation appeared to be approximately 50% lower compared to wild type cells (unpublished results). However, brief cell cycle analysis did not reveal an apparent block in a specific phase. More research is necessary to unravel the proliferation problem of HR23B-/- cells. Mouse cells that lack both HR23A and HR23B suffer from a more severe proliferation problem that could be overcome during extreme long culturing [96] suggesting that additional adaptation is necessary to allow cell cycle to proceed "normally" in these cells.

3.6.3 *The UPS function versus the NER function*

In NER the Rad23 proteins function in lesion recognition as part of a complex together with XPC/Rad4 and CEN2 [203]. In both yeast and mammals Rad23 proteins protect Rad4/XPC form 26S-mediated degradation. Cells that lack Rad23 proteins indeed have lower levels of Rad4/XPC [96,120]. The mechanism of this protection remains unclear and seems to oppose to its role in the UPS. A possible explanation is the difference in affinity of HR23 proteins for XPC and the affinity of the 19S for XPC. Actually, XPC is probably not recognized as a target for degradation by the 19S proteasome. Moreover it is not clear if HR23 proteins in complex with XPC are still capable of binding to the 19S cap. An alternative explanation of XPC stabilization would be the prevention of its ubiquitylation that presumably would lead to its degradation. Overexpression of Rad23 indeed has an inhibitory role on degradation [224,225]. However, the reported ubiquitylation of XPC is not associated with degradation [118]. More research is necessary to understand the Rad23-dependent regulation of XPC/Rad4 levels. The UBL domain but not the UBA domains of Rad23 is essential for its function in repair, suggesting that the interaction to the 19S is necessary to fulfill its task in NER [226,227]. Over expression of Rad4 in a Rad23 null background partially rescues the UV sensitivity of Rad23 mutants in yeast whereas mouse mutant cells that lack both HR23A and HR23B could be rescued [96,122]. A difference that is most likely explained by the mild sensitivity of HR23A and HR23B DKO cells in the mammalian system. The (partial) rescue indicates that the improper functioning of Rad4/XPC causes some if not most of the sensitivity of cells lacking Rad23. In living cells XPC fails to significantly immobilize on damaged DNA in HR23A and HR23B DKO cells (chapter 6). In addition, *in vitro* HR23B or HR23A increases the affinity of XPC for damaged DNA [121]. So apart from the stabilization of XPC/Rad4 Rad23 proteins are essential for a proper recognition or immobilization of XPC, which is essential for GG-NER. Extreme over-expression of XPC in the absence of Rad23 proteins might stimulate NER somewhat but for a fast and efficient initiation of NER Rad23 proteins are essential.

3.7 uH2A

Histone ubiquitination is one of the first discovered ubiquitination events and one of the first known histone modifications [228]. They are the most abundant monoubiquitin conjugates in higher eukaryotes though their function is not entirely understood. In yeast H2B (lys123) ubiquitylation is the dominant form, while in mammals H2A (lys119) ubiquitylation is more abundant, although H2B (lys120) ubiquitylation can be found as well. The amount of histone ubiquitylation varies per species and per tissue. Roughly, 5-15% of H2A and 1% of H2B is ubiquitylated in mammals and 10% of H2B in yeast. Yeast lys123H2B mutants have mitotic and meiotic defects illustrating the necessity of this histone modification for proper cell division [164]. Histone H2B ubiquitylation (uH2B) is mediated by

the Rad6 E2 conjugase [164] with or without the E3 ligase Bre1 [229,230]. In yeast, uH2B is enriched in transcriptionally active chromatin and coincides with the elongating RNAPII [231-233]. Rad6-mediated H2B ubiquitylation and deubiquitylation is indeed involved in transcription activation [234-236].

Yeast lys123H2B mutants are defective in activation of the Rad53 checkpoint after treatment with DNA damaging agents. Moreover, they fail to arrest upon UV- or MMS-induced DNA damage [163,237]. Interestingly, these mutants are not directly sensitive in a one dose UV survival experiment [164]. Moreover, H2B ubiquitylation participates in the stabilization/formation of DSBs during meiosis [238]. We recently showed the increase of uH2A upon UV-induced DNA damage. This ubiquitylation event is strictly dependent on functional NER, moreover we showed that ATR but not ATM is necessary for UV-induced histone ubiquitylation as well. In summary this strongly suggests that histone ubiquitylation plays an important role in the DNA damage response (DDR).

Strikingly, in mammals it is still not fully understood what the exact function is nor what the trigger is for this ubiquitylation. No direct correlation of uH2A between hetero- or euchromatin has been found. Both the silenced X chromosome and the XY body in meiosis are enriched in uH2A [239,240]. Recently, a role of uH2A in polycomb gene silencing has been shown [239,241,242]. It is however unknown if all the uH2A present in the chromatin is functioning in polycomb-mediated gene silencing. H2A ubiquitylation involved in polycomb silencing is mediated by the PRC1 complex that contains three E3 ligases namely: Ring1A, Ring1B and Bmi1. *In vitro* analysis revealed that Ring1b is the actual catalytic subunit, although both Ring1A en Bmi1 contribute to H2A ubiquitylation *in vivo* [239,243]. Mice deficient in Ring1b suffer from cell cycle inhibition and arrest during gastrulation indicating that Ring1b is essential [244]. The E2 Rad6, involved in H2A and H2B ubiquitylation is duplicated in evolution. These two enzymes are most likely redundant except for their roles in gametogenesis [245,246]. Histone H2B ubiquitylation is mediated by the human homologue of Bre1 [247].

Ubiquitin involved in histone modification is in a dynamic equilibrium with other ubiquitin pools [248] suggesting that there is a DUB for ubiquitylated histones. Recently this DUB was indeed identified (Elisabetta Citterio unpublished results). Next to enzymes involved in the (de)ubiquitylation of histones it will be of interest to see if there are proteins specifically binding to ubiquitylated histones. Thereby bridging possible downstream effectors of histone ubiquitylation to the chromatin.

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Chapter 4

DNA damage:
Lets get ubiquitylated.

DNA damage: Lets get ubiquitylated.

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Ubiquitin and ubiquitylation have acquired increasing attention in many molecular biological research areas, ranging from proteasomal degradation to signaling and regulation of DNA damage responses [1]. The Nobel Prize awarded in 2004 to pioneers in this field further boosted the interest in the covalent attachment of this small polypeptide to an overwhelming number of different proteins. Within this 'ubiquitin era' several new branches on the growing tree of ubiquitin modifications have sprouted – one of these being the DNA damage response (DDR) factors where many proteins need to be activated or altered in a highly orchestrated manner.

To accommodate the rich spectrum of damage types each cell is confronted with, as well as their varying localization and timing during the cell cycle a complex array of genome maintenance mechanisms have evolved, including DNA repair pathways and damage signaling systems [2]. Classically, nucleotide excision repair (NER) deals with bulky lesions while base excision repair (BER) is mainly involved in simple sugar and base modifications and single strand breaks. Homologous recombination and non-homologous end joining repair double strand breaks. Mismatch repair handles coding errors arising during DNA replication. Replication forks that become stalled on DNA lesions are resolved by either template switching and recombination, or by temporarily invoking the action of alternative polymerases to perform translesion synthesis. Similarly, transcribing RNA polymerases require specialized functions to overcome blockage at DNA lesions. Obviously, delicate adjustment and coordination of repair, transcription and replication are essential as well as close interaction with the cell cycle checkpoints and emergency responses such as apoptosis to manage genomic stress and its complex response.

The different DDR pathways collectively counteract the biological consequences of genotoxic insults. However, 'every advantage has a certain disadvantage' (citation after J.Cruijff, former Dutch soccer player), intrinsic properties of part of these DDR enzymes which directly act on the DNA template may perturb genetic-homeostasis by uncontrolled actions. Certain repair/genome activities potentially may interfere with the DNA metabolism under non-challenging conditions and need to be restricted in their action. An example is given by the specialized DNA polymerases dedicated to translesion synthesis (TLS), which are optimised for bypassing bulky lesions during DNA synthesis, however at the expanse of their fidelity as compared to processive polymerases. Obviously, a too big contribution of these polymerases to normal replication would lead to enhanced mutagenesis, thus action of these polymerases should be restricted and only being recruited to replication forks when lesions are encountered. In addition, DNA damage recognition proteins have affinity for certain lesions but usually for undamaged DNA as well, creating a potential hazardous situation. Post-translational modifications such as ubiquitylation have turned out to play a key role in this regulation, both to control and to achieve swift adaptation. Several, strategies are applied to control fortuitous or illegitimate actions, ranging from relocalization, to altered activity and even proteolytic degradation to terminate their action. Their evolutionary benefit is obvious, given the large number of critical steps in genome maintenance that are regulated in this manner (see fig. 1). Here we

will discuss recent progress in the field of ubiquitin-related modification in the DNA damage response, by summarising a few key publications.

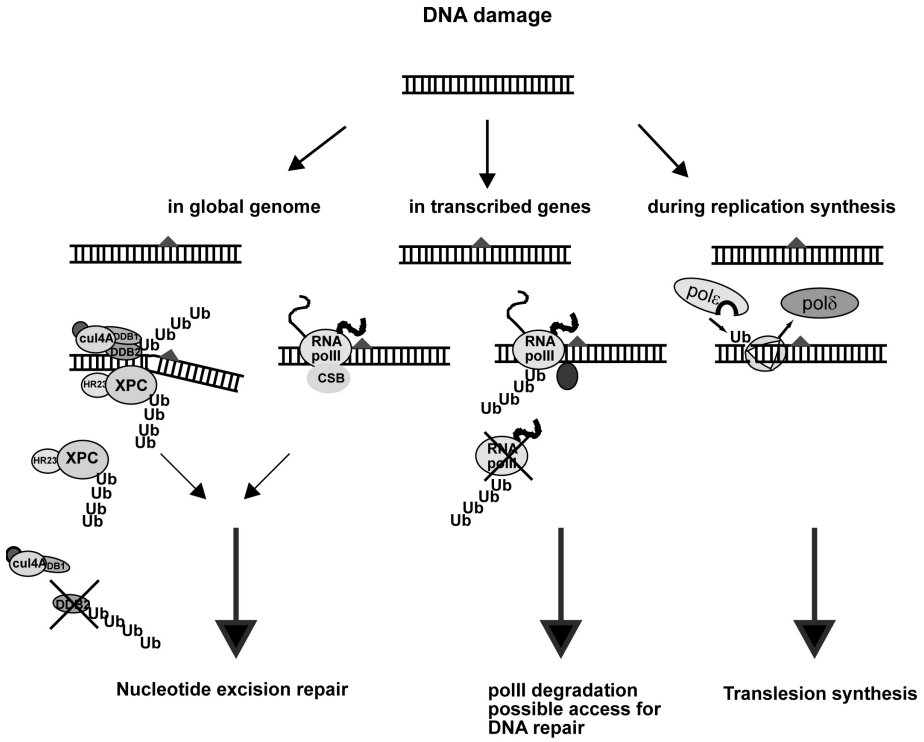

Fig 1 Some of the described-UV induced ubiquitination events.
An UV-induced DNA damage in the non-transcribed part of the genome and in the non-transcribed strand is typically recognized by the XPC/HR23B/Cen2 recognition complex with the help of the UV DDB complex. UV-DDB resides in a supercomplex containing the E3 ligases cul4a and roc1 and the cop9 (CSN) signalosme. The CSN displays deubiquitination activity. Upon UV, the UV-DDB complex dissociates from the CSN, DDB2 gets poly-ubiquitinated and binds to the DNA damage. This mediates the binding of the XPC complex which is poly-ubiquitinated by the UV-DDB E3 ligase activity as well. After subsequent recruitment of TFIIH and other NER factors, XPC and UV-DDB dissociate from the DNA. Free poly-ubiquitinated DDB2 is degraded whereas poly-ubiquitinated XPC is not. Lesions induced in the transcribed strand are mostly recognized by the stalled RNA polymerase. With the help of the CSA and CSB proteins the TFIIH and other NER factors are recruited resulting in a repaired lesion. However, upon long stalling RNApolII is poly-ubiquitinated by the E3 ligase Rsp5 and subsequently degraded. Un-repaired- or lesions induced in the S phase of the cell cycle cause a stalling of the DNA polymerse. If so the PCNA clamp becomes mono-or poly-ubiquitinated. Many TLS polymerases, including polymerase eta contain an ubiquitin interacting domain that faciliates the interaction of monoubiquitylated PCNA and the polymerase.

The Ssl1/p44 subunit of TFIIH is an ubiquitin ligase.

Ubiquitin conjugation to substrates is catalyzed by a three-step enzymatic mechanism. First the 'E1 ubiquitin activating' enzyme transfers ubiquitin in an ATP-dependent manner to a subsequent 'E2 ubiquitin conjugating' enzyme that catalyses the covalent attachment of ubiquitin, either with the help of the 'E3 ligase' (RING domain, subclass) or by transferring the ubiquitin to the E3 (HECT domain, subclass) after which the E3 conjugates the ubiquitin to the substrate [3]. Ubiquitin conjugation may elicit widely different actions on the protein substrate, such as refolding, or remodelling, altering enzymatic action, influence intracellular sorting as well as breakdown by the ubiquitin proteasome system (UPS).

Among the recent publications that integrate the DNA damage response with the UPS, the identification of an ubiquitin ligase activity (E3 ligase) within the transcription/repair factor TFIIH by Takagi and colleagues [4] is an intriguing and as yet enigmatic finding. The TFIIH complex is composed of ten subunits [5,6]. It is essential for RNA polymerase II (RNAPII) initiation and for NER [7], it plays a role in RNA polymerase I driven transcription [8,9] is involved in nuclear receptor-mediated transcriptional regulation [10], and likely functions in cell cycle regulation [11]. The spectrum of intrinsic enzymatic activities comprising ATPases, helicases and kinase functions required to achieve TFIIH's multifunctionality has now been broadened to ubiquitin ligase, carried out by the core TFIIH subunit Ssl1 (the yeast ortholog of mammalian GTF2H2 hereafter called p44).

Within TFIIH both Ssl1/p44 and the CAK components Tfb3/MAT1 contain the consensus amino acid sequence indicative for E3 RING ligases [12]. Takagi et al (2005) showed that 'core' complex (missing the trimeric CAK component) harbours indeed E3 ligase activity. In addition, isolated recombinant Ssl1 possesses E3 ligase activity, although reduced as compared to the intact TFIIH complex. Apparently, presence of the other TFIIH core components especially the Tfb4 (p34) subunit stimulate this function. Mutation studies showed that E3 activity depends on an intact RING finger domain (RNF) of Ssl1.

This E3 ligase activity appeared not to be essential for transcription, since cells carrying mutated *Ssl1* can still grow, although at a lower rate and in a temperature-sensitive fashion. However, these mutant cells showed increased sensitivities towards the DNA damaging agents UV and MMS, suggesting a role of the Ssl1 ligase in the cellular response to DNA damage. Surprisingly, the authors did not observe a clear necessity of ssl1-E3 ligase activity for NER *in vitro*. This observation/notion is contrary to the expectation, by the known pivotal role for the entire TFIIH complex in this process [13]. This apparent contradiction may reflect the limitations of the applied *in vitro* NER assay. A reduction of the NER efficiency by less than 50% (as observed by Takagi and co-workers in the survival assays) can still cause a severe carcinogenic condition, as presented by mice and humans lacking the GGR-specific *DDB2(XPE)* gene [14-16]. Note that purified UV-DDB (DDB1 and DDB2/XPE complex) does not significantly enhance mammalian NER capacity *in vitro* [17]. On the other hand, Takagi et al found evidence for an indirect action of the TFIIH-associated E3 on NER-proficiency: transcriptional profiling of E3-deficient *Ssl1* mutants Takagi showed a reduced DNA damage-dependent (MMS) transcriptional regulation of certain DNA repair genes. The target substrate for the newly identified E3 is currently unknown. Nor the direct consequence of interference with this enzyme since the prime course for the change in the transcriptional response was not uncovered.

It is not yet clear how this novel E3 ligase activity fits in other links between TFIIH and ubiquitin. For instance, the mammalian XPB (Ssl2 in yeast) helicase can interact with the AAA-ATPase rpt6 (Sug1) a subunit of the proteasomal 19S complex on the basis of a yeast dual hybrid screen [18]. Over-expression of rpt6 show a strong RNA synthesis inhibition and chromatin collapse suggesting a role in transcription [18]. In yeast rpt6 (Sug1) and rpt4 (Sug2), as subunits of the 19S particle, are indeed involved in transcription elongation [19,20]. The 19S particle is mostly known for its function in the ubiquitin-dependent 26S-mediated proteasomal degradation. Polyubiquitylated proteins are recruited to be de-ubiquitylated, unfolded and translocated into the 20S catalytic particle by the 19S particle after which actual proteolysis is performed [3]. As yet, the mechanistic link between the role of the 19S particle and the role of TFIIH in transcription is not well understood. TFIIH is essential for initiation whereas the 19S particle rather appears to be involved in elongation.

In yeast, the 19S components rpt6 and rpt4 facilitate the link between H2B ubiquitination and H3 K4 and K79 di- and tri-methylation necessary in discriminating active and inactive genes. Yeast cells mutated in rpt6 fail to methylate K4 and K79, although H2B ubiquitylation stays intact [21]. Yeast cells carrying the H2B ubiquitin mutant (htb1-K123R) lack the UV-induced phosphorylation of rad53, a protein involved in postponing DNA replication after UV treatment. This links H2B ubiquitylation to checkpoint signaling after UV-induced DNA damage [22]. Very recently, we indeed identified a novel DNA damage dependent ubiquitylation of histones upon UV treatment, an increase in ubiquitylated H2A that most likely plays a role in damage signalling by its ATR-dependency (Bergink et al, Genes and Development, 2006, *in press*). Histone ubiquitylation is performed by the E2 conjugase activity of the Rad6/Bre1 complex. Rad6 (the first identified link between DNA damage response and ubiquitylation [23]), also needed for phosphorylation of rad53, is the E2 responsible for ubiquitylating of the polymerase clamp PCNA in the control of TLS (see below). An additional role in the DNA damage response is likely since Rad6 is the canonical E2 enzyme for histone ubiquitylation [24]. It will be interesting to investigate whether the change in expression pattern upon DNA damage that was suggested by Tagaki et al, is indeed associated by a change in the histone makeup.

Within a highly speculative model: DNA damage may cause the TFIIH E3 ligase activity to alter the ubiquitylation status of H2B. Subsequently, the 19S particle will be recruited via the XPB-Rpt6 interaction, which will mediate the methylation of K4 and K79 of H3 leading to DNA damage-induced transcriptional changes, as observed by Takagi et al. However, other scenarios can be envisaged as well. The authors (Takagi et al) mention that it will be of interest to investigate whether mutations in the Ssl1 ortholog might be associated to human diseases as was found for other TFIIH mutations [25]. It should be noted however, that in humans the p44 locus is duplicated [26] making it extremely unlikely that patients will exist suffering from impaired p44 E3 ligase activity assuming that such a mutated p44 will be recessive.

DNA damage-dependent ubiquitylation of RNA polymerase II

Another target for ubiquitylation implicated in transcription and known to be differentially modified as a response to DNA damage, is RNAPII itself [27]. DNA damage in the transcribed strand of active genes causes stalling of RNAPII resulting in recruitment of the DNA repair machinery. Long term stalling, either by DNA damage [28,29] or by other means of elongation pausing [30] induces polyubiquitylation

and subsequent degradation by the 26S proteasome of the largest subunit of RNAPII (Rpb1). Polyubiquitylation of Rpb1/RNAPII is dependent on the yeast gene Def1. Although Def1 is in complex with the transcription-coupled repair gene Rad26 (yeast counterpart of CSB) the latter is not essential for Rpb1/RNAPII polyubiquitylation in yeast [27,31]. In contrast, the DNA damage-induced ubiquitylation of RNAPII is dependent on both CSA and CSB in humans [32]. Both the E2 (Ubc5/Ubc4) and E3 (Rsp5) were identified as the enzymes that catalyse RNAPII ubiquitylation [28,30]. Polyubiquitylation of Rpb1 was more efficient on the immobilized ternary elongation complex consisting of DNA template, RNAPII and nascent RNA than on free RNAPII or promoter-bound polymerase [30]. This difference is determined by the phosphorylation status of RNAPII's C-terminal repeat domain (CTD). The ubiquitylation of RNAPII is inhibited by phosphorylation of the serine 5 residue CTD, a specific phosphorylation associated with early (promoter-bound) transcription. Ser2 phosphorylation of the CTD, associated with elongating RNAPII, is however competent/adequate for ubiquitylation. In contrast, ser5-phosphorylated RNAPII ubiquitylation after cisplatin-induced DNA damage has recently been shown as well, possibly explained by an alternative (non lys48) ubiquitin tree [33]. Altogether, this cooperative action of distinct post-translational modifications appears to allow proteasome targeting of RNAPII when elongation is hindered but prevent unwanted breakdown at the transcription-initiation stage and thus provide more selectivity.

Ubiquitylation of damage recognition factors

Recently, two independent reports describe the UV-dependent ubiquitylation of the DNA damage sensor XPC [34,35]. This ubiquitylation is mediated by the UV-DNA damage-binding (DDB) complex, since polyubiquitylation did not occur in cells from XP group E patients (mutated in one of the complex partners, DDB2) while it is not affected in any of the other NER-deficient XP fibroblasts [35]. The heterodimeric complex UV-DDB consists of the DDB2 (p48) and DDB1 (p127) subunits [36] and stimulates XPC-mediated recognition of DNA lesions [37]. UV-DDB was found to be part of a larger complex, which harbours ubiquitin ligase activity, containing cullin4A, Roc1 and all the components of the COP9 signalosome (CSN) [38]. This CSN complex possesses de-ubiquitylation activity (DUB) normally counteracting (poly) ubiquitylation activity [38] by the Roc1 subunit, the actual E3 ligase [39]. After binding to UV-damaged DNA the CSN dissociates from DDB2-E3 complex, thereby releasing the ubiquitylation suppression. Subsequently, the XPC heterotrimeric complex (XPC, Homolog of Rad23 and cen2 [40,41]) is recruited, followed by the polyubiquitylation of both DDB2 and XPC. Although both recognition complexes are polyubiquitylated when bound to lesions, the consequences of this event are remarkably different for each. Polyubiquitylated DDB2 dissociates from the damage and is rapidly degraded, but remarkably, polyubiquitylated XPC is not [35]. Instead, polyubiquitylated XPC displayed an increased affinity for both damaged as undamaged DNA. XPC apparently remains modified after release from the repair site, since polyubiquitylated XPC was found to exist both as DNA-bound as well as not bound to DNA. A higher affinity of polyubiquitylated XPC for damaged DNA is thought to boost the efficiency of the entire NER reaction.

UV-induced post-translational modification of XPC is further complicated by SUMOylation, on top of polyubiquitylation [34]. Contrary to ubiquitylation this SUMOylation is dependent on the further progression of the NER reaction, since in fully NER-defective XP-A cells it does not occur.

Here, polyubiquitylated XPC is rapidly degraded, suggesting that SUMO-1 modification protects XPC from proteasomal processing. The target residues in XPC of the ubiquitylation and sumoylation and the identity of the polyubiquitin branches are not known yet. Polyubiquitylated XPC species were found to reduce at four hours after UV irradiation [35], which matches with the time required to repair most of the UV-induced 6-4 photoproducts (6-4PP), the main target for early GG-NER. This suggests that XPC-ubiquitylation is most abundant when a maximal GG-NER activity is required. Since polyubiquitylated XPC was found a few hours after UV-irradiation, together with the notion that this has increased affinity for DNA lesions, this suggests a short-term adaptation strategy by making XPC temporarily more competent to act in NER. This response is superposed on a background level of repair of the major photoproduct, the cyclobutane pyrimidine dimer (CPD), which occurs at a much slower rate [42].

How sumoylation temporarily protects XPC from the proteasomal degradation invoked by such a strong polyubiquitylation signal, remains to be elucidated. Previously, it was shown that XPC complex partners HR23B (or HR23A) protect XPC from proteasomal degradation [43] and also enhanced XPC's repair capacity *in vitro* [44] and *in vivo* (our own unpublished observations, chapter 6). Recently, Nishi and coworkers showed that binding of Cen2 enhances repair-efficiency of XPC as well [45]. Apparently, a complex interplay between SUMOylation, ubiquitylation and the binding partners of XPC is required to determine the stability, affinity and action of XPC on DNA lesions. One could speculate, that damage-dependent post-translational modifications are necessary to suppress illegitimate binding to different types of DNA-structure aberrations, to which this protein has intrinsic affinity [46-48], in order to avoid futile initiation of the NER reaction.

The delicate regulation of this NER initiator extends beyond post-translational modifications, as XPC expression appears to be controlled by a complex set of different regulatory mechanisms at the transcriptional level in a p53-dependent fashion [49,50]. Clearly, cells need to keep critical control on the level of XPC; further studies on the behaviour of this protein (and defined mutants of it) in living cells are expected to provide more insight in how all these mechanisms are regulated and coordinated in space and in time.

Stalled replication forks and ubiquitylation

Perhaps the best-explored DNA damage-dependent ubiquitylation is the differential modification of the proliferating cell nuclear antigen (PCNA), which forms a clamp on the parental DNA to support the action of DNA polymerases. Lysine 164 of PCNA can be SUMOylated, monoubiquitylated or polyubiquitylated [51,52]. The polyubiquitin tree on PCNA is mediated by the lys63 of ubiquitin, that has been suggested to function in DNA repair [53,54]. Polyubiquitylated PCNA in yeast is functioning in error-free, damage-avoiding replication, whereas monoubiquitylated PCNA leads to error-prone translesion synthesis. SUMOylation of PCNA on lys164 is likely involved in suppressing unwanted recombination during S phase by the recruitment of the Srs2 helicase [55,56].

The interplay of two ubiquitin-conjugating complexes, RAD6 and MMS2/UBC13 and two ubiquitin ligases, RAD5 and RAD18 determine the ubiquitylation status of PCNA [23,52,57-60]. RAD6 and RAD18 are essential for DNA damage-induced monoubiquitylation of PCNA. Upon monoubiquitylation MMS2/UBC13 and rad5 mediate PCNA polyubiquitylation [52]. Although

monoubiquitylated PCNA has also been observed in human cells [52,61], polyubiquitylation and sumoylation have not been reported in mammals. Monoubiquitylation of PCNA is essential for polymerase η-mediated translesion synthesis (polymerase η (polη) is one of the TLS polymerases, able to efficiently bypass UV-induced lesions). In fact polη, but not polymerase δ, specifically binds monoubiquitylated PCNA [61,62]. Most, if not all polymerases, have a PCNA-interacting domain, whereas only some polymerases harbor different variations of ubiquitin-interacting domains as well [63]. This suggests that PCNA ubiquitylation forms the switch between normal synthesis polymerases and lesion bypass polymerases. Differential modifications of PCNA facilitate the hand of transactions of the different polymerases.

PCNA has yet another essential function in the damage response. During normal S phases Cdc10-dependent transcript1 (Cdt1) is degraded to ensure that re-replication does not occur. Cdt1 is polyubiquitylated on the chromatin by the Cul4-DDB1-Roc1 complex. Although Cdt1 binds DDB1 directly, polyubiquitylation is initiated by the binding of Cdt to PCNA [64,65]. Upon DNA damage Cdt1 is degraded as well, possibly to inhibit entry into S [66,67]. For DNA damage-induced Cdt1 degradation the PCNA interaction is essential as well [65,68]. This shows that posttranslational modifications of PCNA or those mediated by PCNA play an essential role in the cellular defence response towards genotoxic insults.

DNA damage-induced signalling responses and ubiquitin

This destructive DDR by the ubiquitin proteasome system (UPS) of DDB2 and Cdt1, i.e. degradation induced by genomic insults, was also observed in two other examples implicated in DNA damage response; p21 and Chk1 both are involved in DNA damage-induced cell-cycle-arrest. At relatively low doses of UV p21 is degraded in an ATR-dependent fashion. Failure to degrade p21 results in repair inhibition [69], although some reports state that p21 degradation does not occur after genotoxic stress [70]. P21-deficient cells are UV-sensitive and defective in a step downstream of PCNA [71]. More recently, DNA damage-dependent degradation of activated Chk1 has been reported. Upon DNA damage the checkpoint regulator Chk1 is activated (by phosphorylation) by the ATM (ataxia-telangiectasia mutated) and Rad3-related protein (ATR) kinase. Activated Chk1 is thought to quickly distribute the notion of genomic stress pan nuclear by a swift redistribution throughout the nucleoplasm, thereby signalling to replication controllers to temporarily halt S-phase progression [72,73]. In time, the alarm bell (activated Chk1) is degraded, possibly to allow resumption of S phase, when lesions are removed [74]. The complex ubiquitylation events in response to DNA damage suggest that a malfunctioning ubiquitylation machinery would reduce survival after DNA damage. Indeed cells that fail to ubiquitylate either by the usage of temperature sensitive E1 or chemical inhibitors fail to repair UV induced lesions efficiently [75].

DNA double strand breaks and ubiquitylation.

Other ubiquitylation events important for an appropriate DNA damage response have been identified, one of which is the Fanconi anemia complementation group D2 (FANCD2) protein. The FANCD2 protein is monoubiquitylated upon DNA damage, this process requires the checkpoint kinase ATR [76-78], which was shown to stimulate homologous recombination repair [79]. One of the components of

the FA-protein complex, FANCL, possesses a RING-finger-like plant domain (PHD) that is essential for FANCD2 mono-ubiquitination *in vivo* [80]. Confusingly, the BRCA1/BARD hetero-dimer was also found to interact with FANCD2 [81] and also possesses strong E3-ligase activity, but this activity seems not to be necessary for FANCD2 mono-ubiquitylation. In stead BRCA1/BARD is required for the subsequent FANCD2 translocation to RAD51 containing foci [81,82]. FANCD2 deubiquitylation mediated by USP1 deactivates the FA pathway [83].

Other proteins involved in the repair of double strand breaks are ubiquitylated as well. BRCA2 levels are regulated by ubiquitylation in a DNA damage-dependent manner [84]. Similarly, ubiquitin- mediated breakdown of Ku70 precedes apoptosis [85]. Moreover both the 19S and the 20S catalytic core of the 26S proteasome are recruited to DSB bound repair proteins and are essential for the repair of DSBs [86].

Different outcomes of ubiquitylation

Many ubiquitylation events and other posttranslational modifications within the DNA damage response as well as their functional implication remain to be discovered. The regulatory nature of ubiquitylation and the relative fast way to induce this process make it very likely that more DNA damage-induced ubiquitylation events will be reported in the near future. Importantly different consequences of ubiquitylation on DDR targets have been reported, such as: (i) the enhancement of the activity or affinity for substrate of a target protein (e.g. XPC); (ii) the switch between cellular pathways (e.g. PCNA); (iii) the termination of a process achieved either by reversing the modification of the protein of action or, more fundamentally, by degrading it. The latter can occur on proteins actively participating within a process, as illustrated by the UPS-mediated degradation of DDB2 and RNAPII, or after thereby terminating a certain response, for example on signal transducers, such as the degradation of Chk1.

Subcellular redistribution, transient interactions and expression levels are known handles to control the action of complex pathways. In the recent years a wealth of information has become available on cellular networks ranging from global transcriptome, interactome and comparable ‘ome’ analyses, providing a more holistic view on cellular homeostasis. Clearly, posttranslational modifications provide the cell an additional tool to control pathway flow in an even more dynamic manner. This appears to be particularly relevant to sudden emergency situations such as occasional high exposure to genotoxic agents. The examples of ubiquitylation of proteins involved in DDR, highlighted here, may reveal a general pattern of organization and coordination of activities by differential posttranslational modifications. Undoubtedly, studies on posttranslational ubiquitylation (dedicated proteomics) will help to unravel the regulation of this complex response; at the same time it is obvious that ubiquitylation is only part of a whole spectrum of posttranslational modifications that is used to establish pathway control and can only be properly interpreted on the background of phosphorylation, sumoylation and other posttranslational modifications that remain to be discovered.

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Chapter 5

DNA-damage sensing by xeroderma pigmentosum group C in living cells

DNA-damage sensing by xeroderma pigmentosum group C in living cells

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Summary
The global genome nucleotide excision repair (GG-NER) pathway removes numerous helix-distorting lesions from the genome, including UV-induced injuries. The initiator of GG-NER, XPC-hHR23B has an intrinsic high affinity for DNA, which is even greater for damaged DNA. The mechanism by which XPC-hHR23B locates DNA damages in the genome is not clear. Here, we report a study on the behavior of XPC in living cells. We generated a cell line that stably expresses at physiological level fully functional GFP-tagged XPC. High-resolution confocal imaging uncovered that XPC-GFP had an inhomogeneous distribution and a high probability to be located at chromatin. Photobleaching experiments combined with computer simulation suggested that the fusion protein is slowed down in its mobility by constantly associating to and dissociating from DNA. The locomotion of XPC was even further retarded when cells were treated with agents that change the DNA. Induction of UV-damages had the most pronounced effect on the mobility of XPC-GFP. The assembly rate of XPC-GFP at pre-steady-state into NER complexes is not dependent on temperature, whereas the disassembly is severely retarded at lower temperature, suggesting that an enzymatic reaction is required for the release of XPC. In comparison to other NER factors, XPC only shortly interacts (~ 2 min) with UV-lesions, arguing for a scenario of dissociation of XPC from the emerging NER complex before the full incision complex is assembled. Moreover, we uncovered a novel regulatory mechanism for XPC. Under unchallenged conditions XPC is continuously exported from and imported into the nucleus, which is impeded when XPC is required in the nucleus, *i.e* when NER lesions are present.

Introduction

DNA is constantly under attack by various metabolic and external factors, such as UV-light [1]. The integrity of DNA is ensured by multiple interwoven DNA repair systems. Nucleotide excision repair (NER) is one of the most versatile repair pathways, which removes a variety of lesions including UV-induced cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts ((6-4)PP) [2]. The biological significance of a functional NER is evident from the severity of clinical features seen in patients, suffering from inherited NER-deficient syndrome xeroderma pigmentosum (XP). Individuals carrying a mutation in one of the seven XP-genes (*XPA* to *XPG*) mainly exhibit cutaneous symptoms, including extreme UV-sensitivity and sun-induced pigmentation anomalies and most importantly an >2000 fold increase in skin-cancer [3].

The highly conserved NER-pathway, is a multistep 'cut-and-patch' process involving the concerted action of about 30 polypeptides. Two sub-pathways exist within NER, differing in mode of damage recognition [2]. The transcription-coupled nucleotide excision repair (TC-NER), focuses on transcription-blocking lesions located in the transcribed strand of active genes [4]. The global genome nucleotide excision repair (GG-NER) eliminates lesions located anywhere in the genome, where the XPC-hHR23B heterodimer serves as a damage detector assisted (for specific types of damage) by the UV-DDB complex [5-7]. Both sub-pathways funnel into the 'core'-NER', which comprises three additional steps, (i) open complex formation and verification, (ii) incision and (iii) DNA synthesis and ligation. The DNA helix around the lesion is melted by the transcription factor IIH (TFIIH) and this open DNA structure is stabilized by the replication protein A and the NER-specific factor XPA. These latter two proteins likely stabilize the open helix conformation and are essential for loading and proper orientation of the two endonucleases ERCC1/XPF and XPG will cut the damaged strand at some distance from the damage. The pre-incision complex was shown to contain TFIIH, XPA, RPA and the two endonucleases, but not XPC-hHR23B [8]. The last events in this multi-step repair procedure are filling of the gap by conventional DNA synthesis and ligation [9].

XPC-hHR23B is the first factor within the GG-NER pathway to bind to lesions, as was shown both *in vitro* and in intact cells [6,10]. Purified XPC-hHR23B complex displays high affinity for undamaged single and double stranded DNA [11,12]. However, competition experiments revealed a preferential binding of the isolated complex to DNA with various induced lesions [13]. It was shown that XPC-hHR23B even binds to small bubble structures with or without a lesion [14]. However, dual incision in an *in vitro* NER assay was only observed when damage was present in the bubble. Suggesting that after binding of the XPC-hHR23B complex to a helix distorted site, the presence of the injured base is verified by additional NER-specific factors prior to dual excision. This multistep procedure ensures a high safety level within the GG-NER pathway, allowing the NER reaction only to proceed when the proper (NER-specific) lesions are encountered. Recently, it has been shown that XPC is polyubiquitylated by the UV-DDB-E3 complex upon UV-irradiation [15]. The exact function of this XPC modification is not fully understood, but it has been suggested that polyubiquitylated XPC has a higher binding affinity for both damaged and undamaged DNA. In addition, recently also UV-induced sumoylation of XPC was reported, which requires both DDB2 and XPA [16].

It has been suggested that the early NER factors, such as XPC-hHR23B are capable of sensing sites that exhibit unfavorable configuration in terms of free energy [17], e.g. helical distortions

due to DNA damage. XPC-hHR23B might thus be probing the DNA helix for these (thermodynamic) instabilities. The XPC-hHR23B heterodimer has been shown to be able to induce a bend in DNA upon binding both at undamaged and damaged sites [18]. The helix of damaged DNA might accommodate the configuration that is induced by binding of XPC-hHR23B, more easily and thereby stabilizing the binding of the heterodimer to the lesion containing DNA. However, the manner in which XPC-hHR23B finds a lesion in the genome, is not clear. DNA binding proteins are thought to locate target sites by two possible mechanisms (reviewed in [19]), (i) proteins could slide along the DNA, i.e. a one-dimensional linear diffusion along the DNA contour, otherwise (ii) translocation of proteins might also occur through three-dimensional space, via diffusion and multiple dissociation/re-association events on the genome.

In order to study the dynamic nuclear distribution in time and space of the XPC protein and to determine how this protein is targeted to DNA lesions in the most relevant context, the living cell nucleus we tagged XPC with the green fluorescent protein (GFP). Using confocal microscopy and applying various photobleaching techniques we investigated XPC-GFP mobility in untreated cells. We were also able to follow the kinetics of the fusion protein within the GG-NER pathway upon damage induction by UV-light, as was previously done for the core NER components XPA [20], TFIIH [21] and ERCC1/XPF [22]. Surprisingly, both the mobility parameters and kinetic engagement of XPC-GFP in NER differs dramatically from the other core NER factors.

Results

Generation and characterization of stably XPC-GFP expressing cell line

To study the spatio-temporal distribution of XPC in live cells we tagged this protein with GFP. GFP with additional C-terminal His_6 and HA tags was fused in frame to the C-terminus of the XPC cDNA (Figure 1A). The fusion gene was stably expressed in SV40-immortalized fibroblasts derived from a XP group C patient (XP4PA-SV cells) carrying a 2 bp deletion in the *xpc* gene, creating a frameshift at position 1483 and the introduction of a premature stopcodon [23,24]. Stable XPC-GFP expressing cells were isolated in which the mean expression level was comparable to the level of endogenously expressed non-tagged XPC in NER-proficient fibroblasts (Figure 1A, compare lane 1 and 3). UV-survival experiments revealed that XPC-GFP corrected the UV-hypersensitivity of XP4PA-SV40 cells to the same level of UV-resistance as in NER-proficient cells tested in parallel (Figure 1B). The performance of XPC-GFP expressed at physiological relevant levels indicated that these cells are *bona fide* source to study the dynamic behavior of this damage-sensing factor. In the quantitative imaging experiments described below, we only use XPC-GFP expressing cells with a near normal level of XPC as judged by comparative immuno-fluorescence [20].

High-resolution confocal laser scanning imaging showed that XPC-GFP is predominantly nuclear in living cells, similar to previous immuno-staining [10,25]. However, only the live cell imaging revealed an inhomogeneous distribution (Figure 1C, left panel). Interestingly, XPC-GFP appeared to co-localize with the characteristic heterogeneous distribution of chromatin in inter-phase nuclei of cultured mammalian cells, visualized by co-expression of the chromatin marker H2B-YFP [26] (Figure 1C, middle and right panels). This indicates that XPC-GFP is omnipresent in nuclei and even enriched in more condensed chromatin areas. A striking association with the highly condensed M-phase

chromosomes was observed in living (Figure 1F) and in fixed cells [25], again contrasting to the distribution of other NER factors during mitosis, which appeared to be excluded from the condensed chromosomes (data not shown).

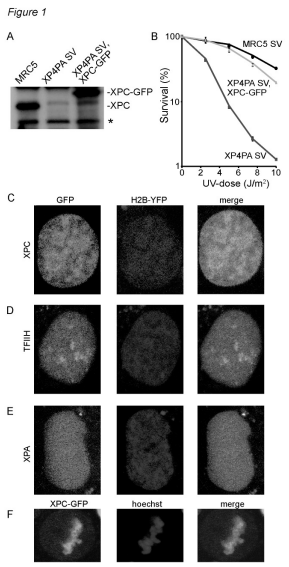


Figure 1. Characterization of the nuclear distribution of GFP tagged XPC, XPA and TFIIH in living cells.
(A) Immunoblot probed with anti-XPC polyclonal of WCE of MRC5 (lane 1), XP4PA SV (lane 2) and population of XP4PA SV cells stably expressing XPC-GFP (lane 3). The asterisk shows a background band that can serve as a loading control. (B) UV survival of MRC5, XP4PA SV and XP4PA SV cells stably expressing XPC-GFP. The log of the percentage of survival is plotted against the dose of UV-C light (J/m^2). (C) Confocal image of a cell stably expressing XPC-GFP (left panel) and transiently expressing H2B-YFP (middle). The merged image is shown in the right panel. (D) Confocal image of a cell stably expressing XPB-GFP (left panel) and transiently expressing H2B-YFP (middle). (E) Confocal image of a cell stably expressing GFP-XPA (left panel) and transiently expressing H2B-YFP (middle). (F) Confocal image of a mitotic cell expressing XPC-GFP. Left panel: GFP-fluorescence, Middle; hoechst staining of DNA, Right: merged image.

Nuclear mobility of XPC-GFP

The peculiar distribution of XPC-GFP suggests that this protein is bound to nuclear structures associated with dense chromatin and does not freely roam the nuclear space as the majority of the other NER factors do in the absence of high concentrations of NER lesions. To investigate the dynamic distribution of XPC-GFP and compare it with the mobility of other NER factors we applied photo-bleaching using different variants of FRAP (fluorescence recovery after photobleaching) [27].

FRAP experiments showed that the fluorescence recovery of XPC-GFP was surprisingly slow as compared to the nuclear mobility of GFP-XPA and TFIIH-GFP (Figure 2A) [20,21]. The effective diffusion coefficient (D_{eff}) was determined by fitting the FRAP curves to mobility simulations (see methods). The D_{eff} of various proteins, GFP, GFP-XPA, ERCC1-GFP/XPF and TFIIH-GFP was plotted against their predicted molecular size on a logarithmic scale (Figure 2B). For the majority of non-engaged NER factors (and non-tagged GFP) a linear relation between D_{eff} and molecular size was apparent, except for XPC-GFP. The calculated molecular mass of XPC-GFP including its binding partners hHR23B and Cen2 [28] is ~230 kDa, though its mobility is significantly slower than TFIIH-GFP (~500 kDa), almost twice the size of the XPC-containing complex. FRAP curves fitted best to Monte Carlo mobility simulations in which a relatively large fraction of XPC-GFP transiently interacts with a less-mobile nuclear component (data not shown). This slow mobility of XPC-GFP was

confirmed using a FRAP variant in which we monitor the entire nucleus (FRAP/FLIP, see M & M and [21], with even a 1.5 times longer redistribution time than TFIIH (Figure 2C). Surprisingly, the nuclear mobility of XPC-GFP was significantly slower when FRAP/FLIP was performed at 27 ^{o}C rather than at 37 ^{o}C (Figure 2D). This temperature shift did not effect the mobility of GFP-XPA, but also retarded TFIIH-GFP (Figure 2D), as was found previously and explained by its engagement in transcription [21]. However, transcription inhibition did not influence the mobility of XPC-GFP (Figure 3E) nor did ATP-depletion influence XPC-GFP mobility (Figure 3F). Fitting FRAP/FLIP data to virtual mobility plots resulting from Monte Carlo computer simulation [22], revealed that best fits were obtained when a large fraction (~90%) with a relative short binding period (~0.5 second) was simulated for 37 ^{o}C with an ~ 4 times increased binding time at 27 ^{o}C. This suggests that the release of the bound fraction to a nuclear structure is largely influenced by temperature. We conclude that nuclear mobility of XPC-GFP under non-challenging conditions is not solely determined by free diffusion, but is also (in part) derived from transient binding to a nuclear immobile structure.

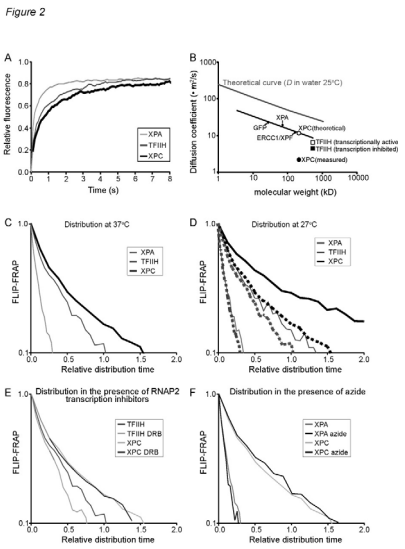


Figure 2. Dynamics of XPC-GFP, TFIIH-GFP and GFP-XPA
(A) Strip-FRAP analysis of untreated GFP-XPA (blue line), XPB-GFP (red line) and XPC-GFP (green line) expressing cells. (B) Calibration curve, log of the effective diffusion rate of several GFP tagged proteins is plotted against the log of predicted molecular weight. The predicted and experimentally obtained diffusion coefficients (*D*) of XPC-GFP are shown. (C) Simultaneous FLIP-FRAP analysis on GFP-XPA (blue line), XPB-GFP (red line) and XPC-GFP (green line) expressing cells at 37^{o}C. (D) Simultaneous FLIP-FRAP analysis on GFP-XPA (blue line), XPB-GFP (red line) and XPC-GFP (green line) expressing cells at 27^{o}C. Also the curves (dashed) obtained at 37^{o}C are shown. (E) Simultaneous FLIP-FRAP analysis on XPB-GFP (red lines) and XPC-GFP (green lines) expressing cells in the presence and absence of DRB, an RNAP2 transcription inhibitor. (F) Simultaneous FLIP-FRAP analysis on GFP-XPA (blue lines) and XPC-GFP (green lines) expressing cells in the presence and absence of Na-azide.

Effect on XPC-GFP mobility of various DNA altering agents

The, for a NER factor, observed atypical nuclear mobility might be explained by its previously reported DNA binding capacity [11]. Although XPC was shown to have a higher affinity for UV-damaged DNA [29], more recent evidence also indicated that variable (non NER-inducing) structural DNA alterations, including small loops bind XPC with a higher affinity than normal B-form helical DNA [14]. To test the possibility whether continuous probing of nuclear DNA retards XPC-GFP mobility we treated the XPC-GFP expressing cells with different agents that influence the DNA structure and subsequently determined the effect on XPC-GFP locomotion. To that aim we selected a number of agents that

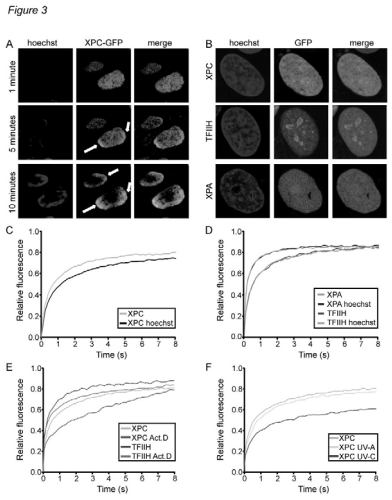


Figure 3. Effect of DNA alterating agents on XPC-GFP locomotion.

(A) Monitoring of the uptake of Hoechst33342 by XPC-GFP expressing cells at different time points. Left panel: Hoechst staining of DNA, Middle; GFP-fluorescence, Right: merged image. (B) Confocal images of a cell expressing XPC-GFP (upper panel), XPB-GFP (middle panel) or GFP-XPA (lower panel) stained with Hoechst33342. Left panel: Hoechst staining of DNA, Middle; GFP-fluorescence, Right: merged image. (C) Strip-FRAP analysis of untreated (light green line) and Hoechst33342 treated (dark green line) XPC-GFP expressing cells. (D) Strip-FRAP analysis of untreated (light line) and Hoechst33342 treated (dark line) XPB-GFP (red lines) and GFP-XPA (blue lines) expressing cells. (E) Strip-FRAP analysis of untreated (light line) and Actinomycin D treated (dark line) XPB-GFP (red lines) and GFP-XPC (green lines) expressing cells. (F) Strip-FRAP analysis of untreated (green line), UV-A light (light blue line) and UV-C light treated (dark blue line) XPC-GFP expressing cells.

directly affect the DNA structure, but known not to induce NER. We first tested the addition of the fluorescent minor groove binding dye Hoechst33342 [30], which allowed us to simultaneously monitor the nuclear uptake of this drug and the effect on XPC-GFP in real time (Figure 3A). Within 5 minutes after addition of Hoechst33342, the nuclear periphery became fluorescent with a gradual decrease towards the nuclear center, reflecting its slow penetration in live nuclei. Remarkably, also XPC-GFP accumulated at areas of high local Hoechst-stained DNA and this accumulation followed the same kinetics as Hoechst33342 nuclear uptake (Figure 3A, and 3B, upper panel). Importantly, the distribution of TFIIH-GFP and GFP-XPA was not influenced by Hoechst33342 incorporation (Figure 3B). FRAP experiments further showed that the mobility of XPC-GFP (Figure 3C), but not of GFP-XPA and TFIIH-GFP (Figure 3D), was reduced by the addition of Hoechst33342. These data suggest that XPC-GFP has a higher preference for DNA harboring the intercalating dye Hoechst33342 than for non-challenged DNA. In addition, the intercalating agent, actinomycin D (ActD) [31] had a similar effect on the nuclear locomotion of XPC-GFP and in this case also on TFIIH-GFP (Figure 3E) but not on GFP-XPA (data not shown). In a recent study we identified that ActD induces an abortive-type of NER complex assembly that became instable and dissociates during TFIIH action (Giglia-Mari et al, 2006 in press). These experiments suggests that distortion of the DNA helix by binding of Hoechst33342 or ActD induces an increased binding of XPC-GFP and that likely the overall slow mobility of XPC-GFP is indeed derived from nonspecific binding to DNA or irregularities in the DNA structure in non-challenged cells. This notion was further corroborated by treating the cells with other non-NER-inducing DNA damaging agents (such as γ -irradiation, which induces single- and double-stranded breaks, interstrand cross-linker mitomycin C, the alkylating agent methyl-methane-sulfonate and UV-A irradiation (that induces mainly oxidative base damages), which all affected with variable degrees the

mobility rate of XPC-GFP (Figure 3F, and data not shown). Not surprisingly treatment with UV-C light had the largest effect on the locomotion of XPC-GFP (Figure 3F).

Activity of XPC-GFP in NER

To determine the active participation of XPC-GFP in NER we compared its nuclear mobility (as measured by FRAP) after applying different doses of a *bona fide* NER inducing DNA lesion (UV-C light) with the unchallenged cells (Figure 4A), as previously described [20-22]. FRAP curves fitted best to a situation in which the transient binding (or temporal immobilization) time of XPC-GFP was significantly extended. The fraction of immobilized XPC-GFP molecules is proportional to the amount of induced damage (applied UV-dose), and saturates around 8 J/m^2 to ~25% of XPC-GFP molecules being immobilized. Since XPC was suggested as one of the early factors required for initiating NER, it is surprising to note that with increasing substrate concentration (UV-damaged DNA) no further depletion of the free nuclear pool of XPC-GFP could be achieved. This observation can only be explained when either not all XPC molecules are competent for binding (inactive) or when another factor preceding XPC binding is limiting. Already two hours after UV-irradiation the immobilized fraction was significantly decreased (in a dose-dependent fashion) and virtually reduced to background levels at 4 hours post-UV (Figure 4B). This relative fast reduction of immobilized XPC has also been observed for ERCC1 and TFIIH [21,22] and further support the notion that with this procedure predominantly the early and robust NER reaction (i.e. removal of 6-4PPs) is monitored and that the relatively slower repair of CPD lesions remains below detection.

Figure 4

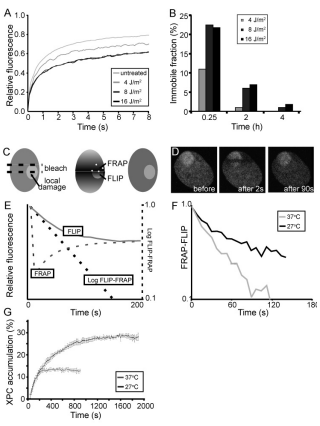


Figure 4. FRAP analysis of UV-C treated XPC-GFP expressing cells.
(A) Strip-FRAP analysis of untreated (green line) and UV-irradiated cells (blue lines) at different UV-doses. (B) UV-dose dependent and time-dependent immobilization of XPC-GFP. Percentage of immobilization is plotted against time for the different UV-doses. (D) Scheme of the FRAP/FLIP procedure on locally damaged areas. A small strip covering half of the local damage and spanning the entire nucleus is bleached at relatively low laser intensity for a period of 2 seconds. Subsequently fluorescence is monitored at regular time intervals in the bleached (FRAP) and non-bleached (FLIP) half of the local damage. (D) Confocal images of a locally irradiated cell expressing XPC-GFP (5 µm pore filter). Left panel: before bleaching, middle panel: directly after bleaching and right panel: 90 seconds after bleaching. (E) The relative fluorescence of the FRAP and FLIP area are shown in time. The log of fluorescence redistribution difference between FLIP and FRAP areas are plotted against time (dotted line). (F) Simultaneous FRAP/FLIP analysis of local damage at 37^{o}C and 27^{o}C. (G) Assembly kinetics of XPC-GFP to a locally damaged area at 37^{o}C and 27^{o}C.

In conclusion, the dose-dependent immobilization of XPC-GFP in combination with the time-dependent decay of immobilization, suggests that this immobilization of XPC-GFP reflects the binding of this protein into NER intermediates (DNA lesion/repair factor complexes).

To determine the residence time or binding time of XPC-GFP within NER complexes, we applied simultaneous FRAP and FLIP on the accumulated XPC-GFP at the locally inflicted DNA damage [20,21]. Briefly, a strip spanning the entire nucleus and covering half of the locally damaged site is bleached (Figure 4C and D). Subsequently, the fluorescence at the bleached (FRAP) and non-bleached area (FLIP) of the local damage is monitored. The difference in relative fluorescence between the FRAP and FLIP area of the local damage is plotted against time (Figure 4E). The time required to obtain full redistribution (>90%) of bleached and non-bleached molecules ($t_{0.9}$) is a measure for the mean residence time of XPC-GFP molecules at NER sites. The measured $t_{0.9}$ of ~100 seconds (Figure 4E), suggests a residence time of ~1-2 minutes, which contrasts to the observed binding time of the other NER factors; XPA, TFIIH and ERCC1/XPF that were bound for 4-6 minutes [20,21]. When the equivalent experiment was performed at 27 ^{o}C, a significant longer residence time of XPC-GFP at the locally damaged site has to be simulated to fit the combined FRAP/FLIP curves (Figure 4F). Also the amount of accumulated XPC-GFP molecules in the damaged area appeared significantly increased as compared to 37 ^{o}C. At 37^{o}C XPC-GFP incorporation into NER complexes reached steady-state within ~4 minutes ([32] and Figure 4G). Interestingly, at 27 ^{o}C the assembly rate of XPC-GFP onto NER lesions was not affected, whereas the time to reach steady-state (k_{on} equals k_{off}) was severely extended to ~20 minutes (Figure 4G). Together these data indicate that the dissociation rate of XPC-GFP from an NER complex is decreased at lower temperatures, but not its association to the lesions.

Dynamic shuttling of XPC-GFP between nucleus and cytoplasm

It is striking to note that the amount and action of XPC is regulated at different levels, both transcriptional and post translational [15,16,33-36]. Careful sequence analysis of the XPC polypeptide even suggested another level of regulating the amount of available XPC; four potential evolutionary conserved nuclear export signals were identified (Figure 5A and B), which suggests that XPC might be exported into the cytoplasm to control XPC levels in mammalian cell nuclei. To further explore this possible novel regulatory mechanism for XPC we first investigated whether indeed XPC-GFP shuttles between cytoplasm and nuclei in living cells. To that aim we fused XPC-GFP expressing cells, using the Sendai virus, and subsequently bleached one nucleus of the poly-nucleated cells and monitored the change of fluorescence of both nuclei in time (Figure 5C). We found a fluorescence recovery of 12% of the original fluorescence level in the bleached nucleus within 25 minutes, i.e. the longest time monitored (Figure 5D, light green line). The majority of this fluorescence recovery is not derived from *de novo* synthesized XPC-GFP, since when we bleach both nuclei we hardly find any fluorescence recovery (Figure 5D, dark green line), the minor increase in nuclear fluorescence is likely derived from import of newly synthesized and /or cytoplasmic XPC-GFP. Note that the steady-state level of XPC-GFP in the cytoplasm is very low, since imaging only revealed a slightly above-background-level of fluorescence. In similar experiments using fused cells that express respectively TFIIH-GFP and GFP-XPA (both also mainly visible in nuclei of living cells [20,21], we do not find this pronounced

fluorescence recovery after bleaching one nucleus (Figure 5E), suggesting that this nuclear/cytoplasmic shuttling is not a common feature of NER factors. Interestingly, this shuttling behavior of XPC-GFP is strongly reduced in UV-irradiated (8 J/m^2) cells (Figure 5F, blue lines). Most strikingly, even after 6-8 hours when XPC-GFP is not significantly involved in NER anymore (Figure 4B) we do not find restoration of XPC-GFP shuttling. This observation argues against entrapment of XPC-GFP in the nucleus by NER sites as a possible explanation for the reduced recovery, but rather suggests a UV-induced modification of the XPC in trans. Similar results were obtained with endogenously expressed XPC when wild type and XPC-deficient human fibroblasts were fused. XPC protein redistribution into the previous XPC-devoid nucleus (derived from the XP-C cells) was significantly retarded after UV-irradiation when compared to non-damaged cells as detected by immunefluorescence (data not shown). These data indicate that this UV-induced regulation of XPC shuttling is not due to a GFP tagging artifact, but also occurs for non-tagged XPC.

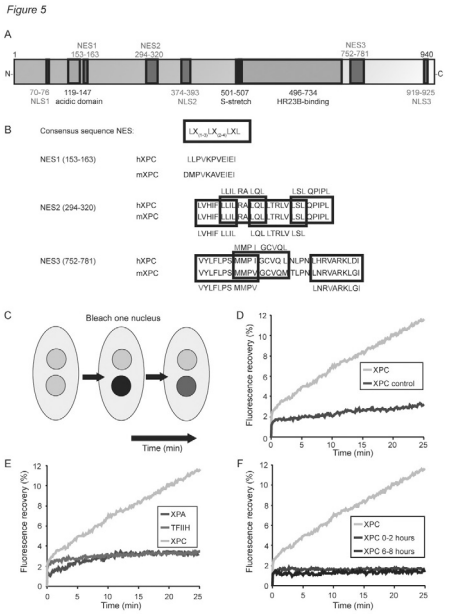


Figure 5. Shuttling of XPC-GFP between nucleus and cytoplasm.
(A) Schematic representation of the XPC polypeptide, showing the different domains. (B) The 3 potential NES sequences present in XPC. (C) One nucleus of the polykaryon is bleached and subsequently the fluorescence in the bleached nucleus is followed in time. (D) The relative fluorescence of XPC-GFP in the bleached nucleus in time (light green line). For the control experiment both nuclei are bleached (dark green line). (E) The relative fluorescence of XPC-GFP (green line), XPB-GFP (red line) and GFP-XPA (blue line) in the bleached nucleus in time. (F) The relative fluorescence of XPC-GFP in the bleached nucleus in time in untreated (green line) and UV-treated (blue lines) cells.

Discussion

The tagging of the XPC protein with the live cell marker GFP and stable expression (at physiologically relevant levels) of the fusion protein in living cells allowed us to study the behavior, regulation, functioning and reaction kinetics of XPC-GFP in living cells under various conditions using high resolution confocal microscopy and photobleaching techniques. The selection of cells that express the fusion protein at physiological relevant levels, i.e. similar to endogenously expressed XPC in XPC-

proficient wild type cells together with the near wild-type UV-sensitivity argued that these cells are a *bona fide* and biological relevant source to examine the dynamic engagements of XPC.

XPC-GFP is localized to chromatin

In contrast to most of the NER factors tested so far (XPA, ERCC1/XPF [22], XPB [21], TTDA [Giglia-Mari et al, 2006 in press] and CSB [37]) XPC appeared non-homogenously distributed in the nucleus (Figure 1C-E). Although TFIIH is also accumulated in the nucleolus and CSB displays irregularly distributed foci, due to specific additional functions of these repair/transcription factors, the co-localization with high local chromatin/DNA concentrations as revealed by co-expressing H2B-YFP (Figure 1C) is significantly distinct from these other factors. This specific sub-nuclear distribution of XPC-GFP has escaped notion in previous immunofluorescence studies, probably due to the applied fixation procedures [25]. Moreover, the high resolution imaging procedure applied in this study further allowed resolution of this specific distribution. The enrichment in DNA dense areas was further corroborated after expressing the fusion protein in mouse embryonal fibroblasts that display, the for mouse cultured cell strains characteristic AT-rich microsatelite concentrations (foci) at which the fusion protein also co-localizes (data not shown and [34]) The association with condensed mitotic chromatin (Figure 1F) further provided evidence that the protein was co-localized even with chromatin at the highest level of condensation and indicated that the fusion protein has a preference to reside in or to associate with chromatin. In addition, the relatively high level of XPC-GFP fluorescence colocalizing with heterochromatin, indicates that XPC-GFP is not only able to access the condensed part of the genome, like TFIIH and XPA, but in contrast to these other NER proteins is also retained there.

Using photobleaching we further showed that the remarkable slow mobility of XPC-GFP in living mammalian cells is likely derived from continues association to and dissociation from an immobile nuclear constituent, likely DNA. The fact that slight (chemical) alterations of the DNA structure significantly retarded the overall nuclear mobility further substantiated this continues DNA probing by XPC, since previous *in vitro* experiments indicated that XPC has an intrinsic affinity for non-damaged DNA [11,12], which increases in the presence of DNA structural aberrations [29,38]. Again, this contrasts to the behavior of the other tested NER factors, which appeared to diffuse freely through the nucleus, without any obvious binding to a relatively immobile nuclear structure. The notion that the overall mobility of XPC-GFP is severely retarded by a 10 °C temperature decrease, suggests that nuclear locomotion of this polypeptide is somehow regulated or influenced by a temperature-dependent process, likely enzymatic. Surprisingly, the dynamics of XPC-GFP do not respond to ATP depletion, suggesting that the energy requiring mobility is not an ATP-dependent process. The reduction of nuclear mobility at lower temperatures in combination with increased binding time to damaged DNA can be explained by a mechanism in which the association to DNA and damaged DNA is not influenced by temperature, as confirmed by the identical slope of XPC binding to damaged DNA, but that the off rate is strongly influenced by temperature.

Recognition of helix distortions

The observed increased binding affinity of XPC-GFP to DNA harboring different structural alterations, such as hoechst33342 (minor groove binding), actinomycin D (intercalating), single- and double-

stranded breaks, interstrand crosslinks, oxidative base damage and bulky helix distorting lesions. This poor selectivity of XPC corroborates earlier biochemical evidence that XPC-hHR23B binds to a broad spectrum of DNA lesions, which disturbed the normal B-form DNA [6,14,39]. However, most of these lesions do not induce a full NER reaction, as was confirmed by the absence of dual incision and repair synthesis in an *in vitro* NER assay [14]. These data suggest that although XPC was recognized as the GG-NER initiating factor (or at least one of the initial factors), binding of XPC only is not sufficient to start NER as was suggested earlier by introducing a two-step NER licensing model; lesion recognition followed by lesion verification [6]. This sophisticated recognition mechanism ensures a high safety level within the GG-NER pathway by allowing the NER reaction only to proceed when a NER-specific lesions is present, thereby preventing spurious and unwanted incisions. Recently, in a study on the association dynamics of TTDA to TFIIH we provided evidence that likely TFIIH has an important role in this verification step (Giglia-Mari, 2006).

Dynamic action of XPC-GFP within NER

It is surprising to note that XPC-GFP revealed a much shorter residence time of ~1-2 minutes within NER complexes as compared to the average binding time of the other measured NER factors (XPA, TFIIH and ERCC1/XPF). This observation points to a scenario where this initiator of GG-NER is not present throughout the entire NER process, but releases from the NER DNA-protein complex in an early stage. It may be speculated that XPC-GFP leaves after one of the subsequent components, *e.g.* TFIIH, XPG or XPA have entered the complex. Recently, some in vitro evidence was provided [40] that confirmed this model in which one of the first factors that enters the NER complex also is one of the first to leave. Earlier studies by Wakasugi and coworkers showed that XPC-hHR23B is not present in a complex that is competent to perform incisions [8]. In addition, recent *in vitro* studies showed that XPC-hHR23B, XPA and RPA do not form a stable complex at the damaged DNA [41]. Mathematical modeling suggested that the early departure of XPC-hHR23B from the NER complex could be beneficial for the repair efficiency [32].

Shuttling of XPC between nucleus and cytoplasm

It is surprising to note that both the amount and the action of XPC are regulated at different levels. First of all, expression of the *XPC* gene appeared to be regulated by a DNA damage-induced and p53-dependent transcriptional regulation [36]. Furthermore, the UV-induced stabilization of the XPC protein, which depends on the association to one of the homologs of RAD23, suggested that also at the post-translational level control of the amount of XPC occurred [34]. Both of these regulatory mechanisms are however surprisingly slow, with the highest UV-dependent XPC induction at time points when the majority of the lesions are removed. This relatively slow damage-induced adaptive response suggests that this process will mainly sensitize cells to respond quicker to a possible subsequent large genotoxic attack. Recently, a new and quicker mode of regulating XPC action was discovered, by the notion that after DNA damage XPC becomes quickly ubiquitylated in a DDB2 (XPE)-dependent fashion [15,16]. This post-translational modification likely enhances the affinity of XPC for damaged DNA and thus reflects an adaptive response that directly regulates NER activity.

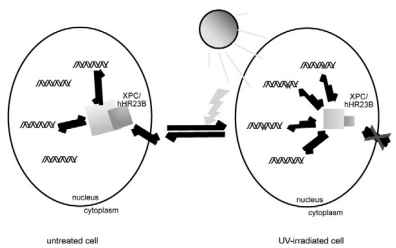
In this manuscript we identified a further sophistication of the intricate XPC regulation, by the notion of a damage-dependent differential nuclear/cytoplasmic shuttling mechanism of specifically XPC. Although the majority of the resident XPC molecules are located in the nucleus, the shuttling equilibrium reduces the steady-state pool of nuclear XPC. Under normal (non-genotoxic stress) conditions XPC continuously shuttles between nucleoplasm and cytosol by the trade-off between nuclear export signals (NES) and nuclear localization signals (NLS) that both are present in the XPC polypeptide (See Figure 5A). Apparently, constitutive high levels of nuclear XPC is less favorable for cells due to its continuous DNA probing which may interfere with essential DNA-transacting processes. The sudden (UV-induced) shift in this equilibrium towards more retention of XPC in nuclei warrants a quick response to a change in the environmental conditions, i.e. the increased amount of the initiating protein likely will aid NER-efficiency.

Remarkably, the XPC-GFP shuttling is still impeded till 6-8 hours after UV-irradiation when the majority of XPC molecules are not involved anymore in NER. Superficially, the enhanced nuclear retention of XPC seems to continue too long, since at this time points the bulk of the 6-4PP lesions were removed and NER slowly progresses to remove the poorly recognized CPD lesions [42]. A possible explanation for this apparent contradiction is that more XPC molecules are required to enhance the probability of locating CPD lesions in the genome, since XPC does not have very high affinity for these injuries [39]. The mechanism that causes this nuclear retention is currently not known. It is tempting to speculate that UV-induced post-translational modifications cause this phenomenon, a likely candidate for this modification is of course the recently observed polyubiquitylation and sumoylation upon UV-irradiation [15,16].

Model for the XPC behavior

In conclusion, we uncovered that under non-challenging conditions the level of XPC in the nucleus is kept under control by it constant shuttling between the cytoplasm and the nucleus. In addition, we found that XPC has an exceptional low mobility due to multiple transient interactions with most likely DNA. Based on these observations we forward the following hypothetical model; the XPC complex might be 'scanning' the DNA (by continuous probing) in search for distortions (Figure 6), when helix distortions are encountered the XPC protein is longer bound, thereby increasing the probability to recruit the subsequent NER factors. Indeed, we found that upon addition of various agents that induce different DNA alterations, i.e single- and double-stranded breaks, interstrand crosslinks, oxidative base damage and bulky helix distorting lesions, overall XPC mobility is strongly retarded which can be explained by extended binding times. However, most of these genomic insults do not form a meta-stabile NER complex, which is required to conclude the NER reaction, indicating that XPC requires additional factors, such as TFIIH and/or XPA to verify the lesion. Only when a *bona fide* NER lesion is encountered by XPC and checked of by its successor a functional NER complex is assembled. When

Figure 6



NER-specific lesions are introduced by UV-irradiation the equilibrium between bound and freely diffusing XPC is shifted even more towards bound molecules (Figure 6, right). Moreover, XPC is prevented from shuttling to the cytoplasm and maintained in the nucleus up to several hours after UV-irradiation.

Material and methods

Cell culture conditions and specific treatments

Cell strains used are XP4PA SV stably expressing XPC-GFP, XPCS2BA SV stably expressing XPB-GFP [21], XP21RO SV stably expressing GFP-XPA [20], and Rad23A$^{-/-}$ Rad23B$^{-/-}$ double knock out mouse embryonic fibroblasts stably expressing XPC-GFP [34]. All cell strains used in this study were cultured in RPMI + hepes (Life technologies) supplemented with 10% fetal calf serum and antibiotics, and maintained in a humidified 5% CO_2, 37 ^{o}C incubator. For DNA staining cells were incubated with 10 µg/ml Hoechst 33258 for 2 hours. Prior to UV irradiation with a Philips TUV lamp (254 nm) at a dose rate of ~ 0.8 J.m^2/s cells were rinsed with PBS. In the cases when cells are locally damaged, an isopore polycarbonate filter (Millipore) containing either 5 or 8 µm diameter pores was used to cover the cells before UV-irradiation [10,43]. After irradiation cells were put back into medium and microscopically examined. For the azide-treatments, cells were incubated for 15 minutes in glucose-free medium (Gibco), with 60 mM deoxyglucose (Sigma) and 0.2% Na-azide (Sigma).

Generation of XPC-GFP-his$_6$HA fusion construct

Full length human XPC cDNA was cloned in frame in an eukaryotic expression vector pEGFP-N3 (Clonetech). A 3' histidine$_6$-hemaglutinine tag was added by insertion of a doublestranded oligo in SspBI-NotI site.

Generation of a stably expressing XPC-GFP cell line

The XPC-GFP fusion construct was transfected to XP4PA SV cells and the cells were selected with 250 µg/ml G418 (Sigma). An UV-resistant population that survived three UV-exposures (4 J/m^2) was isolated.

Confocal Microscopy

Three days prior to microscopic experiments, cells were seeded onto 24 mm diameter coverslips. Imaging and FRAP were performed on a Zeiss confocal laser scanning microscope LSM510meta and LSM 410 (Zeiss, Oberkochen, FRG), equipped with a heatable scan stage (37 ºC) respectively. Images were recorded with a 488nm Ar-laser and a 515-540 nm bandpass filter. Lateral resolution was 104 nm. The simultaneous monitoring of GFP tagged NER factors and H2B-YFP (gift of Dr. K. Mattern) was performed using the Zeiss confocal laser scanning microscope LSM510meta.

Fluorescence Recovery after Photobleaching

Diffusion measurements were performed by FRAP analysis at high time resolution (strip-FRAP). A strip spanning the nucleus was photobleached for 200 ms at 100% laser intensity. Recovery of

fluorescence in the strip was monitored with 100 ms intervals at 1% laser intensity. The effective diffusion coefficient (D_{eff}) of TFIIH-GFP was obtained by calculating relative intensity FR_{diff} (t) = (I_t-I_0)/(I_∞-I_0), where I_∞ is fluorescence intensity (FI) after complete recovery, I_0 is FI immediately after bleaching, and I_t is FI during monitoring. D_{eff} was estimated by minimizing $\sum[FR_{diff}(t)- FT(t)]$, where FT is a theoretical equation for one-dimensional diffusion: $FT(t) = 1 - (w^2 * [w^2 + 4\pi t D_{\text{eff}}]^{-1})^{-½}$. Immobile fractions were calculated as $N_{\text{immobile}}/N_{\text{tot}} = 1 - FR_{imm}(\infty) * (1-N_{\text{mobile, bleached}}/N_{\text{tot}})^{-1}$, where FR_{imm} = (I_t-I_0)/($I_{t<0}$-I_0) and $I_{t<0}$ is fluorescence before bleaching and $N_{\text{mobile, bleached}}/N_{\text{tot}}$ is the fraction of mobile molecules bleached by the pulse. The latter is ~30% in our set-up as determined by bleaching experiments on free GFP. In simultaneous FRAP/FLIP experiments a strip at one side of a nucleus was bleached at 20% laser intensity for 8 s. Fluorescence was then monitored in the bleached and unbleached side of the nucleus and the difference was plotted against time [27].

Simultaneous FRAP/FLIP

Simultaneous FRAP/FLIP analysis to determine the mobility of fluorescent molecules in cell nuclei [21]. A small area at one side of a nucleus is bleached at relatively low laser intensity for a relatively long period of time (10 s) (Figure 5B). Subsequently fluorescence is monitored at regular time intervals (4 s) in the bleached area and in a region at the opposite side of the nucleus. The (log) of fluorescence redistribution (relative fluorescence) difference between FRAP and FLIP area is plotted against time (s) (Figure 5C). The steepness of the line determines the mobility of the fluorescent molecules.

FLIP on polykaryon cells

XPC-GFP expressing cells were fused using 500 TAU of Sendai virus. Three days after fusion one nucleus of a polykaryon was completely bleached using relatively low laser intensity for a period of 4 seconds (Figure 5C). Subsequently the fluorescence in the bleached nucleus is monitored at regular time intervals (10 s). The fluorescence regain (relative fluorescence) in the bleached nucleus is plotted against time (min).

Recruitment of XPC-GFP to locally irradiated cells

As previously described [32,44] XPC-GFP expressing cells were locally UV-irradiated using a 5 µm pore filters on the LSM510 microscope. Immediately after irradiation, the accumulation of XPC-GFP was monitored with regular time intervals (20 seconds) for several minutes.

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Chapter 6

Rad23 facilitates XPC binding to damaged DNA and dissociates from the immobilized complex.

Rad23 facilitates XPC binding to damaged DNA and dissociates from the immobilized complex.

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Introduction

Nucleotide Excision repair (NER) is a versatile DNA repair mechanism that repairs a wide variety of helix-distorting lesions including those induced by sunlight (i.e. UV) (Hoeijmakers 2001). In NER, lesion recognition is either performed by the stalled RNA polymerase II (RNAPII) or by the XPC complex with the help of the UV-DDB complex (Sugasawa et al. 1998). Recognition by RNAPII and subsequent repair occurs only in the transcribed part of the genome and is therefore called transcription-coupled NER (TC-NER). The XPC complex on the other hand recognises lesions throughout the entire genome, this way of recognition is designated as the global genome NER (GG-NER) sub pathway. Following recognition, the transcription factor IIH (TFIIH) complex unwinds the helix in an ATP-dependent manner (Egly 2001). Subsequently, XPA and RPA bind/recruit and orient the two endonucleases XPF/ERCC1 and XPG for the excision of a 25-30 nucleotide patch containing the lesion (de Laat et al. 1998). The resulting single strand gap is filled in by one of the DNA polymerases facilitated by PCNA and RFC, the final ligation is performed by a ligase (Gillet and Scharer 2006).

Obviously, damage recognition is the most important step of repair that also partly determines the rate and efficiency. Within GG-NER, the XPC complex initiates lesion discrimination. Xeroderma pigmentosum patients, carrying mutations in the XPC gene develop with a high incidence skin tumours on sun light-exposed areas of the skin. XPC knock out (K.O.) mice and to a minor extent XPC heterozygous mice, exhibit an age-dependent increase in spontaneous mutagenesis (Wijnhoven et al. 2000). Following UV-B exposure XPC K.O. mice develop with a very high incidence skin cancers. Moreover, XPC heterozygous animals have a higher predisposition to skin cancer in time as well, a phenomenon referred to as allelic insufficiency (Cheo et al. 2000). Typically, a large number of human skin and lung cancers have allelic loss of the XPC gene (Hollander et al. 2005). In addition certain single nucleotide polymorphisms are associated with higher skin and lung cancer risks (Blankenburg et al. 2005; Nelson et al. 2005). All these findings emphasize the biological relevance of a proper functioning damage recognition complex.

In yeast the XPC homologue, Rad4 interacts with Rad23. XPC purified from HeLa cell extracts was found to co-purify with the Homologue of Rad23 B (HR23B) and to a lesser extent with HR23A (Masutani et al. 1994). These observations suggest that the association of HR23A or –B to XPC is evolutionary conserved and that they are linked in their function in NER. Yeast Rad23 mutants are UV-hypersensitive, but single HR23A and HR23B mouse K.O. cells are not UV-sensitive. However, cells missing both HR23A and HR23B are UV-sensitive to a similar extent as the XPC mutants, suggesting a redundancy in NER for the HR23A and HR23B homologues (Ng et al. 2003). Studies of these double knock out (DKO) cells revealed a lower level of XPC protein, suggesting an essential role of these proteins in stabilizing XPC (Ng et al. 2003). Indeed, also in yeast the RAD23 protein is important for stabilising RAD4 (Lommel et al. 2002). Purified XPC alone binds to damaged-DNA *in vitro*. Importantly, the affinity increased when purified HR23B or HR23A was added (Sugasawa et al. 1996). This observation indicates that HR23A and HR23B besides their stabilizing function also play a role in the efficient binding of XPC to the damaged DNA. Recently, Cen2 was identified as a

third component of the (human) XPC-complex (Araki et al. 2001). Like RAD23 proteins, CEN2 exerts a regulatory role in the initiation step of GG-NER (Nishi et al. 2005).

In addition to an involvement in NER, the Rad23 proteins are implemented in the ubiquitin dependent 26S-mediated protein degradation pathway. In this ubiquitin proteasome system (UPS) proteins are ubiquitylated by the subsequent actions of E1 the activating enzyme, followed by an E2 conjugating enzyme and finally by an E3 ligase (Hershko and Ciechanover 1998). Polyubiquitylated proteins are targeted to the 26S proteasome and subsequently degraded. It is important to note that not all ubiquitylated proteins are targeted for degradation, ubiquitylation also plays an important role in many cell processes by altering activities, binding-partners of targeted substrates or subcellular sorting (Di Fiore et al. 2003). Proteins can either be mono- or polyubiquitylated by subsequent ubiquitin conjugation on an internal lysine residue of an already conjugated ubiquitin. Several different polyubiquitin trees exist as well. It is generally believed that polyubiquitin trees, that consist of a polyubiquitin tree branched on the lysine 48 of ubiquitin are targeted for degradation (Pickart and Fushman 2004). Rad23 proteins contain at the amino terminus an ubiquitin like (UBL) domain, capable of binding to the 26S proteasome and two ubiquitin association (UBA) domains. Recently it was shown that the carboxy terminal UBA domain has a specific affinity for lys48 chains (Raasi et al. 2004; Varadan et al. 2005). It has been further suggested that Rad23 proteins are shuttles to target substrates to the proteasome (Verma et al. 2004). Since Rad23 proteins are involved in both DNA repair and in protein degradation a direct link between these two, provided by Rad23 has been proposed (Schauber et al. 1998).

It was put forward that the Rad23 proteins are involved in the regulation of the recognition step within NER. Regulation of the initial step is clearly of critical importance but the molecular mechanism underlying the Rad23-dependent regulation is unfortunately poorly understood. In order to gain more insight in the recognition mechanism and to study whether the Rad23 homologues have possible other roles in DNA repair rather than only stabilizing XPC, we set up a system to follow HR23B in living cells. Using fluorescently tagged proteins and sophisticated live cell microscopy, we demonstrated that the Rad23 proteins are required to efficiently load XPC on NER lesions. Spectroscopic analysis of differentially tagged HR23B and XPC confirmed binding of both in living cells. Most interestingly, we also found that HR23A and HR23B dissociate from XPC upon UV-irradiation, thereby possibly facilitating an efficient recognition and repair.

Results

HR23A and HR23B immobilize XPC on DNA damage in living cells.

In view of the fact that purified XPC has a lower affinity for damaged substrate in the absence of HR23A or HR23B (Sugasawa et al. 1996) we wondered whether HR23A and/or HR23B influence the behaviour of XPC in living cells as well. By over-expressing XPC-GFP in DKO cells we were able to study the dynamic behaviour of XPC in living cells using photobleaching techniques (Houtsmuller and Vermeulen 2001) in the absence of both HR23A and HR23B. In view of the intrinsic instability of XPC in DKO cells (Ng et al. 2003) we choose to transiently over-express XPC-GFP in DKO, wild type and XPC K.O. mouse embryonic fibroblasts (MEFs). Cells were selected that expressed XPC-GFP to a

comparable level as a rescued human XP-C cell line stably expressing XPC-GFP on the basis of fluorescent signal. The

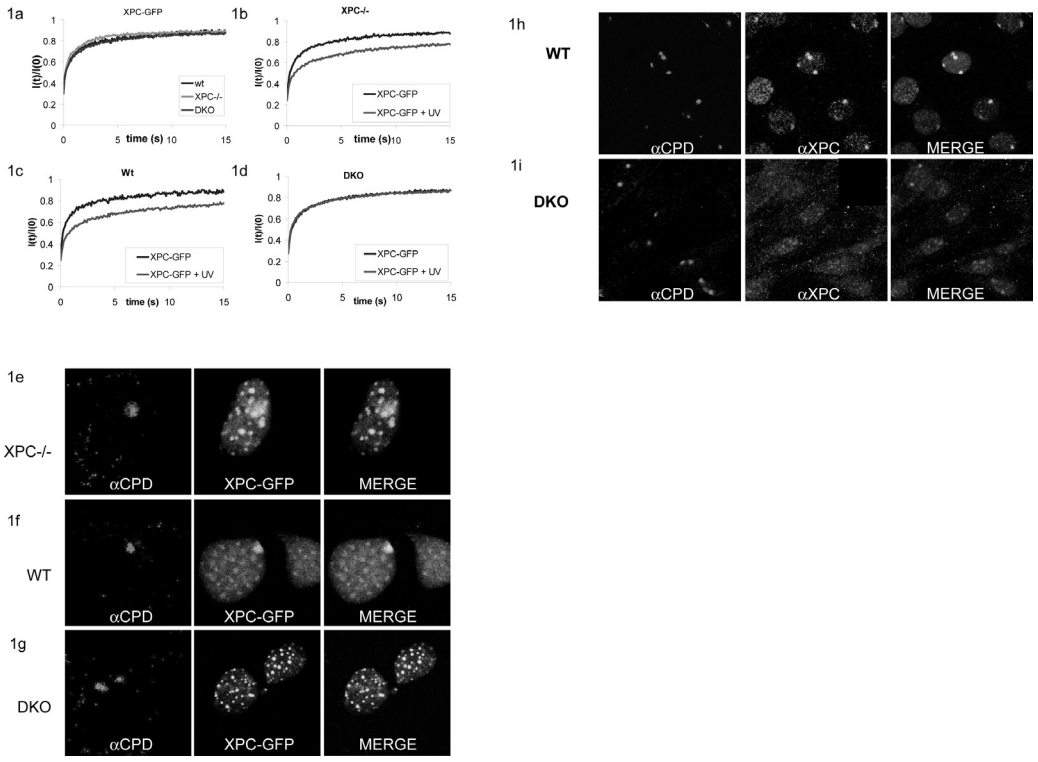

Figure1 HR23A and HR23B immobilize XPC on DNA damage in living cells**.**
a-d) FRAP analysis of XPC-GFP in the absence and presence of UV-damage. **a)** In the absence of DNA damage no apparent difference in the mobility rate of XPC-GFP could be detected when expressed in either XPC K.O. wild type and DKO cells. **b)** Upon UV treatment XPC-GFP ectpoically expressed in XPC K.O. cells immobilizes in comparison to untreated cells. **c)** Similar, ectopically expressed XPC-GFP immobilizes after UV-induced DNA damage in wild type MEFs **d)** Ectopically expressed XPC-GFP in DKO cells fails to immobilize upon UV treatment. **e-i)** Immunofluorescent analysis of XPC and XPC-GFP at local DNA-damaged areas in different genetic backgrounds. Upon UV treatment over-expressed XPC-GFP accumulates at local damaged sites as is indicated by the presence of CPDs in XPC K.O. **(e)**, wild type **(f)** but not in DKO **(g)** cells. Endogenous XPC (middle panel) accumulates at local damaged sites as is indicated by the presence of the CPDs (left panel) in wild type **(h)** cells but not in DKO **(i)** cells.

mobility of XPC-GFP is lower than expected based on its molecular size and compared to other NER factors, most likely due to a frequent association to and dissociation from DNA (Hoogstraten et al., manuscript in preparation, chapter 5 of this thesis). In addition, XPC-GFP mobility was identical in all three murine cell lines (i.e. wild type, XPC K.O. and DKO) and was comparable to the behaviour in human cells (**fig. 1a**). Thus, the relative slow mobility is not influenced by the absence of HR23A and B, suggesting that XPC can still bind the DNA in a non-specific manner. After UV-irradiation (16 J/m^2), inducing suddenly a high dose of DNA-lesions, mobility was significantly reduced, likely by prolonged binding to damaged-DNA in XPC-/- K.O. (**fig. 1b**) and in two independent wild type (**fig. 1c,** and data not shown) MEF-cell lines. In contrast, in DKO cells XPC-GFP failed to immobilize after UV irradiation (**fig. 1d**), suggesting that in the absence of HR23 proteins XPC is not competent to bind/immobilize to UV-lesions. The inability of XPC-GFP to bind to damaged-DNA was confirmed *in situ* by immunostaining. XPC-GFP clearly accumulated on local DNA damage (inflicted by irradiating through a microporous filter of 5 µm (Mone et al. 2001; Volker et al. 2001)) in both XPC K.O. (**fig. 1e**) and wild type (**fig. 1f**), but not in DKO MEFs (**fig. 1g**). In addition, even the low level of endogenous XPC present in the DKO cells failed to accumulate on local DNA damage (**fig. 1i**) while it did so in wild-type cells (**fig. 1h)**. Both immunohistochemistry as well as the mobility studies indicated that XPC has lower (undetectable) affinity for damaged DNA in the absence of either HR23A or HR23B. These data obtained in intact cells are in line with earlier *in vitro* studies in which a lower affinity for damaged-DNA for the single XPC as compared to XPC in complex with HR23B was found (Sugasawa et al. 1996). This clearly shows that HR23A and B besides stabilizing XPC also alter the affinity of XPC for damaged DNA.

Low levels of HR23B are sufficient for successful NER.

To study the behaviour of HR23B in NER and participation in damage binding we tagged mHR23B at its carboxy terminus with the yellow fluorescence protein containing an additional FLAG tag (HR23B-YFP-FLAG). The fusion protein was stably expressed in the DKO cell line using hygromycin as a selectable marker. Since endogenous HR23B proteins levels are far more abundant than XPC levels (Sugasawa et al. 1996; van der Spek et al. 1996) we aimed to obtain a low expressing cell line that still functioned in NER. Clones were selected by virtue of their ability to rescue the UV-sensitive phenotype of the DKO cells. The UV hypersensitivity was completely complemented in several independent clones (**fig. 2a**) using both a colony and a tritium-incorporation based UV survival assay. Immunofluorescence analysis showed that the reduced endogenous mXPC levels were increased to comparable levels as wild-type amounts (**fig. 2b**). Both, the increase in mXPC level as well as the rescue of the UV-hypersensitivity, indicated that the mHR23B-YFP-FLAG protein is functional in NER. The fluorescence of these NER-rescued clones was extremely low. Immunoblot analysis of whole cell extracts revealed an almost undetectable expression level (**fig. 2c** lane2). Cells that were kept under constant hygromycin selection were far more fluorescent. A band of the expected size was visible on immunoblot analysis in whole cell extracts of these cells (**fig. 2c** lane1). The fusion protein is present in the low expressing cells since we were able to immunoprecipitate a band of the correct size using the flag epitope (**fig, 2d**). Although determination of the exact expression level of the fusion protein in the stable cell lines was complicated due to the fact that the antibodies that recognize HR23B

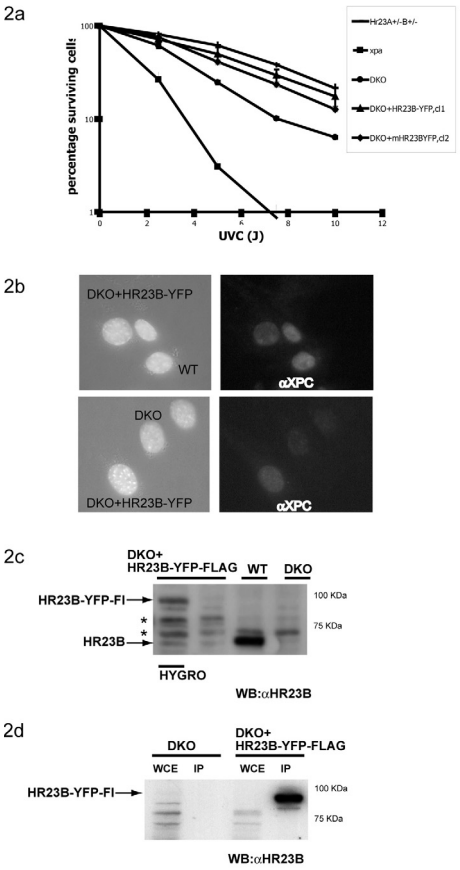

Figure 2 Low levels of HR23B are sufficient for successful NER.
a) UV survival assay of stabile expressing clones. Despite of the low expression levels HR23B-YFP-FLAG expressing clones are not UV sensitive while the parental DKO cell line is. **b)** Immunofluorescent analysis of XPC levels in wt, DKO and HR23B-YFP expressing clones. Endogenous XPC levels are increased in cells expressing HR23B-YFP-FLAG (indicated by the arrow) in comparison with DKO cells (lower panel) to a similar level as wild type cells (upper panel). **c)** Western blot analysis of low and high expressing cells. An increase in HR23B-YFP levels is detected if cells are kept under selection (compare lane 1 with lane2). Although the expression of endogenous HR23B still seems higher. Asterisks indicate aspecific crossreacting bands. **d)** Immunoprecipitation of HR23B-YFP-FLAG. The full-length HR23B-YFP-Flag protein was efficiently immunoprecipitated (as indicated by the arrow) using the carboxy-terminal FLAG-tag indicating that the construct is expressed albeit at low levels

probably have a considerably lower affinity for the tagged version compared to the non-tagged version, expression levels of the tagged HR23B are most likely far below the levels in wild type cells (compare lane 1 and 2 with lane 3 in fig2c). However, UV hypersensitivity of the DKO cells was fully rescued despite of these low expression levels, this would suggest that low levels of HR23A/B are sufficient for a functional NER. Moreover, this indicates that the cell line that we have generated at least does not overexpress the functionally tagged protein and thus represent a biological relevant source to study Rad23 protein dynamics in NER.

HR23A and HR23B do not accumulate at local DNA damage.
Local UV irradiation of the stably expressing mHR23B-YFP cells caused a clear and abundant accumulation of endogenous mXPC as was described previously (Volker et al. 2001), again showing the functionality of the HR23B-YFP fusion protein. In contrast, no mHR23B-YFP-FLAG could be

detected on the local DNA damage in spite of the low expression levels (**fig. 3a**). Also in living cells we could not detect any accumulation of mHR23B-YFP-FLAG within a locally damaged spot induced by a pulsed 800 nm laser (Meldrum et al. 2003), while XPC-GFP and other tested NER genes clearly did (data not shown and Dinant, manuscript in preparation). Importantly, none of the antibodies recognising endogenous mHR23B revealed any accumulation at locally damaged areas, contrasting to the endogenous XPC staining at the damaged sites (**fig. 3b**). A transiently expressed mHR23A-GFP^2-MYC construct in DKO cells also failed to reveal any accumulation at local UV-damage (data not shown). We conclude that HR23B and HR23A are not accumulating at local UV-induced DNA damage while XPC clearly is. This points to a dissociation of the XPC/HR23B complex upon UV treatment. Note that endogenous mHR23B, transfected mHR23B-YFP-FLAG and mHR23A-GFP^2-MYC were all homogeneously expressed in the cytoplasm and the nucleus, while the nucleoli are free of detectable protein. This localization fits with the function of the HR23 genes in 26S-mediated proteasomal degradation and previous studies (Hershko and Ciechanover 1998; Katiyar and Lennarz 2005).

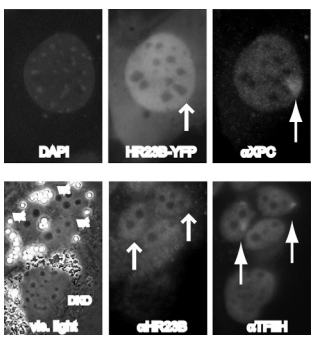

Figure 3 HR23A and HR23B do not accumulate/immobilize at DNA damage.
a-b) Analysis of HR23B-YFP and HR23B at local damaged-DNA spots. **a)** HR23B-YFP (middle panel) is not accumulating at local damaged sites (indicated by the arrow) while its complex partner XPC (right panel) is. **b)** Similar, endogenous HR23B (middle panel) fails to accumulate at local DNA damage (see arrow) as is indicated by the accumulation of the TFIIH-subunit p62 (right panel). The antibody used is specific since virtually no staining can be detected in DKO cells (small beads, left panel) while a clear signal is visible in wild type cells (large beads, left panel).

Since a large fraction of the proteins involved in NER are bound to chromatin after UV treatment and are resistant to triton X-100 treatment we tried to enhance the visualization of a possible accumulation by a simultaneous fixation and triton X-100 wash as described previously for NER proteins (Volker et al. 2001). Although XPC accumulation became even more profound, mHR23B-YFP-FLAG remains undetectable. In addition, also immunodetection of endogenous mHR23B was reduced when the same procedure was applied (data not shown). This suggests, that contrary to the expectation, HR23B is not bound to NER-lesions, whereas under similar conditions XPC is. This further implies that the composition of the XPC/HR23B complex is affected after UV.

HR23B-YFP is not immobilizing on UV-induced DNA damage in living cells.

The previous data all suggest that in the presence of UV-damage the equilibrium of XPC with its separate subunit, HR23B, is changed towards a more loosely associated HR23B. To investigate this possible dissociation in living cells we performed a series of dynamic studies. Similar studies revealed a clear UV dose-dependent (transient) immobilization of all NER proteins tested thus far including,

ERCC1, XPB, XPA, TTDA, CSB, PCNA (Houtsmuller et al. 1999; Hoogstraten et al. 2002; Rademakers et al. 2003; van den Boom et al. 2004; Essers et al. 2005) and XPC (Hoogstraten et al, manuscript in preparation; and fig 1). In striking contrast to the other NER factors HR23B did not immobilize upon UV treatment (**fig. 4a**). The absence of an UV-induced immobilization is in line with the absence of HR23B at DNA damage spots and with a possible dissociation from XPC. The dynamic behaviour of mHR23B-YFP in cells with higher expression levels (i.e. the cells that were kept under constant hygromycin selection, see fig 2c) before and after UV was similar to the lower expressing cells (**fig. 4b**). Since the low expressing cells were fully functional in NER this indicates that, despite the amount, HR23B is not immobilising after UV.

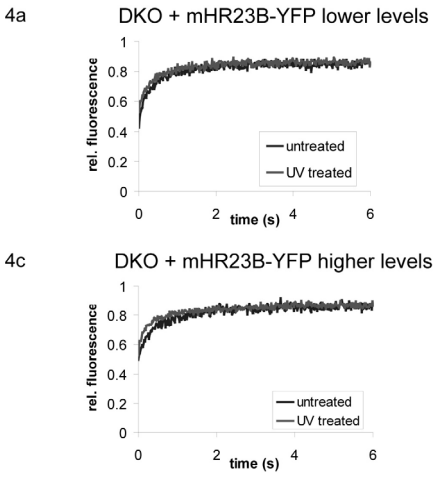

Figure 4 HR23B-YFP is not immobilizing upon UV treatment.
a) FRAP analysis of HR23B-YFP before and after UV-induced DNA damage. HR23B-YFP is not immobilizing after UV treatment, instead a small increase in the diffusion was observed. **b)** FRAP analysis HR23B-YFP in cells with a higher expression level. The dynamic behavior of HR23B-YFP is not altered, with or without UV treatment, by the higher expression level.

The XPC/HR23B complex dissociates upon UV induced DNA damage

To test whether mHR23B binding of XPC is influenced by the presence of DNA damage in living cells, we transiently transfected hXPC-GFP in higher expressing mHR23B-YFP cells to measure fluorescence energy transfer (FRET) between GFP and YFP, which is an indication for protein-protein interactions. Spectral imaging and subsequent spectral unmixing were used to separate GFP and YFP signals (Dinant et al., manuscript in preparation). To determine FRET we used the acceptor photo bleaching procedure, which employs a quick bleaching of the YFP (acceptor) fluorescence and thereby abrogate energy transfer from GFP (donor) to its acceptor (YFP). When protein couples are in such a conformation which allowed efficient FRET photobleaching of YFP (the acceptor bleach), will subsequently result in an increase of the donor (GFP) emission. Using this strategy we were indeed able to detect a clear FRET signal in living cells between XPC-GFP and HR23B-YFP (**fig. 5a**). The efficiency of the energy transfer was 36% compared to the FRET between a GFP-YFP fusion construct normalized to a 100%. This indicates that under non-challenging conditions HR23B and XPC indeed do bind in living cells, confirming the previous *in vitro* data (Masutani et al. 1994). To test

whether the presence of DNA damage influences this binding we irradiated XPC-GFP and HR23B-YFP expressing cells with 16 J/m^2 of UV-C. A striking drop in FRET to only 13% was measured (**fig. 5a**). This reduced FRET can be explained by either dissociation or a conformational change. To test if the decrease in FRET was caused by a change of the stochiometry of the XPC/HR23B complex we immuno-precipitated (IP-ed) mHR23B-YFP-FLAG from a stable expressing clone before and after UV-treatment and analysed the amount of co-IP-ed endogenous mXPC. Immunoblots were stripped and re-probed with HR23B antibody. A clear reduction of binding between mH23B-YFP-FLAG and mXPC was observed after UV-irradiation **(fig 5b)**. This also shows that the tags do not affect association between mHR23B-YFP-FLAG and mXPC. Neither mHR23B-YFP nor mXPC was detected after immuno-precipitation using FLAG beads in DKO cells, indicating that our immuno-precipitations were specific **(fig. 5b)**.

Since *in vitro* we measured a clear dissociation we conclude that the decrease in FRET efficiency is likely caused by dissociation rather than by a conformational change. Both *in vitro* and in living cells we found a dramatic drop of the amount of XPC and HR23B in complex after DNA damage, pointing to a change in the stochiometry of the HR23B/XPC complex upon UV.

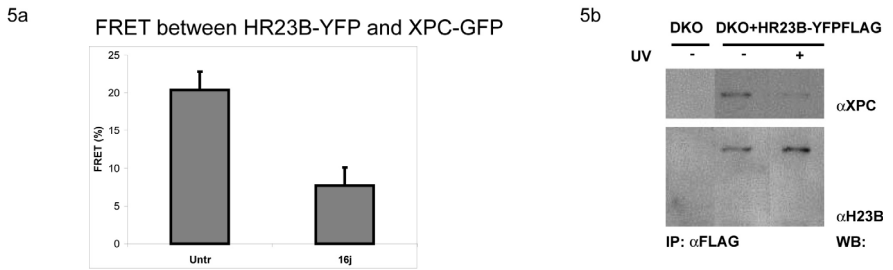

Figure 5 The XPC/HR23B complex dissociates upon UV induced DNA damage
a-b) XPC/HR23B complex composition analysis in living cells and *in vitro*. The FRET efficiencies between XPC-GFP and HR23B-YFP is 21% indicating that HR23B and XPC are in close proximity e.g. bind. Upon UV treatment the average FRET efficiency drops to 7%. **b)** HR23B-YFP associates with endogenous XPC since XPC could be co-immunoprecipiated using the FLAG tag of HR23B-YFP-FLAG.less XPC could be co-immunoprecipitated after UV treatment although the amount of immunoprecipitated HR23B slightly increased.

Discussion

RAD23 proteins dissociate from XPC upon UV

Using both *in vitro* studies and live cell imaging techniques, we found an unexpected change in the stoichiometry of the XPC/HR23B complex upon UV-induced DNA damage. Firstly, we showed that HR23A and HR23B proteins do not accumulate at local UV-damaged nuclear areas, while XPC does. Secondly, we failed to see any UV-induced immobility of biologically active HR23B-YFP in living cells, whereas under the same conditions XPC-GFP clearly shows immobilization, like other NER factors. Thirdly, complex formation between HR23B-YFP and XPC-GFP as determined by FRET, significantly

reduces after binding of XPC-GFP to damaged DNA. Finally, we observed that the amount of HR23B bound to chromatin did not increase after UV-irradiation whereas the amount of XPC bound to chromatin was clearly enriched after this treatment. These data lead us to conclude that HR23B and XPC dissociate when bound to damaged DNA.

RAD23 homologues alter the properties of XPC in living cells.

In both the mammalian system and in yeast it has been shown that cells lacking RAD23 homologues XPC/RAD4 protein levels are extremely reduced (Lommel et al. 2002; Ng et al. 2003). Together with our finding that HR23B (and probably HR23A) dissociate from XPC after binding to NER lesions one could agree that the major function of HR23B or HR23A is to stabilise XPC by protecting it from degradation. For this purposes only weak or transient interactions might be sufficient. However, in a cell free assay the affinity of purified XPC for damaged DNA significantly increases after adding purified HR23B or HR23A (Sugasawa et al. 1996). Furthermore, over-expression of RAD4 in yeast only partly suppresses the UV sensitivity of *rad23* mutant cells (Xie et al. 2004), suggesting an additional role for the Rad23 proteins in NER besides stabilizing XPC. In our life cell set up we demonstrated a striking difference between UV-induced immobilization of XPC in the absence or presence of both RAD23 homologues i.e. no UV-induced immobilization of XPC when none of the rad23 proteins were present. Furthermore, XPC-GFP and endogenous mXPC failed to accumulate on locally inflicted UV-damaged sites in the absence of both HR23A and HR23B. Importantly, no difference in the dynamic behaviour of XPC-GFP in untreated wild type, XPC-/- or DKO cells could be observed. The intrinsic slow mobility of XPC-GFP in non-damaged cells was explained by a continuous association/dissociation of this protein to DNA (Hoogstraten et al, manuscript in preparation, chapter 5). This leads us to conclude that in the absence of HR23, non-damaged DNA probing by XPC is not affected, but strikingly it fails to immobilize on true NER substrates. Since XPC still binds to DNA in the absence of HR23 proteins both *in vitro* and in living cells it is most likely that HR23A and HR23B function in the formation of a stable anchorage of XPC in the early NER complex.

XPC and HR23B after NER

Our co-immunoprecipitations were performed on chromatin-free extracts. After UV treatment, some of the XPC is present in the nucleoplasm and should be in principle available for binding to HR23B (Hoogstraten et al., manuscript in preparation, chapter 5; and fig1). We observed a decrease in the amount of XPC complexed to HR23B after UV-irradiation, indicating that, after UV, XPC is tethered to the chromatin fraction, whereas HR23B remains in the non-bound fraction. Recently, two independent groups showed that XPC is mono- and polyubiquitylated upon UV treatment in a DDB2-dependent manner (Sugasawa et al. 2005; Wang et al. 2005). XPC was ubiquitylated in the chromatin fraction, while an even more profound ubiquitylation could be observed in the chromatin-unbound fraction (Sugasawa et al. 2005). Both HR23B/XPC dissociation and XPC ubiquitylation occur after UV irradiation. Therefore it could be speculated that the ubiquitylation of XPC inhibits the binding to HR23B by a possible steric hindrance or that dissociation of HR23B might be essential for the ubiquitylation of lesion-bound XPC. HR23B is a protein that specifically binds to polyubiquitin chains, especially K48 chains, which are usually targeted for degradation. Since XPC seems not to be

targeted for degradation (Sugasawa et al. 2005), it is therefore likely that the polyubiquitin chain of XPC is not formed by lys48 branches and will not bind to HR23B. DNA damage-induced polyubiquitylation of DDB2 on the contrary does result in degradation (Nag et al. 2001; Rapic-Otrin et al. 2002). It is therefore a possibility that upon UV HR23B, being released from XPC at the damaged site, facilitates DDB2 degradation.

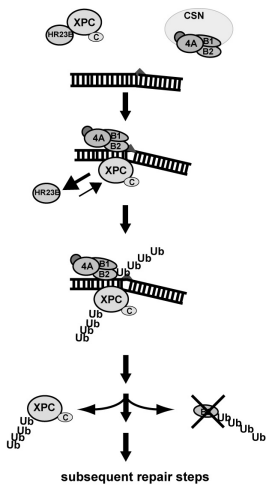

Figure 6 proposed model
The UV-DDB complex and the XPC-complex bind both to helix-disturbing lesions. HR23B and XPC encounter lesions as a complex but for the binding of XPC to those lesions HR23B dissociates. The Cullin4a and Roc1 ubiquitin ligase attached to the UV-DDB complex ubiquitylate both DDB2 and XPC. Polyubiquitylated DDB2 is prompted for degradation. On the contrary polyubiquitylated XPC is not degraded. The modified XPC has an increased affinity for both damaged as undamaged DNA thereby most likely facilitating subsequent repair reactions. In time polyubiquitylated XPC is either degraded or deubiquitylated, the latter is of importance since XPC with a “too high” affinity for DNA might influence other DNA transacting processes such as transcription and replication. DDB2 levels are restored by a p53 transcriptional induction of the DDB2 gene.

Dissociation between HR23B and XPC upon DNA damage facilitates efficient damaged DNA binding.

The DNA binding domain of XPC is localized between amino acid 607 and 742. Interestingly, HR23A or B bind to XPC between residues 496 and 734 (Uchida et al. 2002). It is therefore tempting to speculate that the dissociation we observed upon UV irradiation is necessary to make the DNA binding domain of XPC accessible. Furthermore, AFM studies showed that XPC bends or stabilizes damaged-induced DNA bending (Janicijevic et al. 2003). Recently, it was also shown that XPC binds to the opposing non-damaged DNA-strand (Buterin et al. 2005). Taking these data together we propose the following model (**fig. 6**). Upon DNA damage induction the UV-DDB2 complex binds to the DNA damage facilitating the binding of the XPC/HR23B complex. HR23B dissociates from the complex thereby changing the complex conformation and facilitating XPC binding to damaged-DNA. Upon binding, XPC may slightly alter the helix confirmation thereby disclosing the lesion, enabling binding of subsequent NER factors. In parallel, the UV-DDB2 complex ubiquitylates DDB2 and XPC, unlike XPC, polyubiquitylated DDB2 is directly degraded whereas polyubiquitylated XPC dissociates from the repair site before the establishment of a full incision complex (Riedl et al. 2003). Interestingly, after UV irradiation, non-bound XPC in the nucleoplasm shows a reduced binding to HR23B and is mostly mono- or polyubiquitylated (Sugasawa et al. 2005). The modified form of XPC displays higher affinity for typical NER lesions thereby making subsequent repair initiations more efficient (**fig. 6**).

Besides a higher affinity for NER-specific lesions also the general affinity of XPC for (undamaged) DNA increases (Sugasawa et al. 2005), thus modified XPC potentially interferes with other DNA transacting processes. It is therefore, of importance that modified XPC will be reversed in time, either by degradation or by deubiquitylation to avoid unwanted binding to DNA, which might interfere with normal other DNA-trans acting processes. Since DDB2 is degraded after an initial round of NER-initiation the amount of modified XPC stays limited. DDB2 levels in human cells are restored by the p53-dependent transcriptional response (Hwang et al. 1999). In this way the amount of XPC and modified XPC is tightly regulated. Such a highly orchestrated regulation might be required for a swift and accurate initiation of NER with a relative low level of futile initiation. Unwanted binding, thereby influencing other DNA-transacting processes has potential hazardous consequences for the homeostasis of the cell.

HR23B is not bridging NER and the proteasome on the DNA

Due to the dual role of HR23A and HR23B genes in DNA repair and proteolytic degradation it has been suggested that NER and the UPS are directly linked (Schauber et al. 1998). Although, there are several distinct links between these two processes, here we show that it is not very likely that HR23B or HR23A are recruiting factors implicated in the UPS system to the repair site. Our data shows that either HR23B is not present at the damaged site or its presence is so brief that our live cell set-up could not detect it. In either way HR23B will not be capable of recruiting a significant amount of any factors of the UPS to the damaged site. The dissociation of HR23B and HR23A from XPC upon UV might very well increase the availability of HR23B and HR23A genes for other processes such as degradation. A higher effective concentration of HR23B might increase the degradation of certain substrates; thereby regulating indirect consequences of UV induced DNA damage.

Material and methods;

Cell culture

Wild type, XPC K.O. and DKO MEFs were cultured as described previously (Ng et al. 2003). Human MRC5 cells were cultured in a mixture of Ham's F10 and Dulbecco's modified Eagle medium (GIBCOBRL) containing 10% fetal calf serum supplemented with antibiotics at 37ºC and 5% CO2. Cells were transfected using FuGENE (Roche) or lipofectamine. For transient expression experiments medium was changed at least one day before the experiment, to avoid toxicity of the transfection reagents. In order to obtain stably expressing mHR23B-YFPFLAG clones, cells were selected using 150 µg/ml of Hygromycine followed by (single cell) FACS-sorting. Cells were differentially labelled with latex beads of different size by adding a suspension of beads to the culturing medium. Prior mixing cells were thoroughly washed to remove free beads (Vermeulen et al. 1991).

Immunofluorescence

Cells were fixed for 10 min in 2% of paraformaldehyde followed by a 0.1% Triton X-100 wash or for 10 min with ice-cold methanol (for HR23B staining). Cells were washed in PBS 0.5% BSA and 0.15%

glycine. Primary antibody stating was performed over night at 4º, after washing secondary antibody staining was performed followed by washing at room temperature (RT). Cells were preserved in Vectashield Mounting Medium containing 4'-6-diamidino-2-phenylindole (DAPI) in order to visualize DNA. Antibodies used are: anti-hXPC (rabbit polyclonal), anti-mHR23B (rabbit polyclonal), anti-CPD (gift from Dr. O. Nikaido, Kanazawa University, Kanazawa, Japan), anti-p62 (gift from Dr. J.M. Egly) and corresponding secondary antibodies (Molecular Probes and Jackson)

Fusion protein expression vectors

Both mHR23Aand B cDNA's were cloned form mouse livers. mHR23B was fused to a YFP-FLAG construct and mHR23A was fused to a GFP^2-MYC construct. Cloning details are available on request. The XPC-GFP-HAhis construct has been described previously (Ng et al. 2003).

Protein analysis

Immunoblots for XPC, HR23A or HR23B were performed as described previously (Okuda et al. 2004). For immunoprecipitations cell were performed in cell lysates. Cell were harvested in lysis buffer (50 mM tris-HCl at ph 7.5, 150mM NaCl, 1% NP-40, 0.55 sodium deoxycholate, 1 mM DTT, 0.25 mM PMSF, proteasome inhibitors and 10 mM idoacetamine) and centrifuged. The supernatant was over night incubated with anti-FLAG beats (Sigma). Beats were centrifuged and washed (two times) in lysis buffer, with an additional wash in wash buffer ((50 mM tris-HCl at ph 7.5, 0.25mM NaCl, 1% NP-40, 0.55 sodium deoxycholate, 1 mM DTT, 0.25 mM PMSF, proteasome inhibitors and 10 mM idoacetamine). Beats were boiled in sample buffer followed by SDS-PAGE and immunoblotting using the indicated antibodies. Immunoblots were incubated for 30 min at 55˚C in stripping-buffer (62.5 mM Tris at ph 8, 2% SDS and 75mM β-mecaptoethanol) followed by several washes and subsequent imunoblotting.

Confocal-microscopy&photobleaching

Confocal laser scanning microscopy images were obtained on a Zeiss LSM 510 META equipped with cell culture microscopy stage. GFP fluorescence imaging was recorded after excitation with a 488 nm argon laser and a 515-540 nm band pass filter while YFP fluorescence imaging was performed using a 514 nm argon laser and a 535-560 nm band pass filter. Fluorescent redistribution after photobleaching was performed as described previously (Houtsmuller and Vermeulen 2001). Fluorescence within a 0.8 µm strip spanning the nucleus was monitored every 20.5 ms using a Zeiss LSM 510 META and a 488 nm argon laser or a 514 nm laser excitation at 0.5% transmission, after 200 measurements this strip was photo-bleached using 1 iteration at 100% transmission and the influx fluorescence was recorded for another 1000 measurements. The obtained fluorescence recovery curves were normalized to the pre-bleach fluorescence set at 1. FRET analysis combined with unmixing techniques was performed as described previously (Dinant in preparation).

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Chapter 7

DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A

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DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A

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Chromatin changes within the context of DNA repair remain largely obscure. Here we show that DNA damage induces monoubiquitylation of histone H2A in the vicinity of DNA lesions. Ultraviolet (UV)-induced monoubiquitylation of H2A is dependent on functional nucleotide excision repair and occurs after incision of the damaged strand. The ubiquitin ligase Ring2 is required for the DNA damage-induced H2A ubiquitylation. UV-induced ubiquitylation of H2A is dependent on the DNA damage signaling kinase ATR (ATM- and Rad3-related) but not the related kinase ATM (ataxia telangiectasia-mutated). Although the response coincides with phosphorylation of variant histone H2AX, H2AX was not required for H2A ubiquitylation. Together our data show that monoubiquitylation of H2A forms part of the cellular response to UV damage and suggest a role of this modification in DNA repair-induced chromatin remodeling.

[*Keywords*: DNA repair; ubiquitin–proteasome system; histone; chromatin; ATR; H2AX; DNA damage response]

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Proper cellular functioning depends on the maintenance of the genetic and epigenetic information. The integrity of these constituents is, however, constantly jeopardized by genotoxic agents both from endogenous sources such as reactive oxygen species and from exogenous sources including radiation. A complex network of DNA damage response (DDR) mechanisms collectively protects genome integrity, including several DNA repair pathways and damage-induced cell cycle checkpoints (Hoeijmakers 2001; Peterson and Cote 2004). The nucleotide excision repair (NER) machinery is involved in the removal of a wide variety of helix-distorting DNA lesions, such as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts, which are introduced by exposure to ultraviolet (UV) light (de Laat et al. 1999). More than 30 proteins are involved in NER. Xeroderma pigmentosum (XP) patients have a NER deficiency due to mutations in DNA repair proteins and typically suffer from photosensitivity and predisposition to skin cancer. Seven NER genes responsible for XP have been identified, designated *XPA* to *XPG* (de Laat et al. 1999). Depending on the manner in which the lesions are recognized, NER can be subdivided in global genome NER (GG-NER) and transcription-coupled NER (TC-NER). Whereas the latter is confined to transcriptional active regions where a lesion-stalled RNA polymerase sets off the NER response (Citterio et al. 2000), GG-NER is initiated by genome-wide recognition of lesions by the consorted action of the UV-damaged DNA-binding (DDB) complex and the XPC/hHR23B/Cen2 complex (Sugasawa et al. 1998; Araki et al. 2001).

In eukaryotic cells, the DNA is tightly packed in chromatin, consisting of highly organized nucleosomes. The tails of histones are subject to a wide range of post-translational modifications such as acetylation, methylation, phosphorylation, and ubiquitylation and play a central role in the regulation of chromatin activity (Jenuwein and Allis 2001). Transcription, replication, recombination, and DNA repair are intimately connected with histone modifications, which regulate accessibility of the DNA as well as recruitment of important factors. In mammals, the C-terminal tail of histone H2A is a promi-

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nent target for ubiquitin resulting in as much as 5%–15% of H2A being monoubiquitylated (Zhang 2003). The ubiquitin ligase Ring2 was recently shown to be the dominant ubiquitin ligase of histone H2A (de Napoles et al. 2004; Wang et al. 2004). Ubiquitylated H2A (uH2A) is linked to condensed DNA and associated with repression of gene expression (Levinger and Varshavsky 1982), X-chromosome inactivation (de Napoles et al. 2004), and polycomb gene silencing (Wang et al. 2004).

A number of histone modifications are implicated in DNA repair including phosphorylation, acetylation, and methylation (Vidanes et al. 2005). It has been suggested that chromatin is remodeled during DNA repair and transits between different stages that subsequently allow factors to access and repair the lesion after which the original state is restored (Smerdon 1991). Moreover, histone modifications play a pivotal role in DNA damage-induced signaling responsible for activating checkpoints that will pause cell cycle progression until the genome integrity has been restored (Green and Almouzni 2002; Vidanes et al. 2005). A well characterized DNA damage-induced histone modification is phosphorylation of the variant histone H2AX (Thiriet and Hayes 2005). H2AX phosphorylation is dependent on the DDR kinases ataxia telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR) that are activated after double-strand breaks, replication blocks, and UV light, respectively (Shiloh 2003). Although phosphorylated H2AX (γH2AX) has originally been identified as a marker for double-strand breaks (Rogakou et al. 1998), it is also induced in a NER-dependent manner in UV-exposed cells (O'Driscoll et al. 2003). In sharp contrast to the plethora of histone modifications involved in regulation of chromatin activity, H2AX phosphorylation is the only presently known NER-dependent histone modification in higher eukaryotes (O'Driscoll et al. 2003).

It has become clear that ubiquitylation plays a pivotal role in DNA repair (Hoege et al. 2002; Kannouche et al. 2004; Sugasawa et al. 2005). To gain more insight in this exciting link, we set up a system for following ubiquitylation in living cells in response to genotoxic stress. Here, we show that UV exposure of human cells causes a significant NER-dependent monoubiquitylation of histone H2A. This novel DNA damage-induced histone ubiquitylation event reveals an additional link between the ubiquitin system and DNA repair.

Results

DNA damage induces accumulation of ubiquitin

In order to study ubiquitylation in living cells in response to genotoxic stress, ubiquitin was tagged at its N terminus with the green fluorescent protein (GFP). The resulting GFP-ubiquitin (Ub) fusion is functionally competent and properly conjugated to target proteins (Qian et al. 2002; Dantuma et al. 2006). This construct was stably introduced in the human melanoma cell line Mel JuSo. We noticed that upon local irradiation of the nuclei of GFP-Ub cells with an intense 405-nm laser, the initial bleaching of the GFP fluorophore in the exposed region was rapidly followed by local accumulation of fluorescent GFP-Ub indicative for recruitment of GFP-Ub to the laser exposed region (Fig. 1A). Accumulation of GFP-Ub after laser exposure coincided with sequestration of PCNA (proliferation cell nuclear antigen), suggesting a link with DNA repair (see below). To study a more defined type of DNA damage, we inflicted local UV-C damage in subnuclear areas (Katsumi et al. 2001). Locally UV-exposed cells revealed accumulation of GFP-Ub at the damaged areas, which were identified by the presence of CPD lesions (Fig. 1B, top) and colocalized with the early NER damage recognition factor XPC (Fig. 1B, bottom). Importantly, a similar staining pattern was observed for endogenous ubiquitin in locally UV-irradiated wild-type fibroblasts (Fig. 1C), indicating that this behavior is not caused by a nonspecific property of GFP-Ub.

To examine whether ubiquitin is recruited to the site of DNA damage through specific conjugation, we used the mutant ubiquitin $Ub^{K0,G76V}$ fused to GFP. In this conjugation-incompetent mutant Ub, all seven lysine residues, which are potentially involved in the formation of polyubiquitin chains, as well as the C-terminal glycine residue that is crucial for ubiquitin conjugation have been replaced by arginines and valine, respectively (Qian et al. 2002; Dantuma et al. 2006). The mutant GFP-$Ub^{K0,G76V}$ failed to accumulate at the sites of lesions induced by local UV irradiation (Fig. 1D) or laser exposure (data not shown). Thus, DNA damage induced by UV or laser light exposure initiates rapid local ubiquitin conjugation.

DNA damage causes monoubiquitylation without proteasome recruitment

To investigate which type of ubiquitylation was responsible for the observed UV-induced response, we used the mutant GFP-Ub^{K0}. This mutant acts as a chain terminator since it lacks lysine residues to further extend polyubiquitin chains. Therefore, this ubiquitin mutant can efficiently participate in monoubiquitylation events while polyubiquitylation is hindered. We found that both GFP-Ub and GFP-Ub^{K0} readily accumulated in laser-exposed regions (Fig. 2A). Time lapse imaging showed similar kinetics for the accumulation of each construct, suggesting that the laser light-induced ubiquitin accumulation represents predominantly monoubiquitylated species (Fig. 2B).

Ubiquitin and the proteasome are intimately linked in many processes including DNA repair (Glickman and Ciechanover 2002; Wang et al. 2005). However, the proteasome interacts primarily with polyubiquitin chains (Thrower et al. 2000). Consistent with the idea that DNA damage triggered mainly monoubiquitylation, we found that 20S proteasomes carrying the GFP-tagged α3 subunit did not accumulate at local DNA damage (Fig. 2C) nor did we observe a colocalization of endogenous proteasomes in immunostainings (data not shown). Moreover, the mobility of the GFP-tagged proteasomes was not significantly altered in the nuclear compart-

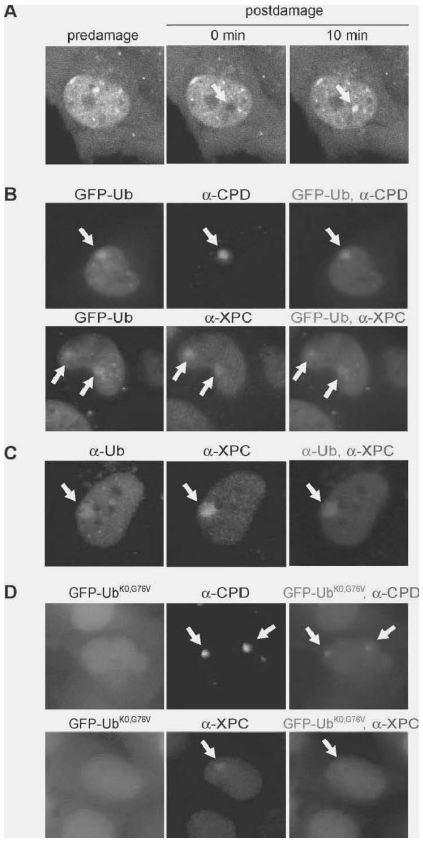

Figure 1. DNA damage induces accumulation of ubiquitin. (*A*) Mel JuSo cells stably expressing GFP-Ub were locally irradiated in the nucleus with a high-intensity 405-nm laser beam. Accumulation of GFP-Ub appeared at the local laser-irradiated spot, indicated by the arrow. (*B*) Mel JuSo cells stably expressing GFP-Ub were locally irradiated with UV-C light. A clear accumulation of GFP-Ub is visible at the local UV-irradiated areas. The arrows indicate the local UV DNA-damaged spot visualized with either the presence of DNA damage by CPD counterstaining (*top middle* panel) or the presence of the NER protein XPC (*bottom middle* panel). Colocalization of GFP-Ub with the DNA damage (*top right* panel) and XPC protein (*bottom right* panel) is shown. (*C*) An antibody recognizing conjugated endogenous ubiquitin revealed accumulation of ubiquitin at local irradiated spots, marked by the presence of XPC protein, in primary human fibroblasts (C5RO). Staining with the ubiquitin-specific antibody (*right* panel), the XPC-specific antibody (*middle* panel), and the merged images (*left* panel) are shown. (*D*) Ubiquitin accumulation at local DNA damage is dependent on conjugation. The nonfunctional mutant GFP-Ub^{K0,G76V} (*left* panel) did not accumulate in Mel JuSo cells at the DNA damage, as detected by CPD (*top middle* image) or the presence of XPC (*bottom middle* image).

ments in response to UV exposure, arguing against substantial recruitment of the 20S proteasome to the lesions (data not shown).

In order to analyze cell cycle dependency of the ubiquitylation response, we cotransfected Mel JuSo cells with GFP-tagged proliferation cell nuclear antigen (GFP-PCNA) and monomeric red fluorescent protein (mRFP)-Ub. GFP-PCNA can be used to distinguish cells in S phase from cells in G1 and G2 phase since it localizes on the replication forks during S phase giving rise to a characteristic punctuate nuclear staining (Leonhardt et al. 2000; Essers et al. 2005). We did not observe any striking differences between non-S-phase and S-phase cells in local mRFP-Ub accumulation (Fig. 2D,E). Notably, also GFP-PCNA, which is involved in various DDR mechanisms, was sequestered in the laser-exposed region, further confirming that the 405-nm laser causes DNA damage. We conclude that the DNA damage-induced monoubiquitylation response occurs throughout the cell cycle.

UV-induced local monoubiquitylation is dependent on functional NER

UV-induced lesions in placental mammals are predominantly repaired in a cell cycle independent manner by NER (Hoeijmakers 2001). Therefore, we investigated whether functional NER was implicated in this process (Fig. 3A). We compared the response of GFP-Ub in wild-type (NER-proficient) and various XP (NER-deficient) fibroblasts. UV-induced ubiquitylation was completely abrogated in two independent XP-C fibroblast cell lines, suggesting that functional GG-NER is required for this ubiquitin modification (Fig. 3B,C; data not shown). The DNA damage accumulation of ubiquitin was also undetectable in XP-A (Fig. 3D), XP-G (Fig. 3E), and XP-F (data not shown) cell lines. The latter two cell lines are deficient in the NER-specific endonucleases that incise the damaged strand 3′ and 5′ of the lesion, respectively. This suggests that the monoubiquitylation occurs during a late NER step.

XP variant (XP-V) patients display a similar pathology as NER deficient patients (Cleaver 2005). However, these patients are not defective in NER but carry instead a mutated DNA polymerase η, one of the translesion (TLS) DNA polymerases involved in replicative bypass of specific DNA lesions (Masutani et al. 1999). In line with a strict NER dependency of the ubiquitylation event, we found that UV exposure of XP-V primary fibroblasts resulted in a similar colocalization of XPC and endogenous ubiquitin on DNA lesions (Fig. 3F), while endogenous ubiquitin did not accumulate on local damage in primary XP-A cells (data not shown).

Global UV irradiation causes NER-dependent immobilization of nuclear ubiquitin

The fact that GFP-Ub conjugation on lesions is clearly traceable in living cells argues that a significant fraction of the GFP-Ub pool may be involved in this process. To obtain quantitative information of the UV-induced ubiquitylation, the overall mobility of the total nuclear ubiquitin pool before and after DNA damage was investigated by photobleaching experiments. In nonchallenged

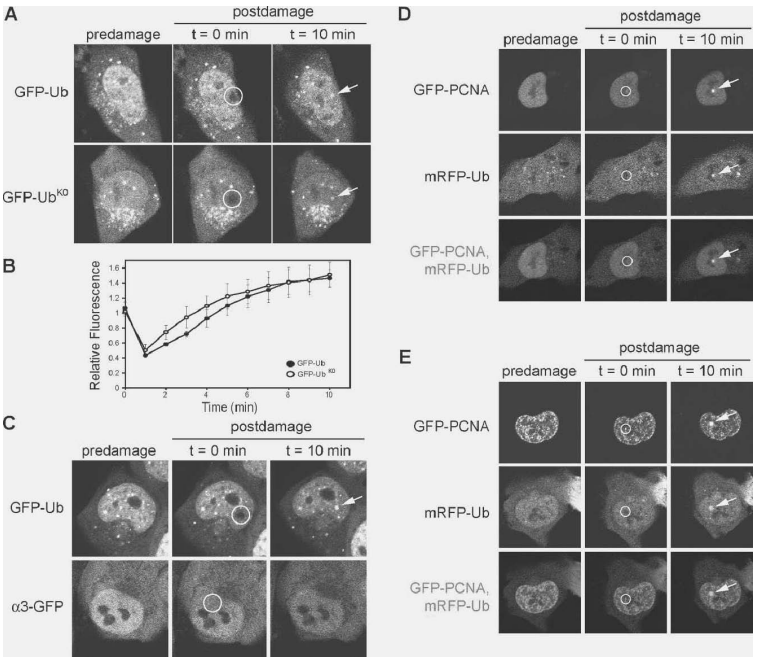

Figure 2. DNA damage causes monoubiquitylation without proteasome recruitment. (*A*) Cells were transfected with either GFP-Ub or the mutant GFP-Ub^{K0}, which can be efficiently monoubiquitylated but hinders polyubiquitylation. Both GFP-Ub and GFP-Ub^{K0} localized at the laser-irradiated areas, which are indicated by the arrows. (*B*) Quantification of fluorescence recovery in the bleached area circled in *A*. Accumulation of GFP-Ub (closed circles) and GFP-Ub^{K0} (open circles) did not differ, indicating that the observed GFP-Ub accumulation is primarily caused by monoubiquitylation. (*C*) Proteasomes tagged with α3-GFP fusion subunit were followed in Mel JuSo cells after high-intensity laser bleaching as in *A*. The circle denotes the bleached spot. The proteasomes did not accumulate at the high laser-irradiated spots. (*D*) The mRFP-Ub fusion accumulates on local laser-induced damage in G1 or G2 cells as was observed by the GFP-PCNA staining. (*E*) Similarly, cells with a typical S-phase-like GFP-PCNA staining were locally damaged using the 405 laser; mRFP-Ub accumulated on these sites.

cells a significant immobile fraction of the nuclear GFP-Ub can be observed by the incomplete recovery of fluorescence in the photobleached area (Fig. 4A). An increase of the immobile pool of GFP-Ub was noted after UV irradiation in a dose-dependent fashion (Fig. 4B). At the maximum dose of 16 J/m^2, we recorded a 43% increase in the levels of immobile GFP-Ub. Importantly, no such UV-induced increase of immobile GFP-Ub was found in NER-deficient XP-A cells (Fig. 4C) and XP-C cells (data not shown), confirming the functional requirement of NER for the local nuclear accumulation of ubiquitin at the damaged area. Cell lines expressing the conjugation-deficient GFP-Ub^{K0,G76V} mutant did not display the nuclear immobile pool nor did we observe a UV-induced immobilization (Fig. 4D).

Histone H2A is the primary target for UV-induced monoubiquitylation

It has been shown that ubiquitylation plays a role in DNA repair (Hoege et al. 2002; Kannouche et al. 2004; Sugasawa et al. 2005). Interestingly, however, none of the previously described UV-induced ubiquitylation targets are likely candidates for the here described monoubiquitylation. XPC and DDB2 are polyubiquitylated following UV-induced DNA damage (Sugasawa et al. 2005). However, ubiquitylation of XPC or DDB2 does not explain our observation since both these ubiquitylation events occur in wild-type and XPA-deficient cells (Sugasawa et al. 2005). Moreover, we were dealing primarily with a monoubiquitylation event (see Fig. 2B). Another potential target would be PCNA, which is both mono- and polyubiquitylated after damage (Hoege et al. 2002; Kannouche et al. 2004). However, monoubiquitylation of PCNA is linked to TLS and not to NER. Furthermore, PCNA monoubiquitylation is unaffected in XP-A and XP-C fibroblasts (Kannouche et al. 2004). In addition, TLS occurs strictly in S phase, whereas the here described ubiquitylation happens throughout the entire cell cycle (Fig. 2D,E)

We argued that given the dramatic effect on the mobility of nuclear ubiquitin, an abundant immobile nuclear protein would be a likely target. Since histones are immobile, rather abundant and a prominent target for monoubiquitylation (Zhang 2003), we investigated whether UV exposure changed the ubiquitylation status of histones. Probing isolated histones from untreated and UV-irradiated wild-type fibroblasts with a ubiquitin antibody revealed a clear increase in a band of 23 kDa corresponding in size to monoubiquitylated H2A (Fig. 5A). The same band reacted with an antibody directed against uH2A (Fig. 5B) and with an H2A-specific antibody (data not shown). Analysis of a tryptic digest from the corresponding band by liquid chromatography tandem mass spectroscopy revealed three peptides originating from ubiquitin and two peptides originating from H2A (Fig. 5C). Immunocytochemical analysis of locally inflicted UV lesions in wild-type fibroblasts with the uH2A-specific antibody confirmed the enrichment of endogenous uH2A at the damaged area (Fig. 5D), while locally damaged areas in XP-A fibroblasts were not marked by this antibody (Fig. 5E).

Normal nondamage-induced monoubiquitylation of histone H2A occurs at Lys 119 (Nickel and Davie 1989).

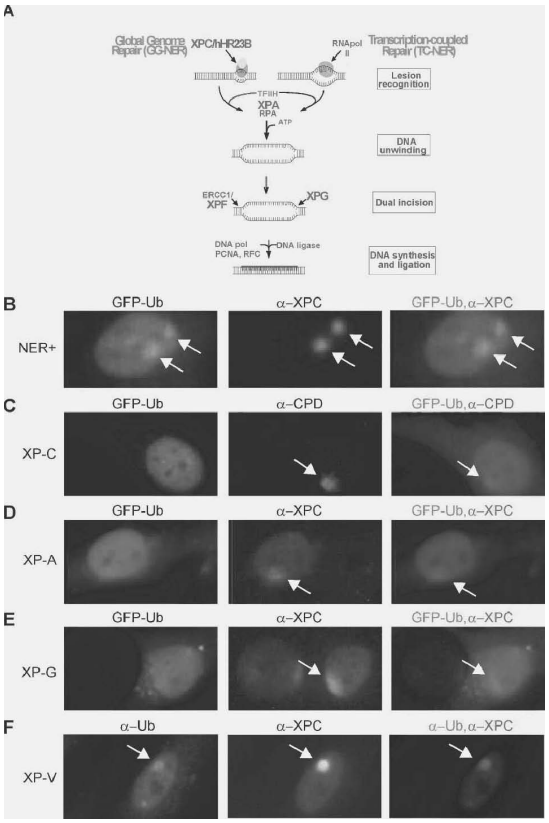

Figure 3. UV-induced local monoubiquitylation is dependent on functional NER. (*A*) A schematic drawing of the NER pathway is shown. NER factor deficiencies that have been tested in this study are indicated in red. NER lesion recognition is predominantly performed by the XPC complex, after which the TFIIH complex and XPA and RPA are recruited, probably proceeded by XPG. The DNA helix is unwound by the TFIIH complex in an ATP-dependent fashion. The two endonucleases, XPG and XPF-ERCC1, excise the lesion. After incision, the GAP is filled in by DNA polymerase δ/ε, PCNA, and RFC and is sealed by ligase I (Hoeijmakers 2001). (*B*) In normal fibroblasts (C5RO-hTERT), GFP-Ub (*left* panel) accumulated at local damaged spots, indicated by the arrow. (*C*) In XP-C human fibroblast (XP21RO-Sv), GFP-Ub (*left* panel) did not accumulate at local damaged spots as detected by CPD (*middle* panel). (*D*) GFP-Ub (*left* panel) did not accumulate in XP-A cells (XP12RO-Sv) at NER active sites as detected by XPC (*middle* panel). (*E*) In XP-G (XP3BR-Sv), GFP-Ub (*left* panel) did not accumulate at local UV-irradiated spots as indicated by the presence of XPC protein (*middle* panel). (*F*) In primary NER proficient XP-V cells (XP1RO), deficient in TLS polymerase η, ubiquitin accumulated at the UV-induced lesions.

To determine whether this lysine residue was also targeted for ubiquitylation upon UV exposure, we expressed Flag-tagged H2A (^{Flag}H2A) and Flag-tagged H2A in which Lys 119 had been substituted by an arginine residue (^{Flag}H2A^{K119R}) and analyzed ubiquitylation of the ectopically expressed proteins. We found that ^{Flag}H2A was monoubiquitylated while ^{Flag}H2A^{K119R} was not (Fig. 5F). More importantly, UV exposure enhanced ubiquitylation of ^{Flag}H2A but did not induce ubiquitylation of ^{Flag}H2A^{K119R} (Fig. 5F). This indicates that the same lysine residue of H2A that is ubiquitylated under nonchallenging conditions is also targeted in DNA damage-induced monoubiquitylation.

The ubiquitin ligase Ring2 is required for DNA damage-induced H2A ubiquitylation

It has been recently reported that Ring2 is the dominant ubiquitin ligase involved in ubiquitylation of H2A on repressed DNA (de Napoles et al. 2004; Wang et al. 2004). To test whether the same ubiquitin ligase is responsible for the DNA damage-induced ubiquitylation, we generated a vector that encoded Ring2-specific RNA interference (RNAi), based on a previously published Ring2-specific RNAi construct (Wang et al. 2004), and an mRFP marker to identify cells that express the RNAi vector.

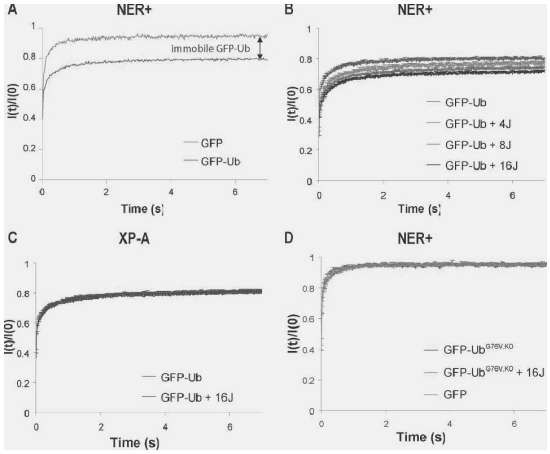

Figure 4. Global UV irradiation causes NER-dependent immobilization of nuclear ubiquitin. (*A*) FRAP analysis of GFP and GFP-Ub in nuclei of wild-type C5RO-hTERT cells. The immobile pool of GFP-Ub is indicated. (*B*) FRAP analysis of GFP-Ub in nuclei of wild-type C5RO-hTERT cells. Cells were left untreated or exposed to 4, 8, and 16 J/m^2 UV light. Approximately 21% of the total nuclear ubiquitin pool was immobilized in wild-type C5RO-hTERT human fibroblasts. UV increased the immobile fraction in a dose-dependent manner to 24%, 27%, and 30% immobilization after 4, 8, and 16 J/m^2, respectively ($n > 24$). Note that the FRAP curve of untreated GFP-Ub cells is the same data set as seen in *A*. (*C*) FRAP analysis of nuclear GFP-Ub in the nuclei of NER-deficient XP-A cells (XP12RO-Sv). Note that the UV damage-induced immobilization was not observed in XP-A cells (mean of 25 cells). (*D*) FRAP analysis of GFP-Ub^{K0,G76V} and GFP in the nuclei of wild-type C5RO-hTERT cells. The dynamics of the mutant GFP-Ub^{K0,G76V} and GFP (mean of 16 cells) in C5RO-hTERT cells were similar. No difference was detected in mobility before and after UV exposure (mean of 16 cells).

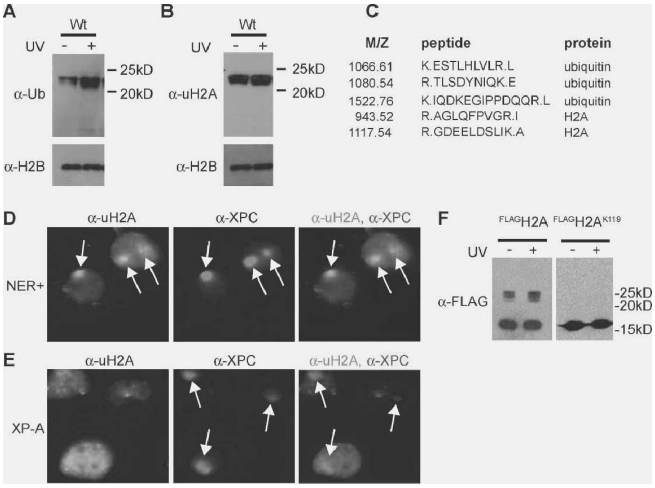

Figure 5. UV irradiation causes NER-dependent ubiquitylation of histone H2A. (*A*) Core histones were purified before and after UV (16 J/m^2) exposure from NER-proficient (MRC5-Sv) and probed with an antibody directed against ubiquitin. An increase in a 23-kDa protein reacting with antibodies against ubiquitin was observed. (*B*) The samples shown in *A* probed with an antibody specific for uH2A. Blots in the *bottom* panels of *A* and *B* were stripped and reprobed with H2B-specific antibody. (*C*) Peptides identified by liquid chromatography tandem mass spectroscopy from the *top* band shown in *A*. MRC5-Sv (NER-proficient) (*D*) and XP12RO-Sv (XP-A, NER-deficient) (*E*) were locally UV-irradiated and stained with uH2A- and XPC-specific antibodies. uH2A accumulated in NER-proficient cells (*top* panels) at the damaged spot but not in XP-A cells (*bottom* panels). (*F*) Core histones were purified before and after UV (16 J/m^2) treatment from cells ectopically expressing ^{Flag}H2A or ^{Flag}H2A^{K119R}. Western blots probed with anti-Flag. An increase in the band corresponding to ubiquitylated ^{Flag}H2A was detected after UV treatment, while no ubiquitylated ^{Flag}H2A^{K119R} before or after UV treatment was detected.

Immunostainings confirmed that this RNAi construct successfully knocked down expression of Ring2 in Mel JuSo cells (data not shown). GFP-Ub-expressing cells transfected with the RNAi construct displayed reduced levels of nuclear GFP-Ub in line with a decrease in ubiquitylated histones (Fig. 6). Importantly, the DNA damage-induced ubiquitylation was strongly reduced in the cells expressing Ring2 RNAi as compared with untransfected cells (Fig. 6). Similar results were obtained in cells with locally UV-inflicted damage (data not shown). Thus, monoubiquitylation of H2A in response to DNA damage is dependent on the H2A ubiquitin ligase Ring2.

H2A ubiquitylation does not require variant histone H2AX but is dependent on ATR

In addition to double-strand breaks also the process of NER can trigger phosphorylation of variant histone H2AX, currently the only known NER-induced histone modification (O'Driscoll et al. 2003). Immunostaining revealed that uH2A and γH2AX often colocalized on the local UV damage (Fig. 7A). Since it is not uncommon that different types of histone modifications are mechanistically linked (Jenuwein and Allis 2001; Zhang 2003), we tested whether ubiquitylation of H2A was dependent on NER-induced phosphorylation of H2AX. To that aim, we expressed GFP-Ub in fibroblast cells derived from H2AX-deficient and wild-type mouse embryos. These cell lines were transiently transfected with the GFP-Ub expression constructs. Both cell lines accumulated GFP-Ub in laser-damaged areas (Fig. 7B). Since these cells completely lack H2AX, we can exclude the possibility that ubiquitylation of H2A depends on phosphorylation of H2AX.

DNA damage-induced signaling is an important event in the control of the cellular response to DNA damage. The kinases ATM and ATR are important players in DDR that are activated upon (DNA) stress and phosphorylate downstream targets (Shiloh 2003). To determine whether these kinases are important for the induc-

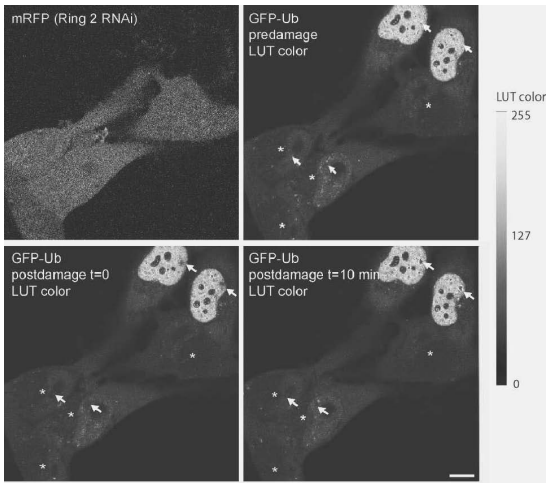

Figure 6. The ubiquitin ligase Ring2 is required for DNA damage-induced H2A ubiquitylation. GFP-Ub expressing Mel JuSo cells were transfected with a plasmid encoding a Ring2-specific RNAi and mRFP. (*Top left* panel) The transfected cells could be identified by mRFP expression (grayscale). This picture shows four representative transfected cells (asterisks) and two untransfected cells. (*Top right* panel) Ring2 RNAi transfected cells had reduced nuclear GFP-Ub levels (LUT [Look Up Table] color). (*Bottom left* panel) Laser damage was induced in two untransfected cells and two RNAi transfected cells (arrows) (LUT color). (*Bottom right* panel) The untransfected cells showed a clear accumulation of GFP-Ub in the laser exposed region whereas the response was very low in both Ring2 RNAi transfected cells (LUT color). The LUT for the color coding is shown at the *right*. Note that blue refers to overexposed on the LUT scale.

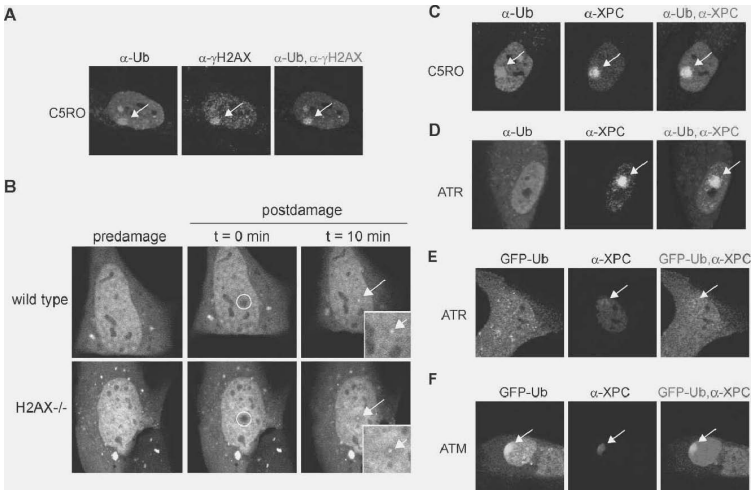

Figure 7. H2A ubiquitylation does not require variant histone H2AX but is dependent on ATR. (*A*) Local DNA damage was caused by UV light in CRO5 cells. Phosphorylated H2AX and accumulation of ubiquitin colocalized on the lesion. (*B*) Wild-type and *H2AX*$^{-/-}$ MEF cells were transfected with GFP-Ub. After 405-nm laser damage, GFP-Ub localized at the laser-irradiated areas, which are indicated by the arrows. Local DNA damage was inflicted in C5RO (NER-proficient) cells and *ATR* mutant cells. In C5RO cells (*C*), but not in *ATR* mutant cells (*D*), endogenous ubiquitin accumulated on the lesions. *ATR* (*E*) and *ATM* (*F*) mutant cell lines were transiently transfected with GFP-Ub. The lesions were identified by XPC staining. In the ATM cells, but not in the ATR cells, GFP-Ub was found enriched on the DNA damage.

tion of uH2A upon UV exposure, we locally inflicted DNA damage in mutant cells of each kinase. Interestingly, whereas UV exposure triggered ubiquitin accumulation in wild-type fibroblast (Fig. 7C), accumulation of endogenous ubiquitin (Fig. 7D) or GFP-Ub (Fig. 7E) was less evident or completely absent in a patient cell line with reduced ATR expression (O'Driscoll et al. 2003). However, in cells deficient in ATM, which is primarily involved in double-strand break-induced signaling, GFP-Ub was still sequestered at the lesions (Fig. 7F). This indicates that DNA damage-induced signaling through ATR stimulates the induction of uH2A upon DNA damage, analogous to the situation for γH2AX.

Discussion

In this study, we show that UV-induced DNA damage induces monoubiquitylation in close proximity to DNA lesions. We identified histone H2A as the primary target in this NER-dependent ubiquitylation response. It appears that the DNA damage-induced monoubiquitylation event is identical to the constitutive ubiquitylation of H2A involved in gene silencing, since the same target lysine residue is ubiquitylated and this ubiquitylation is dependent on Ring2, the ubiquitin ligase responsible for the bulk of H2A ubiquitylation.

To our knowledge, ubiquitylation of H2A is, next to phosphorylation of the variant histone H2AX, the second covalent modification of histones that is induced in an NER-dependent manner in mammalian cells. There are several striking similarities between DNA damage-induced γH2AX and uH2A. First, both histone modifications are in UV-exposed, non-S-phase cells strictly dependent on functional NER (O'Driscoll et al. 2003). Second, the DNA damage signaling kinase ATR is required for both histone modifications. Third, similar to γH2AX, H2A ubiquitylation spreads over a confined distance (see below), which is in the same order of magnitude as γH2AX (Shroff et al. 2004).

One of the most striking features of this UV-induced ubiquitylation is the extent of the modification. It is well established that ubiquitylation of several repair proteins is triggered by UV exposure; none of these ubiquitylation events passes the detection limit in our live cell imaging experiments. Although XPC, DDB2, and PCNA poly- and monoubiquitylation still occur upon DNA damage in NER-deficient XP-A cell lines, we were unable to detect accumulation of ubiquitin at the DNA lesions in these mutant cells. This is in itself not surprising given the vast amount of ubiquitylated proteins in the nuclear environment. The UV-induced ubiquitylation of H2A, on the contrary, induces accumulation of ubiquitin that can be readily monitored in living cells by fluorescence microscopy. The observation that global UV exposure significantly immobilizes ubiquitin in the nucleus underscores the massive nature of this event. Based on the amount of immobilized ubiquitin upon global UV irradiation and the rate of NER-mediated lesion removal, we have estimated that for each repaired DNA lesion 50–150 H2A molecules are monoubiquitylated (Supplemental Material). This would correspond with nucleosomes being modified over a region of 10–30 kb for each lesion.

Two independent lines of evidence in our study clearly support that DNA damage H2A ubiquitylation occurred downstream from the assembly of the NER machinery. First, immunostainings show that UV-induced damage causes local accumulation of uH2A in NER-proficient cells but not in any of the NER-deficient cells, including cell lines with mutations in NER factors that act late in the reaction. Second, FRAP analysis revealed that global UV damage causes NER-dependent immobilization of a substantial pool of ubiquitin in living cells, in line with ubiquitylation of an abundant immobile nuclear target (e.g., histones). Notably, this UV-induced immobilization of ubiquitin was not observed in XP-A cells. While this paper was under review, Kapetanaki et al. (2006) reported that the UV-irradiation induced a quick reduction of uH2A and that the ligase complex DDB1–

$Cul4A^{DDB2}$ was required to restore levels of uH2A at lesions, which is believed to be important for lesion recognition. Our data do not support a role for the DDB1–$Cul4A^{DDB2}$ ubiquitin ligase in the UV-induced histone H2A ubiquitylation described herein. We observed that UV-induced H2A ubiquitylation is absent in XP-A, XP-F, XP-G, and ATR cells whereas it has been reported that ubiquitylation of other targets at the NER site of DDB1–$Cul4A^{DDB2}$ still occur in these cells (Sugasawa et al. 2005). Moreover, we show that H2A ubiquitylation is induced after UV exposure and that this process is dependent on the ubiquitin ligase Ring2. It is however not excluded that different H2A ubiquitylation events are connected to the NER pathway, one mediated by DDB1–$Cul4A^{DDB2}$ and occurring early in NER and a Ring2-mediated induced in a late NER step of which the former has escaped notification in our system possibly due to its very transient existing.

Studies in yeast suggest a role for histone ubiquitylation in G1/S checkpoint signaling after UV-induced DNA damage (Giannattasio et al. 2005). Notably, in the yeast nucleosome the histone H2B is the primary ubiquitylation target instead of H2A as in mammalian cells. Yeast strains expressing the mutant histone $H2B^{K123R}$ that cannot be ubiquitylated are viable and not overtly sensitive to UV light (Robzyk et al. 2000). However, UV-induced phosphorylation of Rad53 is abrogated in the $H2B^{K123R}$ strain resulting in a failure to block DNA replication (Giannattasio et al. 2005). Thus, it is tempting to speculate that γH2AX and uH2A are part of the same post-repair epigenetic histone code involved in DNA damage signaling. Importantly, we found that ubiquitylation of H2A does not require the presence of H2AX, suggesting that these modifications are independently regulated. Given the similarities between ubiquitylation of H2A and phosphorylation of H2AX and the pivotal role of γH2AX in double-strand break repair (Thiriet and Hayes 2005), it will be interesting to study whether ubiquitylation is restricted to NER or also plays a more general role in different forms of DNA repair.

The novel link between histone ubiquitylation and UV-induced DNA damage revealed in this study highlights the importance of nucleosome remodeling and modifications in NER. A better understanding of the significance of post-translational histone modifications in NER may shed some light on the enigmatic link between the status of histones, chromatin remodeling, and DNA repair.

Materials and methods

Cell culture

We generated stable cell lines by transfecting C5RO-hTERT (human NER-proficient fibroblast) and the SV40 immortalized human fibroblasts XP20MA-Sv (XP-C), XP12RO-Sv (XP-A), and Mel JuSo with the GFP-Ub and GFP-$Ub^{K0,G76V}$ constructs. Stably expressing cells were isolated after subsequent selection of neomycin resistance and selection of GFP expression by flow cytometry-activated cell sorting. GFP-Ub and GFP-Ub^{K0} were transiently expressed in HeLa, MRC5-Sv (NER-proficient human fibroblast), XP21RO-Sv (XP-C), XP3BR-Sv (XP-G), and XP2YO-Sv (XP-F) cells. Cells were cultured in a mixture of Ham's F10 and Dulbecco's modified Eagle medium (GIBCO-BRL) containing 10% fetal calf serum (FCS) supplemented with antibiotics at 37°C and 5% CO_2. Primary human fibroblasts, C5RO, C7RO (both wild-type human fibroblasts), XP25RO (XP-A), XP1RO (XP-V), AT2RO (ATM), and GM18366 (fibroblasts with low level of ATR protein, obtained from Coriell Institute), used for immunofluorescence, were cultured on coverslips in Ham's F10 medium containing 15% FCS and supplemented with antibiotics. Murine $H2AX^{-/-}$ and wild-type embryonic fibroblasts cell lines were generated from crosses of heterozygotes $H2AX^{+/-}$ (Celeste et al. 2002) (gift from Dr. André Nussenzweig, National Cancer Institute, Bethesda, MD) (approved by ethical committee in Stockholm; permission no. N118/05). Treatment with UV light was performed with 254-nm (UV-C) light using a germicidal lamp at the indicated doses. Prior to local and overall UV irradiation, cells were rinsed with phosphate-buffered saline. For local UV irradiation, cells were grown on coverslips and were covered with 5- or 8-μm pore-containing membranes (Millipore) and irradiated with 65 J/m^2.

Constructs

The previously described RNAi sequence for Ring2 (Wang et al. 2004) was inserted in the BglII and HindIII site of pSUPER-mRFP. This vector is a variant of the RNAi-expression vector pSUPER (Brummelkamp et al. 2002). The sequence of the expressed RNAi is 5′-TTCCAAAAAGGCTAGAGCTTGATAATAATCTCTTGAATTATTATCAAGCTCTAGCCGGG-3′ (Ring2 target sequence underlined). Wild-type ubiquitin (Ub), ubiquitin in which all lysine residues had been substituted by arginines (Ub^{K0}), and ubiquitin in which all lysine residues and the terminal glycine residue had been substituted by arginines and a valine, respectively ($Ub^{K0,G76V}$), were cloned in the vector EGFP-C1 (Clontech) using the restriction sites Asp718 and HindIII. The GFP-PCNA construct is a gift from Dr. Jeroen Essers (Erasmus Medical Center, Rotterdam, The Netherlands). ^{Flag}H2A was obtained by subcloning GFP-H2A (a gift from Dr. Pierre-Yeves Perche, INSERM U309, Institut Albert Bonniot, La Tronche cedex, France) in pcDNA3.1Flag. The codon encoding Lys 119 in ^{Flag}H2A was substituted for an arginine codon with QuikChange XL site-directed mutagenesis (Stratagene) using the following primers: K119R Fw: 5′-CGTGCTACTGCCCAAGAgGACCGAGAGCCACCACAAGG-3′; Rv: 5′-CCTTGTGGTGGCTCTCGGTCCTCTTGGGCAGTAGCACG-3′.

Immunofluorescence microscopy

Cells were fixed with 2% paraformaldehyde in the presence of 0.2% Triton X-100. For uH2A antibody staining, cells were incubated with 0.5% Triton X-100 prior to fixation (de Napoles et al. 2004). Samples were processed as described previously (Rademakers et al. 2003). Immunofluoresecent images were obtained by the Aristoplan Flu 134,795 (Leitz). The following antibodies were used: anti-hXPC (rabbit polyclonal anti-human XPC), anti-CPD (gift from Dr. O. Nikaido, Kanazawa University, Kanazawa, Japan), anti-uH2A (Upstate Biotechnology 05-678), 20S sampler pack (Biomol), anti-ubiquitin (FK2; Affiniti), anti-γH2AX (Upstate Biotechnology 07-164), anti-Ring2 (gift from Dr. M. van Lohuizen, The Netherlands Cancer Institute, Amsterdam, The Netherlands), and corresponding secondary antibodies (Molecular Probes and Jackson Laboratory).

Live cell confocal laser scanning microscopy

Confocal laser scanning microscopy images were obtained on a Zeiss LSM 510 META equipped with cell culture microscopy

stage. GFP fluorescence imaging was recorded after excitation with a 488-nm argon laser and a 515–540-nm band-pass filter. Fluorescent redistribution after photobleaching was performed as described previously (Houtsmuller and Vermeulen 2001). Fluorescence within a 0.8-µm strip spanning the nucleus was monitored every 20.5 msec using a Zeiss LSM 510 META and a 488-nm argon laser excitation at 0.5% transmission; after 200 measurements this strip was photobleached using one iteration at 100% transmission and the influx fluorescence was recorded for another 1000 measurements. The obtained fluorescence recovery curves were normalized to the prebleach fluorescence set at 1. For live cell imaging of laser-induced DNA damage, cells were irradiated in a spot using 100% 405 nm for 2000 iterations. Directly after irradiation cells were monitored every minute for 10 min. Fluorescent intensities were measured using ImageJ software. Accumulation was calculated by averaging the fluorescent ratio between exposed and nonexposed areas in the nucleus.

Histone purification

Cells were harvested 30 min post-irradiation (16 J/m^2 of UV) or mock treatment by scraping and subsequent centrifugation in ice-cold phosphate-buffered saline. Histone1 and high-mobility group proteins were isolated using 5% perchloric acid extraction. Extraction was repeated three times; the supernatants were separated from the pellet by centrifugation. From the remaining pellet core histones were dissolved in 0.4 M HCl; extraction was repeated three times. Pellet and supernatant were separated using centrifugation. Pooled supernatants were precipitated using 25% trichloroacetic acid. After precipitation pellets were washed with acetone and dried using a speedvac (Giancotti et al. 1985). All steps were performed at 4°C. All buffers contained protease inhibitor cocktail (chymostatin, leupin, antipain, and pepstatin A) and 10 mM iodoacetamide to inhibit deubiquitylation activity. Samples were run on 15% polyacrylamide gels and transferred to 0.1-µm pore nitrocellulose membranes. Membranes were incubated with anti-H2A (Upstate Biotechnology 07-146), anti-H2B (Upstate Biotechnology 07-266), anti-ubiquitin (FK2), or anti-Flag (Sigma) followed by corresponding secondary antibody incubation (Amersham NA934, DAKO P0161, or Biosource AMI4604) and detected using enhanced chemiluminescence (Amersham).

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The number of ubiquitylated H2A molecules was calculated based on two plausible assumptions. 1) The immobile fraction as observed by FRAP (**Fig. 4A**) is predominantly caused by histone monoubiquitylation, mostly consisting of H2A (the H2B ubiquitylation is ignored for its contribution since only ~1% of H2B is ubiquitylated (West and Bonner 1980)) and 2) the relative incorporation of GFP-Ub on H2A is comparable to the relative levels of endogenous ubiquitylation on H2A. The maximum number of H2A molecules within these cells can be estimated by calculating the maximum amount of nucleosomes present in diploid mammalian cells, which is: ~$6*10^9$ nucleotides [human genome size] divided by 200 nucleotides [i.e. amount of DNA wrapped around one nucleosome including the internucleosome spacer] leaving ~$3*10^7$ nucleosomes accommodating ~$6*10^7$ H2A molecules. The total amount of ubiquitylated H2A is 5-15% (Jason et al. 2002).This comes down to 3-$9*10^6$ uH2A molecules. Upon overall UV exposure (16 J/m^2), there is an approximate 43% (from 21% to 30% in FRAP experiments) increase in uH2A, resulting in a total amount of 4.3-$12.9*10^6$ uH2A molecules. From which 1.3 to 3.9 *10^6 uH2A are directly caused by NER (total level of uH2A after UV subtracted the normal level of uH2A). Since the UV induced ubiquitylation of H2A is strictly dependent on functional NER, ubiquitylation will only take place at sites repaired by NER. Within the used time frame (30 min) and the used dose (16 J/m^2) roughly $25*10^3$ lesions are repaired by (Suquet et al. 1995). This corresponds to between 52 and 156 uH2A molecules per repaired lesions. Every 200 base pairs contains one nucleosome. Assuming that every adjacent nucleosome contains 1 uH2A (2 uH2A molecules per nucleosome are rare (Levinger and Varshavsky 1980)), the uH2A signal spreads between 10.4 and 31.2 Kb away from the lesion.

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Chapter 8

The DNA repair-ubiquitin associated HR23 proteins are constituents of neuronal inclusions in specific neurodegenerative disorders without hampering DNA repair.

The DNA repair-ubiquitin associated HR23 proteins are constituents of neuronal inclusions in specific neurodegenerative disorders without hampering DNA repair.

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Abstract

Intracellular inclusions play a profound role in many neurodegenerative diseases. Here we report that HR23B and HR23A, proteins that are involved in both DNA repair and shuttling proteins to the 26S proteasome for degradation, accumulates in neuronal inclusions in brain from a mouse model for FXTAS, as well as in brain material from HD, SCA3, SCA7, FTDP-17 and PD patients. Interestingly, HR23B did not significantly accumulate in tau-positive aggregates (neurofibrillary tangles) from AD patients while ubiquitin did. The sequestration of HR23 proteins in intracellular inclusions did not cause detectable accumulation of their stable binding partner in DNA repair, XPC. Surprisingly, no reduction in repair capacity was observed in primary human fibroblasts that over expressed GFP-polyQ, a polypeptide that induces HR23B-positive inclusions in these transfected cells. This illustrates that impairment of the ubiquitin proteasome system (UPS) by expanded glutamine repeats, including the sequestration of HR23B, is not affecting NER.

Introduction

In mammals two Homologues of the yeast gene Rad23 exists, designated HR23A and HR23B. Both have a function in DNA repair and in the ubiquitin proteasome system (UPS) (Schauber, *et al.* 1998). In DNA repair, part of the HR23 proteins are in complex with XPC and function in the DNA damage recognition step of the global genome sub-pathway of nucleotide excision repair (GG-NER), which recognizes and removes helix-distorting lesions from the entire genome (Ng, *et al.* 2003, Sugasawa, *et al.* 1998, Sugasawa, *et al.* 1997). Both HR23A and HR23B stabilize the XPC protein *in vivo* and increase the affinity for damaged DNA of XPC *in vitro* (Li, *et al.* 1997, Lommel, *et al.* 2002, Ng, *et al.* 2003, Sugasawa, *et al.* 1996). It has been demonstrated that Rad23 interacts with the 26S proteasome and with ubiquitylated proteins as well (Elsasser, *et al.* 2004, Saeki, *et al.* 2002). In addition to a repair function, HR23B is also essential for development as indicated by the dramatic phenotype of the HR23B knock out mouse, including impaired embryonic development and high rates of intrauterine death. In contrast, mouse models defective in XPC develop relatively normal and only exhibit a profound NER deficiency (Cheo, *et al.* 1997, Ng, *et al.* 2002, Sands, *et al.* 1995). HR23B knock out mice are smaller, display facial dysmorphology and male infertility but are GG-NER proficient due to the functional redundancy with HR23A (Ng, *et al.* 2002). In contrast, HR23A knock-out mutants are indistinguishable from wild type, because of complete functional compensation by HR23B (Ng, *et al.* 2003).

Ubiquitylation of proteins is a general protein modification involved in many processes such as endocytosis, transcription, antigen presentation and protein degradation (Glickman, *et al.* 2002, Ravid, *et al.* 2004). Ubiquitylation of target proteins is performed by the sequential action of the ubiquitin-activating enzyme (E1), one of a series of ubiquitin-conjugating enzymes (E2) in combination with a specific ubiquitin ligase (E3). Ubiquitin is usually covalently attached to internal lysine residues of the target protein. Since ubiquitin contains several internal lysines itself, a polyubiquitin tree can be formed (Glickman, *et al.* 2002). It is generally believed that proteins containing a lysine 48-branched ubiquitin tree are destined for degradation by the 26S proteasome. Proteins that contain an ubiquitin-interacting domain like the ubiquitin-associated domain (UBA) recognize ubiquitylated proteins creating new interactions and subsequent actions (Verma, *et al.* 2004). Interestingly, both HR23A and HR23B contain besides an amino terminal ubiquitin-like domain (UBL), known to interact with the proteasome (Elsasser, *et al.* 2002, Saeki, *et al.* 2002), also two UBA domains. Recently it has been shown that the carboxy terminal UBA domain of yeast Rad23 has a relatively high affinity for lysine 48 polyubiquitin trees (Raasi, *et al.* 2004, Ryu, *et al.* 2003, Varadan, *et al.* 2005), whereas Rad23 itself is protected from degradation by an intrinsic stabilization signal (Heessen, *et al.* 2005). This, together with the capacity to interact with the proteasome makes HR23A and HR23B ideal as shuttles/chaperones for proteins to be degraded by the proteasome. In fact, it has been shown that Rad23, and others, are shuttles of the 26S proteasome (Varadan, *et al.* 2005, Verma, *et al.* 2004). Misregulation of protein degradation via the UPS is directly or indirectly involved in many human diseases including malignancies, auto immune diseases and neurodegenerative disorders (Glickman, *et al.* 2002).

The formation of inclusions in specific parts of the brain is a pathological hallmark of many neurodegenerative diseases (Ciechanover*, et al.* 2003). Actually, the specific localization and the composition of these inclusions has been utilized as a diagnostic criterium of several neurodegenerative disorders, including frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), Parkinson disease (PD), Huntington disease (HD), Spinocerebellar ataxia (SCAs), and fragile X associated tremor/ataxia syndrome (FXTAS). Major constituent of the inclusions is often a disease-specific mutant protein (e.g. tau, α-synuclein, huntingtin) or mutant mRNA (e.g. FMR1 mRNAs in FXTAS). For instance, an important group of neurodegenerative disorders are the polyglutamine diseases characterized by neuronal intranuclear inclusions that consist of accumulations of insoluble aggregated polyglutamine-containing proteins. Next to mutant protein, UPS components and molecular chaperones have been detected within the inclusions, including ubiquitin and the 20S catalytic core complex of the proteasome.

Many neurodegenerative diseases share these ubiquitin-positive structures; however, their origin and function in relation to the pathology remain unclear. Although, ubiquitin-positive inclusions are the pathological hallmark of many neurodegenerative disorders it might very well be that they are secondary responses to different causes (Ciechanover*, et al.* 2003). Inclusions have been found to positively influence cell viability supporting a protective function for the inclusions (Arrasate*, et al.* 2004, Bowman*, et al.* 2005). Therefore it might be that they arise as a consequence of cellular stress, thereby trying to solve a toxicity problem in the cell. Indeed, it has been shown that accumulation of mutant proteins containing long polyglutamine tracts can cause impairment of the UPS leading to cellular stress in living cells (Bence*, et al.* 2001). In neurodegenerative diseases such as HD and SCA 1-7, expanded polyglutamine tracts in target proteins are known to be the primary source of the inclusions (Ciechanover*, et al.* 2003). Purified 26S proteasomes fail to digest these long repeats and release them instantly (Venkatraman*, et al.* 2004). Consequently, polyglutamine-containing (mutant) proteins accumulate in time, especially in non-dividing neurons, ultimately leading to neuronal cell death. In mouse models mimicking human SCA7 no proof for UPS impairment prior to or during onset of the disease was found, suggesting that the impairment is a secondary consequence (Bowman*, et al.* 2005).

The issue whether the formation of inclusions is a cause or an effect of toxicity is of great importance for understanding the pathogenesis of these diseases. Knowledge of the formation and of the constituents of inclusions, regardless if they have a positive or a negative influence on cell viability, can ultimately lead to the development of novel therapeutic interventions. Since HR23A and HR23B have a prominent role in shuttling proteins to the proteasome we wondered if HR23A and/or HR23B participate in the formation of inclusions.

Material and Methods

Western blotting

Wild type and HR23B-/- mouse embryonic fibroblasts were lysed as described previously (Okuda*, et al.* 2004). HR23B protein was detected using either fresh or pre-absorbed rabbit polyclonal antibodies against HR23B. Pre-absorption was performed by incubation of the fresh serum with nitrocellulose paper containing homogenates of HR23B-/- cells during overnight at 4^{o} C. Horseradish peroxidase

(HRP)-labelled anti-rabbit IgG serum was used as secondary antibody, allowing chemiluminescence detection with ECL (Amersham).

Immuno-neuropathology

Human brain autopsy was carried out within 24 hours after death according to legal and ethical guidelines. The brain was fixed in 10% buffered-formalin solution for 6 weeks. In addition, brain tissue from the expanded-CGG mice and E13.5 embryos from WT and HR23B KO mice (Ng*, et al.* 2002) were fixed in 3% paraformaldehyde and further processed. Paraffin-embedded sections of all brain regions underwent routine staining with haematoxylin-eosin (HE), Bodian and Congo red. From well-diagnosed cases on both clinical and neuropathological criteria (FTDP-17, PD, HD, SCA3, SCA7 and AD) specific brain regions known to contain inclusions were further analyzed for both ubiquitin and HR23B localization. In addition, brains were dissected from a mouse model for FXTAS (Willemsen*, et al.* 2003) and immediately fixed in 3% paraformaldehyde for 24 hours followed by routine paraffin embedding.

Immunohistochemistry with rabbit antibodies directed against HR23B, HR23A (Okuda*, et al.* 2004), XP-C (van der Spek*, et al.* 1996) or ubiquitin (1:500, Dako, Glostrup, Denmark) was performed according to standard protocols. Briefly, sections were deparaffinized, microwave treated and blocked for endogenous peroxidase activity. Subsequently, sections were immuno-incubated with the primary antibody for 16 hours, followed by a secondary step with swine anti-rabbit Igs conjugated with HRP (1:100, DAKO, Glostrup, Denmark). Visualization was performed with 3,3, diaminobenzidine as substrate.

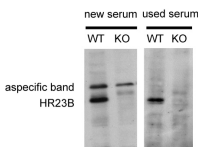

Figure 1. **Increase in the specificity of the anti HR23B serum after pre-incubation.** Immunoblots from wild type and HR23B-/- homogenates were stained with either non-used or pre-absorbed anti-HR23B serum. Besides the HR23B specific band a higher migrating cross-reacting band can be detected if new serum is used (left panel). The cross-reacting band cannot be detected when pre-absorbed serum is applied (right panel).

Microinjection and DNA repair synthesis

Normal human fibroblasts (C5RO) were fused using Sendai virus. Fused cells were microinjected with either GFPpolyQ or GFP as described previously (Vermeulen*, et al.* 1994). After injection the cells were incubated for 6 days at 37°C in standard F10 medium to recover from the injections and to form inclusions. Fluorescent (GFP) images were obtained with an Olympus IX70 microscope, PlanApo 60×/1.40 Oil immersion lens (excitation with 455-490 nm and long pass emission filter >510 nm.

After microinjection and fluorescent image recording, DNA repair capacity was determined by measuring unscheduled DNA synthesis (Vermeulen*, et al.* 1994). Human fibroblasts were UV-irradiated (16 J/m^2, 254 nm), labeled for 2 hours using [^{3}H]thymidine (20μCi/ml) and fixed for autoradiography. Autoradiographic grains above the nuclei of injected polykaryons were counted and compared with the number of grains above nuclei of normal fibroblasts (C5RO) non-fused, assayed in parallel.

Results

Specificity of anti-HR23B serum.

In order to test whether HR23B is a component of inclusion bodies we applied an immunohistochemical approach. Our polyclonal anti-HR23B serum showed several cross-reacting bands when used on immunoblots. After multiple usages these aspecific bands vanished while the specific HR23B band did not (**Figure 1**). To increase the specificity of the serum it was pre-absorbed prior to the actual staining. This pre-absorbed antiserum was used for our further immunohistochemical experiments. Next, the pre-absorbed HR23B antibody was applied on paraffin sections from wild type and mHR23B knock-out E13.5 embryos(Ng*, et al.* 2002). In wild type embryos, an overall staining was detected in almost all cell types (**Figure 2a,c**), whereas HR23B knockout embryos were devoid of detectable HR23B labeling (**Figure 2b,d**), illustrating the monospecificity of our pre-absorbed antibody.

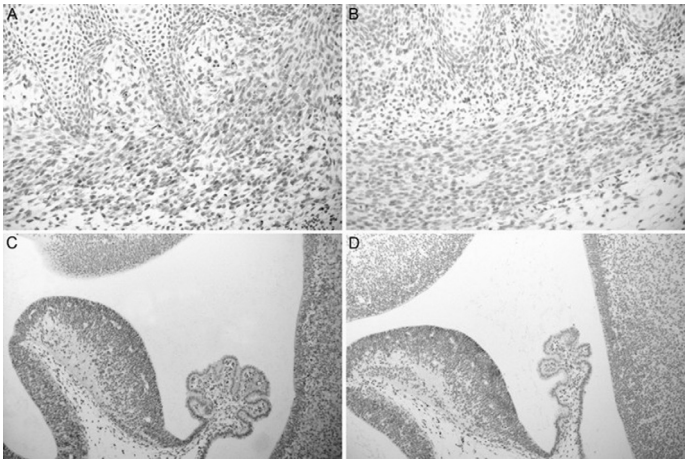

Figure 2. **Specificity of the pre-absorbed anti HR23B serum.** Paraffin sections from E13.5 embryos from WT (A and C) and HR23B (B and D) KO mice were stained with pre-absorbed anti HR23B serum. In WT embryos HR23B is expressed in almost all the cells at relatively low levels. Figure A shows HR23B expression in the myotoom (M). B shows HR23B expression in the choroid plexus (CP) within central part of lumen of fourth ventricle. Similar areas in HR23B KO embryos (B and D) are devoid of HR23B expression. C=cartilage primordium, Ce=intraventricular portion of cerebellar primordium.

HR23B and HR23A accumulate in neuronal inclusions of FXTAS mice.

Subsequently, the participation of HR23B proteins in the formation of inclusion bodies was analyzed in a mouse model of FXTAS using paraffin-embedded brain sections (Willemsen*, et al.* 2003). Previously, it has been shown that ubiquitin-positive intranuclear inclusions accumulate in a time-dependent manner throughout the brain of FXTAS mice with very high number of ubiquitin-

positive inclusions in neurons located in specific brain regions, including n.parafascicularis. In addition, these inclusions contained molecular chaperones (HSP 40) and the 20S catalytic core complex of the proteasome (Willemsen*, et al.* 2003). To determine whether HR23B protein is co-localizing with these neuronal intranuclear inclusions in the FXTAS mouse model an immunohistochemical analysis was performed. HR23B was abundantly present within the intranuclear inclusions throughout the brain in a similar pattern as described for the ubiquitin-labeled inclusions (**Figure 3a**). In addition, cells harboring inclusions showed an overall increase in total cellular HR23B levels compared to wild type mice (**Figure 3a** compare cells with arrows, containing inclusions with any other neuron). A similar labeling pattern was observed in all tested samples using HR23A-specific antibodies although the intensity of the staining was weaker (**Figure 3a,** inset for FXTAS brain; other human samples data not shown). We concluded that both HR23A and B proteins are present in relatively large quantities in FXTAS-related intranuclear inclusions.

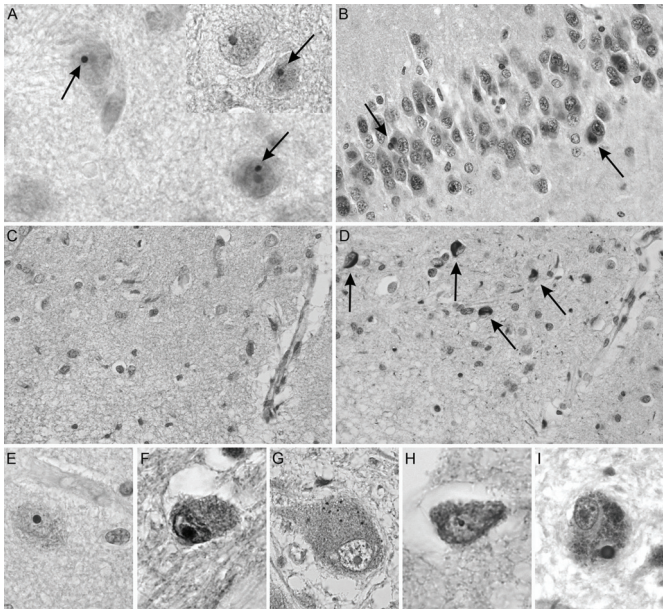

Figure 3. **HR23B accumulates in inclusions related to a variety of neurodegenerative disorders.** Paraffin brain sections from a FXTAS mouse model (A), FTDP-17 (B), AD (C and D), HD (E), SCA3 (F and G), SCA7 (H), and PD (I) were analyzed for the presence of HR23B-positive inclusions. **A)** HR23B-positive neuronal intranuclear inclusions (arrows) in the n. parafascicularis from a 100-week-old FXTAS mouse. The inset shows HR23A-positive inclusions in the same area (arrows) **B)** Similarly, HR23B is present in Pick bodies (arrows) in the hippocampus of an FTDP-17 patient. **C)** HR23B is absent in intra-cellular neurofibrillary tangles of an AD patient, while ubiquitin immuno-labelling of a succeeding section (D) did show the presence of ubiquitin-positive neurofibrillary tangles (arrows). **E)** Neuronal intranuclear inclusions in the n. caudatus from an HD patient did stain positive for HR23B. **F)** HR23B is present in neuronal intranuclear inclusion in pontine neurons of an SCA3 patient. **G)** In the same sections HR23B was also present in small cytoplasmic inclusions in pontine neurons that were devoid of intranuclear inclusions. **H)** In cortical brain sections of a SCA7 patient HR23B did accumulate in neuronal intranuclear inclusions. **I)** Finally, Lewy bodies present in neurons from the substantia nigra from a PD patient are HR23B-positive labelled.

To further gain insight whether the observed accumulation is limited to FXTAS-related inclusions and to study whether the co-localization has any pathological relevance we stained brain sections from patients diagnosed with various neurodegenerative diseases. In brain from an FTDP-17 patient, HR23B was abundantly present in cytoplasmic Pick bodies (**Figure 3b**) in a similar way as was described for ubiquitin.

Characteristic for the neuropathology of brains from AD patients are extra-cellular amyloid plaques and intra-cellular aggregates of hyperphosphorylated tau protein (neurofibrillary tangles; NFT). NFT are known to stain positive for ubiquitin as well (Leigh*, et al.* 1989). However, HR23B did not accumulate to a detectable level in intracellular tangles in the cortex from an AD patient (**Figure 3c**), whereas ubiquitin staining was positive in an adjacent section (**Figure 3d**) illustrating that HR23B accumulation in ubiquitin-positive aggregates is not an aspecific event. Moreover, this excludes a hypothetical cross-reactivity of our anti-HR23B antibody with ubiquitin.

Expanded glutamine-coding CAG repeats in huntingtin that accumulates in intranuclear inclusion bodies in specific brain regions during lifetime, predominantly striatal neurons, causes HD. Next to ubiquitin (data not shown); HR23B was present within these intranuclear inclusions (**Figure 3e** for n. caudatus).

SCAs are a clinically heterogeneous group of disorders. Several identified mutations correspond to expansions of trinucleotides repeats, including CAG repeats in SCA1, SCA2, SCA3, SCA6, SCA7, SCA17 and DRPLA. These expanded CAG repeats can be found in different target genes like ataxin-3 and ataxin-7 in SCA3 and SCA7, respectively. Previously it has been shown that HR23B and ataxin-3 interact with each other *in vitro* using a yeast two-hybrid screen (Wang*, et al.* 2000). Intranuclear inclusions could predominantly be detected in neurons located in the pons of SCA3 patients. Mutant ataxin-3 accumulates in these intranuclear inclusions along with ubiquitin, other components of the UPS system and molecular chaperones (Chai*, et al.* 1999). Here we show *in vivo* the presence of HR23B within these pontine neuronal intranuclear inclusions (**Figure 3f**). In addition, we could detect small HR23B-positive cytoplasmic inclusions in pontine neurons that were devoid of intranuclear inclusions (**Figure 3g**). Also in cortical brain sections of a SCA7 patient, HR23B-positive intranuclear inclusions were present (**Figure 3h**). Finally, a clear accumulation of HR23B was observed in Lewy bodies in neurons of the substantia nigra of a PD patient (**Figure 3i**).

GFP-polyQ expression induces aggregate formation but does not reduce NER capacity.

Since HR23B and HR23A are known to associate with the NER protein XPC (Masutani*, et al.* 1994) we wondered whether the accumulation of HR23B caused a sequestering of the XPC protein as well. No XPC-positive inclusions could be observed in paraffin sections of the brain in the FXTAS mouse model using immunohistochemistry with monospecific antibodies against XPC (Ng*, et al.* 2003, van der Spek*, et al.* 1996) (**Figure 4a,left**) while in a succeeding section ubiquitin did (**Figure 4a, right**). Although XPC staining, predominantly nuclear, was present in normal quantities in neurons of the FXTAS mice compared to wild type mice (data not shown) we cannot exclude an impairment of the DNA repair capacity of the XPC/HR23B complex. To test the influence of inclusions on NER, both dependent as well as independent of HR23B, we measured the repair capacity in cells harboring inclusion bodies. For this purpose we made use of a GFP-polyQ construct known to induce inclusion

formation in living cells (de Pril*, et al.* 2004). Unscheduled DNA synthesis, which is an established measurement for global genome NER capacity (Hoeijmakers*, et al.* 1990), was used in fused normal human fibroblasts micro-injected with cDNA encoding either GFP-polyQ or GFP alone. DNA repair capacity was determined by measuring DNA damage-induced DNA synthesis, or 'unscheduled DNA synthesis (UDS)'. In this assay cultured cells (here primary human fibroblasts) were UV-irradiated (inflicting DNA damage) and subsequently grown in the presence of tritium-labeled thymidine. The incorporated radioactively labeled DNA synthesis marker can be visualized by autoradiography. Quantification of repair capacity is than performed by counting the autoradiographic silver grains above the nuclei. In order to avoid false positive S-phase cells (due to the incorporation of tritiated thymidine during normal replicative DNA synthesis), cells are fused using Sendai virus, three days after fusion fibroblasts do not replicate anymore. Six days after injection, cells were UV-irradiated with a NER-saturating dose of 16J/m^2. This time point has been chosen because of the presence of a maximum number of inclusions. After a longer period (more than six days) we observed massive cell death. Prior to irradiation, pictures of microinjected cells were taken. In some, but not all, nuclear inclusions were clearly visible (**Figure 4b**). Strikingly no difference in unscheduled DNA synthesis was observed between cells injected with cDNA expressing GFP, GFP-polyQ or non-injected cells (**Figure 4b, and Table1a**). Neither a difference in UDS was detected between cells expressing GFP-polyQ harboring nuclear inclusions or not (**Figure 4b and Table1b**). Thus, high expression of polyglutamine repeats has no detectable effect on NER capacity. Importantly, the inclusion bodies induced by GFP-polyQ expression did stain positive for HR23B using the pre-incubated serum (**Figure 4c, middle**).

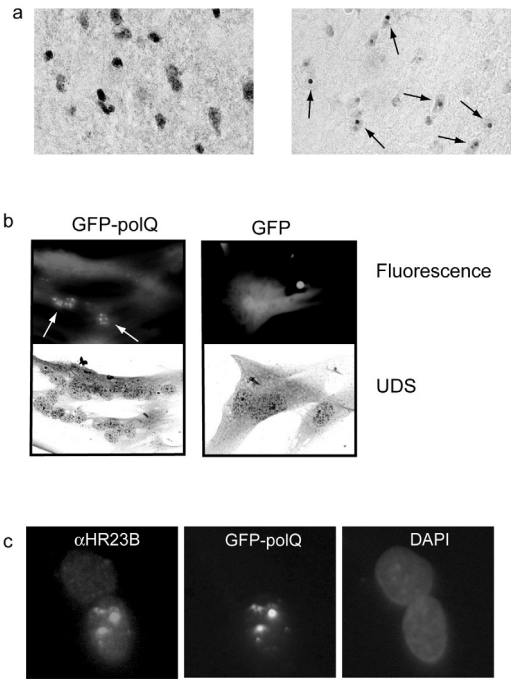

Figure 4. Inclusion formation is not influencing NER repair capacity. **A)** Paraffin section of a FXTAS mouse was analyzed for the presence of XPC using an affinity purified XPC serum. No detectable accumulation of XPC in inclusions was observed (left panel), while ubiquitin-positive inclusions (arrows) were present in a succeeding section (right panel). B**)** Cells that were injected with GFP-polyQ (top left panel) did form visible inclusions (arrows) while GFP injected cells (top right panel) did not. Repair capacity of these GFP-polyQ (below left panel) injected cells did not change compared to GFP (below right panel) and non-injected cells. C**)** HR23B (middle panel) accumulates in GFP-polyQ aggregates (left panel).

Discussion

HR23B is present in neuronal inclusion of some specific neurodegenerative disorders.
The exact cause and role of the UPS in the formation of inclusions in many distinct neurodegenerative disorders are not defined yet. Here we report the accumulation of HR23B (and HR23A) in neuronal inclusion bodies in a mouse model for FXTAS, and in patient material from several but not all neurodegenerative disorders, including FTDP-17, HD, PD, SCA3 and SCA7. Interestingly, HR23B was not detected in NFT in brain tissue from AD. Antibodies recognizing ubiquitin are widely used for a post mortem confirmation of diagnoses for one of these neurodegenerative diseases. The localization of ubiquitin-positive inclusions (brain region, cell type, nucleus or cytoplasm) is specific for different groups of neurodegenerative diseases. Here, we report a new neuropathological hallmark, the presence of HR23B within inclusions, in some specific neurodegenerative disorders. In contrast to ubiquitin the HR23B immunolabelling could discriminate between AD and the other neurodegenerative disorders studied here. The observation that HR23B is not detected in ubiquitin-containing NFTs in AD might point to a different nature of these deposits in comparison to the inclusion bodies in other diseases tested here. However, the origin of the inclusions in the other neurodegenerative diseases examined in this study does not share a common root cause as well. The origin may vary from different mutant proteins to mutant mRNAs as the primary cause. For example, inclusions in FTDP-17 brains, a neurodegenerative disorder caused by mutations in the Tau gene, show a positive staining with the phosphorylation-dependent anti-Tau antibody AT8 (Spillantini*, et al.* 1998). The same antibody also shows a positive labelling of the characteristic intracellular NFTs in AD patients. Although hyperphosphorylated tau is accumulating in both these deposits only those from FTDP-17 patients stain positive for HR23B. Different cellular aspects of cells harboring inclusions may explain why HR23B is not or at low abundance accumulating in all deposits. Nevertheless, we conclude that HR23B is a significant constituent of neuronal inclusions in some specific neurodegenerative disorders, including FTDP-17, HD, FXTAS, SCA3, SCA7 and PD. Further research should focus on the presence of HR23B in inclusions/deposits of other neurodegenerative disorders that were not tested here.

A

	Non-injected	**GFP**	**GFP-polyQ**
UDS (grain/nucleus)	58	57	55
SEM	2	2	2
N (number of nuclei)	12	17	56

B

	with inclusions	**without inclusions**
UDS (grain/nucleus)	57	58
SEM	2	2
N (number of nuclei)	16	12

Table 1
a) Quantification of unscheduled DNA synthesis (UDS) in non-injected, GFP injected and GFP-polyQ injected cells. **b)** Quantification of UDS in GFP-polyQ injected cells with and without inclusions.

A possible role for HR23B in the formation of neuronal inclusions.

Inclusion formation either as a cause or a consequence of the impairment of the UPS is of importance for the understanding of many neurodegenerative diseases. Since both UBA domains of HR23B are capable of binding polyubiquitylated proteins (Raasi*, et al.* 2004, Ryu*, et al.* 2003) a logical explanation for the observed co-localization with ubiquitin is the sequestering of (poly)ubiquitylated proteins in general. However, this is difficult to rationalize with the absence of HR23B in ubiquitin-positive NFTs in neurons from AD patients, in spite of the notion that expression levels appeared normal in AD patient brain. Alternatively, a more fundamental role of HR23B in the formation or maintenance of these inclusions can be hypothesized. The accumulation of HR23 proteins in inclusions either as an effect of a cellular state or more functional in the cause of these structures can be of mechanistic significance. The notion that HR23B is not sequestered in all inclusions is a possible clue for future experiments to unravel these mechanisms. Next to the presence of HR23B in, ubiquitin positive, nuclear neuronal inclusions we observed HR23B positive cytoplasmic structures in pontine neurons of a SCA-3 patient. These structures did not stain positive with our ubiquitin antibodies. Although we do not know the origin or the function of these structures it might be that these HR23B positive cytoplasmic inclusions are a prelude in the formation of the more familiar nuclear inclusions associated with SCA-3. Moreover, besides the clear accumulation of HR23 proteins in the inclusions we observed a general increase in the HR23B level in cells displaying inclusions as well. Although no correlation between HR23B protein levels and proteasomal activity *in vivo* has been made this suggests that the UPS is indeed impaired. Extreme overexpression of either HR23A or HR23B in living cells is known to inhibit 26S proteasome-mediated breakdown (Ortolan*, et al.* 2000, Raasi*, et al.* 2003). High levels of HR23A or HR23B might mask the polyubiquitylated substrates from the perturbed function of proteasomes, thereby relieving part of the proteasomal pressure. If so, it might be beneficial to alter HR23A or HR23B levels in target tissues. Future research monitoring the effect of either a lack or a surplus of HR23 proteins on inclusion formation is essential to understand the role of HR23A and HR23B in inclusion formation. Due to the complexity of the formation of these structures in time it is essential to study the mechanism in an *in vivo* model. *In vitro* systems bypass the time factor and are mostly performed in an inaccurate cellular system of a pathologically irrelevant target tissue.

Polyglutamine induced aggregates do not hamper NER.

Interestingly, no difference in the repair capacity of cells microinjected with a cDNA encoding a polyglutamine repeat was observed. The construct used here is known to cause inclusion formation in time. Indeed we could demonstrate the presence of HR23B-positive inclusions in some but not all nuclei of the microinjected cells. This is in line with the expectations, since inclusions develop in time, it is therefore hard to predict when an inclusion will be formed. . Here, we used a system that forces high expression of the polyglutamine repeats known to cause impairment of the UPS and frequently resulting in inclusion formation (Bence*, et al.* 2001, de Pril*, et al.* 2004). Recently, it has been shown that in ubiquitylation-defective cells NER is suppressed (Wang*, et al.* 2005). Ubiquitylation was inhibited by using temperature sensitive E1 or chemicals that block the 26S proteasome. Inhibition of the ubiquitin system has a serious effect on the homeostasis of the cell. This and the notion that

several specific ubiquitylation events have a known stimulating effect on NER make the effect of ubiquitylation deficiency on NER not surprising (Hoege*, et al.* 2002, Sugasawa*, et al.* 2005). Here we show that despite the proteasomal stress and the presence of HR23B-positive inclusions, NER repair capacity as measured by unscheduled DNA synthesis was normal. This clearly indicates that NER is functionally normal in these cells regardless of the proteasomal stress and the sequestration of HR23B within inclusions. Either the impairment does not influence the homeostasis too much or NER occurs in cells despite of a high stress level. Apparently there is no connection or influence of neurodegeneration associated inclusion formation and NER despite of the sequestering of one of the NER factors in these structures. In line with the uncoupling of DNA repair and polyglutamine induced proteasomal stress is the notion that polyglutamine diseases are in general not correlated with elevated cancer risks, which are associated with hampered GG-NER.

Complementary to ubiquitin-positive inclusions, we report here that the presence of neuronal HR23B-positive inclusions in specific brain regions is a new neuropathological hallmark of several neurodegenerative diseases, including FTDP-17, HD, PD, SCA3 and SCA7. Interestingly HR23B did not accumulate detectably in neurofibrillary tangles from an AD patient, whereas ubiquitin did, suggesting disease-specificity. Although HR23B is associated with DNA repair we demonstrate that the NER repair capacity in living cells with impaired UPS is unaffected. In conclusion, HR23B seems to have a dual cellular function, that is, a specific role in NER and a role in the ubiquitin-proteasome degradation pathway. Furthermore, our results indicate that both cellular functions are operational independently.

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Chapter 9

Discussion and future perspectives

Discussion and future perspectives

Since most of the experimental data are discussed throughout the different chapters, in this part the future perspectives concerning the three main issues of this thesis: DNA damage-induced histone ubiquitylation, the function of HR23 proteins in repair and the function of HR23 proteins in general, will be discussed. The common denominator between these is the regulation of cellular pathways via posttranslational modifications, specifically ubiquitylation. As discussed in the introduction during certain processes, especially in genome maintenance, specialized enzymes have to be activated both fast but in a controlled and restricted fashion to prevent fortuitous actions. Regulation via posttranslational modification seems to be a commonly used solution for this dilemma.

UV-induced histone ubiquitylation.

It is generally accepted that histone modifications are important events that (partly) determine or adjust the performance of many, if not all DNA-transacting processes, including DNA repair/genome maintenance mechanisms. Currently, only a few DNA damage-induced histone modifications have been reported. Only one of these modifications is established in a repair-dependent manner (i.e. the phosphorylation of H2AX) [1]. In chapter 7 a novel UV-induced histone H2A ubiquitylation is described as a second repair-dependent histone modification. Obviously, this new finding raises many questions concerning this ubiquitylation event. These questions refer to both mechanistic details as well as the broader biological impact of this event.

Firstly, the quantity of increase in uH2A after DNA damage in time needs to be established. Insight in the time course and the quantitation of this modification will help to implement and interpret the role of this UV-induced histone ubiquitylation in the biological context. Although, we have clearly shown that the rapid UV-induced ubiquitylation of H2A appeared to depend on NER, an interesting question that can be raised whether other DNA repair mechanisms also induce a similar response? Severely delayed ubiquitylation within XP-A cells at local damaged sites suggest a NER-independent event. Counterstaining with PCNA suggested that these cells went through S phase, a cell cycle phase with alternative genome maintenance pathways (e.g. TLS, template switching). A third important issue is the enzymology involved in this event. Although, knock down of the polycomb E3 ligase Ring1b resulted in a failure to ubiquitylate H2A after DNA damage suggesting that this is the responsible E3 ligase, more research is required to further establish this activity. Ring1b is the only E3 ligase of H2A described so far, knock down of this enzyme resulted in a general reduction of uH2A levels. Moreover these cells displayed morphological changes and cell growth inhibition [2,3]. Therefore it might be that the absence of DNA damage-induced histone ubiquitylation in Ring1b knock down cells is due to the general stress situation in these cells. The DNA repair enzyme Rad6 is the canonical E2 conjugase of H2B in yeast. Rad6 and the rabbit homolog both can ubiquitylate H2B and H2A *in vitro* [4]. It seems likely that HR6A and/or HR6B are E2 conjugases of H2A ubiquitylation in mammals. However, the role of HR6A and HR6B in histone ubiquitylation in mammals is still not clear. It has never been shown *in vivo* if HR6A and /or HR6B are indeed involved in histone ubiquitylation. The repair sensitivity and the (assumed) role in histone ubiquitylation suggest that they also might be involved in DNA damage-induced H2A ubiquitylation. Though this still has to be experimentally proven. It is likely that the damage induced ubiquitylation of H2A needs to be reversed. Usually

reversion of ubiquitylated substrates is performed by specific deubiquitylation (DUB) enzymes. It is important to determine which DUB is responsible for the removal of DNA damage-induced H2A ubiquitylation. Recently, the mammalian DUB for “normal” uH2A has been identified (unpublished results Elisabetta Citterio). Knock down of this enzyme resulted in “spontaneous” phosphorylation of H2AX and formation of DSBs. This suggests that it is involved in stress signaling either by inducing DNA damage directly, by for example hindering the DNA transacting processes (e.g. replication) or due to high levels of uH2A that possibly mimic a cell cycle arrest signal. Thus implying that the alteration of the histone code itself forms a signal that somehow activates stress signaling pathways.

This brings us to the fourth and most important question concerning DNA damage-induced H2A ubiquitylation: what is the biological function? Although there are strong clues that DNA damage-induced H2A ubiquitylation indeed functions in DDR there is no firm experimental evidence, in mammalian cells, yet. In yeast, H2B rather than H2A ubiquitylation is apparent, there is a clear function of H2B ubiquitylation in DDR as shown by mutant analysis [5]. In mammals we showed that H2A ubiquitylation is largely dependent on the DDR-kinase ATR. The phenotype of the uH2A DUB knock down suggests activation of DDR as well. So if we assume that DNA damage-induced ubiquitylation of H2A indeed signals to downstream pathways such as DNA damage-induced signaling, an obvious (fifth) question is: How? In general posttranslational protein modifications including ubiquitylation induce the binding of other proteins. A wide range of domains capable of interacting with (poly)ubiquitylated proteins have been described [6]. Usually proteins harboring these domains integrate ubiquitylation events into downstream (signaling) events [7]. So there presumably is another protein that can interact with uH2A, which activates/recruits downstream events/targets. Most likely this protein harbors an ubiquitin-interacting domain.

A related and important issue is if there is a consequence of the DNA damage induced –massive- ubiquitylation on the entire cellular UPS? Using life cell dynamic studies we show that 16J/m^2 of UV irradiation causes an increase in immobile-ubiquitin of 43% (from 21% immobile before UV to 30% immobile after UV). Using a more physiological relevant dose of 4J/m^2 we measured a 14% increase (from 21% immobile to 24% immobile after UV) in the immobile fraction. It has been shown that the conjugations of the different targets of ubiquitin are in a dynamic equilibrium with the free pool of ubiquitin [8]. Furthermore the amount of free ubiquitin is relatively low [9]. For example, after proteasome inhibition histones are massively deubiquitylated most likely due to the accumulation of ubiquitin in polyubiquitin trees [8]. Using photobleaching techniques this equilibrium has been shown in living cells as well [9]. So, if an increase of polyubiquitylated proteins causes a decrease in uH2A, what is then the effect of an increase in uH2A, caused for example by UV irradiation? Following the logic of a dynamic equilibrium it can be expected that other ubiquitin-dependent processes are affected. Since the UV-induced immobilization of nuclear ubiquitin is dependent on functional NER these processes should be unaffected in NER-deficient cells. Although it is hard to predict which of the ubiquitin-dependent processes might be affected, the degradation of targets of the 26S proteasome is a likely candidate. Certain degradation-model substrates exist that accumulate upon proteasomal stress, due to a fluorescent tag the accumulation of these can easily be followed in living cells [10]. Unfortunately, these model substrates only accumulate upon severe proteasomal stress. It is not to be

expected that DNA damage-induced immobilization of ubiquitin on histones causes such an extreme lack of available ubiquitin resulting in the acquired proteasomal stress.

DNA repair and DNA damage-induced signaling

As suggested above DNA damage processing by repair or other genome maintenance pathways in general might lead to an alteration of the histone code that functions in the DNA damage-induced signaling pathways. In this way the cellular response to DNA damage is integrated with repair/avoidance of these damages. A clear benefit is that the damaged DNA-induced signaling pathway themselves doesn't have to sense all the different types of (genomic) stress but only a common intermediate of the DNA repair or damage avoidance pathways. One likely candidate for this intermediate is the ssDNA-bound RPA complex. ATRIP, a complex partner of ATR, is known to bind to ssDNA-bound RPA complexes and subsequently activate ATR-mediated DNA damage-induced signaling [11]. RPA still accumulates at local damaged sites in XP-A cells [12], while UV-induced signaling does not occur in XP-A deficient and Rad14 (yeast homolog of XPA) deficient yeast cells [13,14]. Thus, it is likely that further processing of the damage (e.g. the cut or the release of the damaged oligo) is necessary to enable ATRIP to bind the ssDNA-bound RPA complex and subsequently activate the signaling response. The ssDNA-bound RPA complex is indeed a common intermediate of several genome maintenance pathways (fig1).

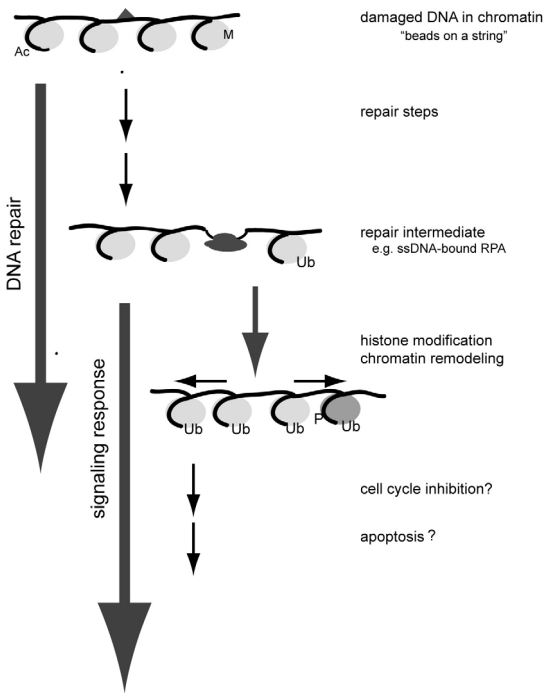

Fig1 proposed model for the interplay of DNA repair and DNA damage induced signaling.
Lesions in chromatin are recognized and repaired by DNA repair mechanisms such as NER. Intermediates of the repair reaction trigger the signaling response. Either an early step of the signaling response or a late repair step differentially modifies the histone code thereby partly determining the outcome of the signaling response e.g. cell cycle arrest, apoptosis etc.

There are obvious cellular advantages for a DNA damage signaling response. Particularly in proliferating tissue the induced cell cycle block is of clear benefit to provide an extended “repair time window”. But also post-mitotic cells can profit from DNA damage-induced signaling. Other genome maintenance pathways can be activated by this response, which might be essential since DNA damage sources usually induce a wide range of physically diverse damages (e.g. bulky lesions, double- and single strand breaks, oxidative lesions, crosslinks) that are resolved by different pathways/mechanisms. Moreover it might be that the damaged cell enters senescence or apoptosis, a process that could be guided by the DNA damage-induced signaling pathways. Several data suggest that a DNA damage-induced signaling response indeed exist in non-proliferating cells (personal communication Marco Muzi-Falconi and [13-15]) although some reports claim the opposite [16].

Histones as a possible sensor for DNA damage-induced signaling can function as a cellular abacus; the amount of signal (i.e. histone alteration) would determine the appropriate response. High genotoxic stress with according high levels of differentially modified histones (e.g. ubiquitylation and H2AX phosphorylation) result in apoptosis while lower levels would induce in cell cycle arrest. As a side effect the alteration can mark the chromatin for future DNA processes as “repaired”. The repair-mediated histone modifications described here could be integrated in such a post-repair chromatin remodeling-response.

Next to a direct histone modification, other types of UV-induced chromatin remodeling take place as well. There is strong biochemical evidence for UV-induced nucleosomal rearrangements [17]. Previously, it has been shown that the chromatin assembly factor 1(CAF1) is recruited to UV-damaged sites both *in vitro* as well as in living cells [18,19], however thus far no overt nucleosomal rearrangement has been demonstrated in living cells. To study in which step(s) and to what extent nucleosomal rearrangement or removal takes place in living cells, local damage techniques can easily be applied. Cells that stably express different fluorescently tagged histones are available [20]. Using different (mutant) cell lines that express fluorescent histones, a UV-C-laser and photobleaching techniques it is possible to determine: if, when, how and how much nucleosomal removal and repositioning takes place. To verify if histone removal at repair sites indeed takes place, the S phase-specific histone H3.1 might be used. This histone isoform is only incorporated during replication; outside S other isoforms are built in. Recently, specific antibodies against H3.1 became available [21], when these are used nucleosomal removal at local UV-damaged sites would result in a negative staining of this antibody.

Function of HR23B outside NER

HR23B K.O. and HR23A and HR23B DKO mice have a phenotype that does not correlate with a function of HR23A and HR23B strictly in NER. Studies in yeast have indeed revealed other functions of Rad23 such as being a shuttle of target substrates to the proteasome [22,23]. In the mammalian system HR23A and HR23B most likely have a similar function. Still, the impact and consequences of misregulated shuttling in mammals, besides the notion of the biological relevance (i.e. the severe phenotype of HR23B K.O. animals) is still a mystery. The absence of a clear NER-deficient phenotype makes the HR23A and HR23B K.O. mouse models in theory an ideal model system to study its additional functions (e.g. shuttle-function) in the mammalian system. However, no apparent phenotype

of the HR23A K.O. mice has been discovered today. Furthermore, the (too) severe phenotype and the high rate of embryonic lethality of the HR23B K.O. mice make this model inaccessible [24]. A solution to make these animals experimentally more available would be the creation of an inducible K.O. system. Additionally, the generation of transgenic animals might be useful since there are indications for a gene dose effect. Using bacterial artificial chromosome (BAC) technology, a combination of these strategies can be made, and is currently in progress (fig2). We have generated a BAC containing the entire genomic HR23B locus. This BAC is able to express a full-length HR23B-YFP gene fusion protein, in addition loxP sites are positioned in such a way that a (conditional) K.O. can be created (inducible knock-out knock-in). This multifunctional recombinant BAC has the following advantages. (1)By injecting the modified-BAC into oocytes transgenic animals can be generated harboring an extra locus (loci) for the HR23B gene, thereby ensuring that the gene is under its own promoter and that regulatory elements throughout the locus remain intact. A similar strategy was successfully used in the creation of p53 transgenic animals [25]. (2)As a parallel strategy the modified-BAC can be transfected into embryonic stem (ES) cells. Transfected ES cells can be selected for either random versus site-directed integration. Since long homology arms increase the targeting efficiency extensively, the BAC that contains the entire locus, can be used as targeting vector, as was previously shown by others [26]. Transferring the BAC into ES cells will result in random integration (of the entire BAC) as well. These cells can be conveniently used to generate transgenic animals by blastocyst injection. This allows selection of gene copy number and correlation with cellular performance.

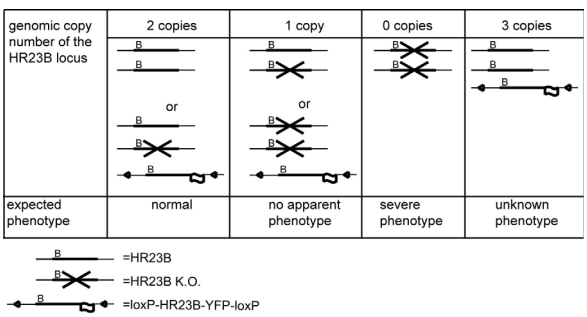

Fig2 number of genomic loci of the HR23B gene and the expected phenotype. The number of loci of the HR23B gene will determine the phenotype of the mouse. Two genomic copies e.g. a "normal" wild-type or a heterozygous K.O. with the extra HR23B-BAC will result in healthy animals. Similar animals with only one allele, e.g. heterozygous K.O. or K.O. with the extra HR23B-BAC, will be viable and relatively normal. Animals with no HR23B alleles will display the known severe phenotype. Finally, animals with the extra HR23B-BAC (so with a total of three genomic loci) have a yet unknown phenotype

(3) By crossing the HR23B BAC-transgenic with the existing HR23A and HR23B K.O. animals and the various available CRE mice, several different HR23B mouse models can be obtained, which will be instrumental to address a number of questions, concerning the function of HR23A and HR23B. For example, mild overexpression (three "genomic copies") will give insight in the function of the HR23B gene. Tissue-specific mild overexpression can be achieved by crossing the transgenic/BAC animals to

tissue-specific CRE mice. In those tissue were CRE is not active the transgene/BAC will remain intact and result in overexpression, whereas the transgene/BAC in the CRE target tissues will be removed. Similar, tissue-specific K.O. will be informative as well (achieved by crossing the HR23B K.O., the transgenic/BAC and CRE mice). (4) The YFP tag functions as a marker that enables the study of the expression pattern in different tissues throughout the life span of these animals. Moreover, protein dynamic studies on HR23B-YFP (expressed under physiological conditions) under normal and different (proteasomal/genome) stress conditions will reveal its dynamic engagements in different processes. Both the tag as well as the mild overexpression situation are helpful tools to resolve the function of HR23B in, for example inclusion body formation as is suggested in chapter 8.

Function of HR23A and HR23B inside NER

In chapter 6 we described that the HR23 proteins are essential for XPC immobilization/binding on damaged DNA. This is in line with i*n vitro* data showing that both HR23A and HR23B have a stimulatory effect on XPC binding to damaged DNA [27]. Moreover in yeast, overexpression of Rad4 (XPC) only partly rescues Rad23 mutant sensitivity [28]. All this emphasizes that Rad23 proteins have an additional function in NER besides the stabilization of XPC/Rad4. Interestingly, we provided evidence suggesting that HR23B dissociates from XPC upon DNA damage induction. In addition to regulation of XPC activity by HR23 proteins also the DDB2-dependent polyubiquitylation appeared to influence its binding properties and possibly repair rates [29]. It is of interest to see what the effect of HR23B on XPC ubiquitylation is. Since both have a stimulatory effect but seem mutually exclusive; before damage induction XPC is in complex with HR23B and not ubiquitylated and after UV XPC is ubiquitylated and not in complex with HR23B. Although the players involved in loading/stabilizing XPC on the lesion have been identified, the exact molecular hand of transactions at lesions is not known: which factor binds, which proteins stay and which are required to facilitate binding of successive NER factors? To answer some of these questions *in vitro* single molecule studies are useful. As a matter of fact these are in progress.

The level of XPC is of biological relevance since it influences mutagenesis, as was shown by the dosage effect of mice that lack one or two XPC alleles [30,31]. In living cells stable overexpression of XPC seems deleterious (unpublished observation), possibly due to uncontrolled binding to undamaged DNA, thereby influencing the DNA transacting processes (see chapter 5). This suggests that the cellular amount of XPC is delicate. However and in seeming contradiction to the above findings dynamic studies showed that XPC immobilization reaches a plateau at roughly 30% of all XPC molecules present in the cell, meaning that 70% of all cellular XPC molecules remain unbound. This suggests that an equilibrium between the amount of XPC probing the genome (i.e. constantly associating and dissociating on undamaged DNA) and the amount of XPC transiently immobilized on NER lesions has been reached. Alternatively, this might indicate that XPC is not the limiting factor for initiation of the NER reaction. Initiation is determined by either another factor e.g. the UV-DDB complex or that the “effective” XPC concentration i.e. XPC that is capable of binding/immobilizing is limited. It is very likely that this “effective” XPC is the modified XPC. In XP-E cells the accumulation of XPC on local UV-irradiated sites is compromised, showing that the UV-DDB complex is necessary for a full and efficient binding of XPC to local UV damage [32]. This implies that the amount of XPC is not

the bottleneck of the repair reaction per se, instead it seems that the efficiency of immobilizing/binding of (modified) XPC to damaged DNA is the critical step. How to influence (e.g. increase the efficiency of) this step is a more difficult problem. Constitutive high expression might be toxic, therefore a touchable or regulatory activity to respond to changing conditions (e.g. chronic exposure to genotoxic insults) are likely more favorable. It might be more beneficial to alter the proteins involved in XPC regulation i.e. its complex partners HR23A or HR23B and Cen2 [33,34] and the UV-DDB complex. Absence of any of these has a negative effect on XPC activity/immobilization on damaged DNA as was previously shown by others and us [29,35,36]. It is hard to predict but certainly of interest to see what the elevation (using for example the above mentioned HR23B-YFP transgenic animal) of these factors would do with the initiation step of NER. Regulation of this step by XPC seems to be a complicated and highly orchestrated event that is influenced at the transcriptional level, posttranslational level, by shuttling and by the stochiometry of the complex.

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List of abbreviations

AAA	ATP-ases associated with a wide variety of cellular activities
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM and Rad3-related
BER	Base excision repair
CAF	Chromatin assembly factor
CAK	Cyclin-activating kinase complex
Cdt1	Cdc10-dependent transcript1
CEN2	Centrin 2
Chk1,2	Check1,2
CS	Cockayne syndrome
CPD	Cyclobutane pyrimidine dimer
CTD	C-terminal domain of the large subunit of RNAPII
DDB1/2	Damaged DNA binding protein 1/2
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DUB	Deubiquitylating enzyme
E1	Activating enzyme
E2	Conjugating enzyme
E3	Ligating enzyme
ERCC1	Human excision repair cross complementing gene 1
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence energy transfer
GFP	Green fluorescent protein
GG-NER	Global genome NER
HA	Hemagglutinin
HECT	Homologous to E6AP Carboxy Terminus
HR	Homologous recombination
HR23A/B	Homologue of Rad23A/B
kDa	Kilo Dalton
MAT1	Ménage-a-trois 1
MEF	Mouse embryonic fibroblast
mRNA	Messenger ribonucleic acid
MMR	Mismatch repair
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
PCNA	Proliferating cell nuclear antigen
pol	Polymerase
(6-4) PP	(6-4) pyrimidine-pyrimidone photoproduct
Rad	Radiation sensitive

RING	Really interesting new gene
RNAPI/II	RNA polymeraseI/II
Roc	RING of Cullin
ROS	Reactive oxygen species
RPA	Replication factor A
Rpn1-2,10	Non-ATPase subunits of the regulatory particle
Rpt1-6	ATPases subunits of the regulatory particle
SCF	Skp1/Cul-1/F-box
SUMO	Small ubiquitin-related modifier
ssDNA	Single stranded DNA
TC-NER	Transcription-coupled NER
TFIIH	Transcription factor IIH
TLS	Translesion synthesis
TTD	trichothiodystrrophy
UBA	Ubiquitin-associated
UBD	Ubiquitin-binding domain
UBL	Ubiquitin-like protein
UDS	Unscheduled DNA synthesis
uH2A	Ubiquitylated Histone2A
UPS	Ubiquitin proteasome system
UV	Ultraviolet light
XP	Xeroderma pigmentosum

Summary

The genetic information stored in the DNA encodes directly or indirectly for all processes important for life. The nucleic acid order of the DNA is (via RNA) translated into proteins. The various proteins have distinct and vital functions that are important for the catalysis of the distinct processes in our cells, furthermore they function in the structures in and outside our cells as well. During the live cycle of every cell a constant challenge from both exogenous as endogenous sources potentially damages both proteins and DNA. Proteins can be easily be replaced by the synthesis of new ones. In contrast however, DNA as the carrier of information itself cannot be replaced and therefore, damage needs to be repaired. Several genome maintenance pathways have evolved that are briefly discussed in **chapter2**. One of these is the nucleotide excision (NER) repair pathway, which repairs bulky lesions including those induced by the short wave length components of sunlight (UV-C). NER is being discussed in more detail, since this is the main topic of research discussed in this thesis. In NER lesion recognition is either performed by the stalling of the RNA polymerase, leading to the so-called transcription coupled repair subpathway (TC-NER) or by the XPC-complex with the help of the UV-DDB complex. The latter recognition mechanism is active throughout the entire genome and is therefore referred to as global genome repair (GG-NER). After recognition the helix surrounding the lesion is unwound and the strand containing the lesion is excised. After excision the remaining gap is filled in using the opposing strand as a template. Although DNA repair/damage avoidance mechanisms are crucial, they can be hazardous as well. The intrinsic affinity for (undamaged) DNA of many components/proteins in these pathways demand restriction and controlling mechanisms to activate genome maintenance pathways. One of the frequently used ways to induce these is by posttranslational modifications such as ubiquitylation. Ubiquitin is a small peptide that can be covalently linked (conjugated) to target proteins. Ubiquitylation as a modification plays a critical role in many processes including targeting of proteins that need to be degraded. Polyubiquitylated proteins are recognized and subsequently degraded by the 26S proteasome. The ubiquitylation machinery and the 26S proteasome degradation apparatus are often referred to as the ubiquitin proteasome system (UPS). The UPS is discussed in **chapter 3**. Some examples of ubiquitylation of genome maintenance factors and their consequences are outlined in **chapter 4**.

In the experimental **chapter 5** the dynamic behavior of the GG-NER recognition factor XPC is described. Previously, using a biochemical approach it has been shown that XPC binds naked DNA with a slightly higher affinity for damaged DNA. We show in living cells, using GFP-tagged and different photobleaching (e.g. FRAP) procedures that XPC moves relatively slow throughout the nucleus, a behavior that could be explained by a frequent binding (and release) to DNA. In a similar fashion as all other NER factors tested thus far, XPC immobilizes on genuine NER lesions including UV-C light. However, XPC also immobilizes on other helix disturbing lesions that do not result in NER, indicating that the NER reaction can be abrogated after the initiation. Moreover, XPC is constantly shuttled back and forth between the nucleus and cytoplasm. Interestingly, Upon UV irradiation XPC shuttling is abrogated. This shuttling might therefore function as a regulatory mechanism of XPC levels in the nucleus. In contrast to XPC one of its complex partners, HR23B fails to immobilize/accumulate on damaged DNA as is shown in **chapter 6**. Furthermore, we show that XPC and HR23B dissociate

upon DNA damage. That HR23B is of importance for recognition of DNA damage is shown by the notion that XPC is not accumulating/immobilizing on DNA damage in the absence of HR23B (or HR23A).

In living cells DNA is wrapped around histone proteins. The modifications on the tails of these histones partly determine the accessibility to the DNA. In **chapter 7** we demonstrate the relative increase in the levels of one of these modifications, namely the ubiquitylation of H2A after UV irradiation. Mutant analysis revealed that this UV-induced ubiquitylation event is dependent on NER. A likely function of this DNA repair mediated histone modification is in the DNA damage induced signaling response. DNA damage induced signaling provokes a cell cycle block, activate (other) genome maintenance pathways and can induce cell death or terminal arrest. The exact trigger for DNA damage induced signaling is as yet not fully understood. Besides repair-dependent histone alterations other indications have been found that support a model where DNA repair, or repair intermediates triggers the signaling.

In the (last) experimental **chapter 8** the accumulation/sequestering of HR23B in neuronal inclusions is shown. HR23B has a dual role in NER and in UPS mediated protein degradation. In the UPS HR23 proteins are supposed shuttles of (poly)ubiquitylated poteins to the 26S proteasome, due to their ability to bind both (components of) the 26S proteasome and (poly)ubiquitylated proteins. Despite of the accumulation of a NER factor (i.e. HR23), neuronal inclusions do not have an effect on NER efficiency. Furthermore XPC is not a constituent of these neuronal inclusions. HR23B accumulated in neuronal inclusions in brain from a mouse model for fragile X associated tremor/ataxia syndrome, as well as in brain material from Huntington disease, Spinocerebellar ataxia3, Spinocerebellar ataxia7, frontotemporal dementia and parkinsonism linked to chromosome 17 and Parkinson disease patients. However, HR23B did not accumulate in tau-positive aggregates (neurofibrillary tangles) from Alzheimer disease patients while ubiquitin did. This suggests that the accumulation of HR23B is not solely due to their affinity for ubiquitylated proteins.

The main focus of this thesis has been on the regulation of NER. For example during the last few years it became evident that posttranlational modifications, shuttling, transcriptional induction, and changes in the stochiometry of the complex regulate the recognition step. In the **discussion** some future perspectives and practical ideas are proposed. The work presented in this thesis resulted in new insights into the NER process both in the recognition step as in post excision events. Furthermore novel NER-unrelated functions of HR23B have been revealed

Samenvatting

De genetische informatie, opgeslagen in het DNA, codeert direct of indirect voor alle processen die belangrijk zijn voor het leven. Vrijwel iedere cel waaruit ons lichaam is opgebouwd bevat hetzelfde DNA, dat uniek is voor het individu. Dit DNA is opgebouwd uit vier verschillende nucleïnezuren waarvan de volgorde via het RNA vertaald wordt naar eiwitten. De verschillende eiwitten katalyseren in onze cellen de processen die essentieel voor leven zijn. Daarnaast functioneren de eiwitten, zowel binnen als buiten onze cellen, in de structuren die ons lichaam bepalen. De lichaamscellen worden daarbij constant getart door bronnen van buiten, of van binnen het lichaam zelf, die de eiwitten en het DNA in de cellen kunnen beschadigen. Eiwitten kunnen vervangen worden door de aanmaak van nieuwe eiwitten. Aangezien het DNA zelf de drager van de erfelijke informatie is, kan dit niet simpelweg vervangen worden. Verschillende DNA herstel- (of genoom onderhoud-) mechanismen zijn geëvolueerd die ervoor zorgen dat de integriteit van het DNA behouden blijft. In **hoofdstuk twee** worden deze DNA herstelmechanismen kort samengevat. Een van deze herstelmechanismen is nucleotide-excisie-herstel (in het Engels aangeduid als nucleotide excision repair, NER) dat voornamelijk DNA schades herstelt, die de helix ruimtelijk verstoren. Deze schades kunnen onder andere veroorzaakt worden door de korte golflengte component van het zonlicht (UV) of door (chemische) componenten in sigarettenrook. Bij NER kan een schade herkend worden door het vastlopen van de RNA polymerase op het beschadigde DNA of de schade wordt onafhankelijk van DNA-replicatie door het XPC-complex herkent met behulp van het UV-DDB complex. De eerste manier van schade herkennen, de transcriptie gekoppelde NER (TC-NER), is slechts in een klein gedeelte van het genoom actief. Immers, niet alle DNA wordt afgelezen (getranscribeerd) door de RNA polymerases. Volgens de tweede manier worden schades daarentegen in het hele genoom herkent, vandaar dat deze de globaal genoom-NER (GG-NER) wordt genoemd. Na de herkenning wordt de DNA helix rondom de schade geopend. De DNA streng, die de schade bevat, wordt daarna weggeknipt. Tegenover het overgebleven enkelstrengs DNA wordt vervolgens nieuw DNA geïncorporeerd.

Alhoewel DNA herstelmechanismen belangrijk zijn voor het overleven van de cel, kunnen ze ook schadelijke gevolgen hebben. De intrinsieke affiniteit voor (onbeschadigd) DNA van veel eiwitten betrokken bij DNA herstel maakt dat er regulatie mechanismen nodig zijn om dit herstel alleen te activeren wanneer het nodig is. Een veel voorkomende manier om dit te bewerkstelligen is het modificeren van bepaalde “sleutel” eiwitten die betrokken zijn bij DNA herstel. Deze modificaties worden posttranlationeel geïnduceerd, dwz nadat de eiwitten gesynthetiseerd zijn. Een voorbeeld van zo’n posttranslationele modificatie is ubiquitylering. Ubiquitine is een klein peptide dat covalent gebonden kan worden aan andere eiwitten. Ubiquitylering is betrokken bij verschillende cellulaire processen zoals: het sorteren van membraan partikels, DNA herstel en eiwitafbraak. Meervoudig geubiquityleerde eiwitten kunnen herkend en afgebroken worden door het 26S proteasome als een complex dat in de cel eiwitten afbreekt. De enzymen, die betrokken zijn bij ubiquitylering en het 26S proteasome, worden samen het ubiquitine proteasome systeem (UPS) genoemd. Het UPS wordt kort behandeld in **hoofdstuk drie**. Enkele voorbeelden van ubiquitylaties van DNA hersteleiwitten en de consequenties daarvan worden besproken in **hoofdstuk vier**.

In het experimentele **hoofdstuk vijf** wordt de intracellulaire beweging van de GG-NER herkenningsfactor, XPC, beschreven. In eerdere studies is met behulp van biochemische technieken aangetoond dat XPC aan “naakt” DNA bindt met een licht verhoogde affiniteit voor onbeschadigd DNA. Met behulp van fluorescent gelabeld XPC en verschillende fotoblekings technieken laten we zien dat XPC in levende cellen relatief traag door de kern beweegt. Dit gedrag wordt hoogstwaarschijnlijk verklaart door het constant binden en weer loskomen van het DNA. Net als alle eerder geteste NER factoren immobiliseert XPC op eigenlijke “NER schades”, zoals bijvoorbeeld UV geïnduceerde schades. XPC immobiliseert ook op andere helix verstoringen welke desondanks niet resulteren in NER. Dit wijst erop dat de NER reactie na de initiatie gestopt kan worden. Daarnaast laten wij een nieuw regulatiemechanisme voor XPC zien. XPC wordt continu tussen de kern en het cytoplasma heen en weer gependeld. Na UV geïnduceerde schade stopt dit pendelen. In tegenstelling tot XPC immobiliseert één van zijn complex partners, HR23B, niet na DNA schade. Evenmin vindt er ophoping van HR23B plaats op lokale schade. Na UV is er inderdaad minder HR32B in complex met XPC. Zonder HR23B is er minder XPC aanwezig in de cel, daarnaast hoopt XPC niet meer op lokale schade op. HR23B stabiliseert XPC dus en is essentieel voor een efficiënte binding aan DNA schade. Het gedrag van HR23B in NER is beschreven in **hoofdstuk 6**.

In levende cellen bestaat het DNA niet als een naakt molecuul maar is het “verpakt” waarbij het om de histone eiwitten is gewikkeld. De uiteinden van deze histone eiwitten kunnen op vele verschillende manieren gemodificeerd worden. Deze modificaties aan de uiteinden van histonen zijn erg belangrijk voor de toegankelijkheid van het DNA voor bijvoorbeeld transcriptie (het aflezen van het DNA). In **hoofdstuk 7** laten we zien dat er na UV een toename is van de ubiquitylering van histone H2A als één van de histone modificaties. Door mutant analyse hebben wij bewezen dat deze UV geïnduceerde histone modificatie afhankelijk is van NER. De functie van de toename in geubiquityleerd H2A na DNA schade moet waarschijnlijk in de DNA-schade-geïnduceerde-signalering-response gezocht worden. Deze response is betrokken bij: het remmen van de cel cyclus, het activeren van (andere) genoom onderhoud mechanismen en het initiëren van apoptosis (gecontroleerde zelfdood) of een terminaal arrest. Hoe de DNA-schade-geïnduceerde-signalering-response zelf geactiveerd wordt is nog niet volledig bevatbaar. Naast de DNA reparatie afhankelijke histone veranderingen zijn er andere aanwijzingen dat DNA herstel de DNA-schade-afhankelijke-signalering-response activeert.

In het laatste experimentele **hoofdstuk 8** wordt de ophoping van HR23B in neuronale insluitingen getoond. HR23B heeft een duale rol in NER en in het UPS afhankelijke eiwit afbraak. In het UPS functioneert HR23B waarschijnlijk als een chaperonne/koppelaarster die (meervoudig) geubiquityleerde eiwitten en het UPS bij elkaar brengt. Dit omdat HR23B zowel aan het 26S proteasome als aan (meervoudig) geubiquityleerde eiwitten kan binden. Ondanks de ophoping in deze neurale insluitingen van een NER factor (HR23B), hebben deze insluitingen geen effect op NER. HR23B ophoping in neuronale insluitingen werd gevonden in het brein van een fragiel X geassocieerd tremor/ataxia syndroom muis model, ook werd het gevonden in het brein van patiënten die of leden aan: de ziekte van Huntington, Spinocerebellar ataxia3, Spinocerebellar ataxia7, frontotemporale dementie en parkinsonisme gelieerd aan chromiosoom 17 of aan de ziekte van Parkinson. Er werd echter geen ophoping van

HR23B in tau-positieve aggregaten (neurofibrillaire tangles/verwarringen) van patiënten die lijden aan de ziekte van Alzheimer gevonden. Dit laatste toont aan dat de ophoping van HR23B in neuronale insluitingen niet alleen bepaald wordt door de interactie met het ubiquitine dat in deze insluitingen is opgehoopt maar dat voor deze ophoping ook andere interacties nodig zijn.

Het hoofdonderwerp van dit proefschrift is de regulatie van NER. De laatste jaren is duidelijk geworden dat de herkenningsstap onderhevig is aan een aantal processen zoals: posttranslationele modificaties, transcriptionele inductie, nucleair en cytoplamsatisch pendelen en een verandering in de stochiometrie van het complex. In de **discussie** worden enkele mechanistische en praktische ideeën geopperd. Het werk dat hier wordt gepresenteerd heeft geresulteerd in nieuwe inzichten in NER, zowel in de herkenning als in de post excisie stappen. Daarnaast werden nieuwe, NER onafhankelijke, functies van HR23B onthult.

Curriculum Vitae

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1988-1995	VWO, Titus Brandsma Lyceum te Oss
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Submitted

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