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Nucleotide excision repair : a multi-step mechanism required to maintain genome integrity

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NUCLEOTIDE EXCISION REPAIR
A MULTI-STEP MECHANISM REQUIRED TO MAINTAIN GENOME INTEGRITY

Jill Moser
2010

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Cover: Immunofluorescent staining showing accumulation of XRCC1 at local UV-induced DNA damage sites in normal human fibroblasts

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NUCLEOTIDE EXCISION REPAIR
A MULTI-STEP MECHANISM REQUIRED TO MAINTAIN GENOME INTEGRITY

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1

INTRODUCTION AND OUTLINE OF THE THESIS

1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

DNA carries the genetic information required for sustained life and reproduction. Hence, DNA damage induced by continuous exposure to both endogenous and exogenous genotoxic agents can have serious consequences for all organisms. Nucleotide excision repair (NER) is a multi-step enzymatic process which recognizes and eliminates a wide spectrum of lesions causing large distortions of the DNA structure such as UV induced lesions and bulky chemical adducts (Pfeifer, 1997; Wogan et al., 2004). The consequences of defective NER are apparent from the clinical symptoms of individuals affected by three disorders associated with reduced NER capacities- Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS) and Trichothiodystrophy (TTD). These disorders are linked by increased sensitivity to UV-irradiation and patients have a greatly elevated cancer incidence (XP) and multi-system defects which can include immunological and neurological disorders (Mitchell et al., 2003).

The aim of the work outlined in this thesis was to identify novel factors and gain new insights into the global genome repair pathway in human cells. Although most core factors are known we are only beginning to understand the complex organization of this repair process and regulation of the various NER factors. In **chapter 2**, we focus on the importance of genome stability and the various cellular defense mechanisms as a response to various types of DNA damage. In addition, the various DNA repair pathways involved in the repair of various types of DNA damage (such as single- and double strand breaks, oxidative lesions as well as UV-induced damage) are briefly described. **Chapter 3** provides a more detailed explanation of NER. In humans, NER is solely responsible for the repair of UV-induced lesions. The consequences of defective NER, as well as the 2 sub-pathways of NER, Transcription-coupled repair (TC-NER) and Global genome repair (GG-NER) are therefore described in detail. In addition we present a model for GG-NER (in mammals) describing the probable sequence of events. This model is based on data from previously published work, as well as on experimental evidence described in this thesis. Genetic information is packaged with histones and other accessory proteins into chromatin. Chromatin remodeling is essential for key cellular processes such as the regulation of gene expression, DNA replication and DNA repair in order to alter the accessibility of DNA. In addition to ATP-dependent chromatin modifications (by chromatin remodeling complexes) numerous covalent modifications of core histones have been identified which include acetylation, sumoylation, methylation, phosphorylation and ubiquitination. These chromatin alterations are described as the "histone code". The importance of chromatin remodeling mechanisms and chromatin modifications is described in **chapter 4**.

The role of damage recognition factor UV-DDB in NER is described in **chapter 5**. UV-DDB was shown to form a stable complex when bound to 6-4PP which allowed subsequent NER proteins such as XPC-hHR23B to accumulate and verify the lesion resulting in efficient 6-4PP repair. In **chapter 6**, the *in vivo* properties of DDB2, Cul4A and XPC are described. The properties of fluorescently labeled DDB2 in wild-type and XPC-deficient cells were analyzed and results suggest that the bulk of DDB2 interacts with lesions independently of XPC. We propose that DDB2 prepares UV-damaged chromatin for the assembly of

NER complexes. Turning our attention to the later stages of NER, the involvement of DNA polymerases and DNA ligases are described in **chapter 7**. Here we show for the first time that DNA Ligase III and its binding partner XRCC1 are involved in the sealing of chromosomal nicks introduced by NER. In fact, 2 distinct ligase-polymerase complexes are functional dependent on the cell-cycle status. **Chapter 8** focuses on the stability of the NER complex and the assembly and disassembly of the various NER components at UV-induced lesions. Here we show that all pre-incision NER proteins with the exception of RPA are able to disassemble from NER complexes and associate with unprocessed sites of damage. In contrast, post-incision complexes together with RPA remain associated with the incomplete NER synthesis sites. We propose a model wherein RPA plays a pivotal role in preventing new incision events when gap filling/sealing is not completed or is disturbed thereby averting further generation of DNA strand breaks that could lead to mutagenic and recombinogenic events with deleterious consequences for cells and organisms. It is known that arsenic acts as a co-carcinogen by inhibiting the repair of UV-induced lesions by NER but the exact mechanism has not been described until now. In **chapter 9** we show that the late stages of NER are affected and in particular we show a specific inhibitory affect of arsenic on DNA ligase III function.

2

CELLULAR RESPONSES TO DNA DAMAGE

DNA DAMAGE

Translation of genetic information into functional proteins as well as the inheritance of genetic code is essential for species survival. Alterations to the DNA induced by endogenous as well as exogenous agents threaten cellular survival by interfering with important processes such as replication and transcription. Spontaneous mutations are those intrinsic to the chemical nature of DNA in an aqueous solution and include deamination and the creation of abasic sites (Lindahl, 1993). Endogenous metabolism of the cell results in accumulation of reactive oxygen and nitrogen species as well as other metabolites which are able to damage DNA (De Bont and van Larebeke, 2004). Mutations are also the consequence of DNA damage induced by either mutagens present in the environment, such as UV radiation, dietary contaminants, air pollutants such as exhaust fumes and numerous chemicals. Exposure to X-rays and chemotherapeutic agents also cause DNA damage (Hoeijmakers, 2001). All organisms are exposed to damaging agents as part of everyday life. It is estimated that endogenous sources induce approximately 50,000 DNA lesions per cell per day in humans (Friedberg, 1995) whereas one hour of sunbathing is estimated to generate around 80,000 DNA lesions per cell (Mullaart et al., 1990). Metabolic cellular processes generate reaction products including active reactive oxygen species (ROS) and nitrogen species which cause oxidative modifications to DNA including base modifications (8-oxo-G, thymine gycols and cyclopurines) as well as single- and double-strand breaks (Cadet et al., 2003). In addition, ultraviolet light (UV) can induce the formation of helix-distorting lesions such as 6-4 photoproducts (6-4PP) and cyclopyrimidine dimers (CPD) (Wood, 1999). Ionizing radiation (IR) generates hydroxyl radicals introducing base damages which can lead to single- or double-strand DNA breaks (Cadet et al., 2003; Hoeijmakers, 2001).

CELLULAR RESPONSES TO DNA DAMAGE

Fortunately, eukaryotic and prokaryotic organisms possess a variety of genome maintenance mechanisms to protect themselves from constant assault by damage-inducing agents. Cells respond to DNA damage by a number of carefully coordinated processes such as, cell cycle checkpoint activation, induction of transcriptional programs, enhancement of DNA repair pathways, and chromatin remodeling and modification mechanisms (Hoeijmakers, 2007). When the level of damage is too severe, apoptosis is initiated. Nevertheless, none of these systems is perfect, and any remaining damage, or any damage repaired incorrectly, may play an important role in the induction of birth defects, the development of diseases such as cancer as well as aging.

DNA DAMAGE CELL CYCLE CHECKPOINTS

The cell cycle is organized into a series of sequential pathways; G1 followed by DNA replication or synthesis phase-S, followed by G2 and finally cell division or mitosis-M. DNA damage checkpoint systems are control mechanisms that delay cell-cycle progression in

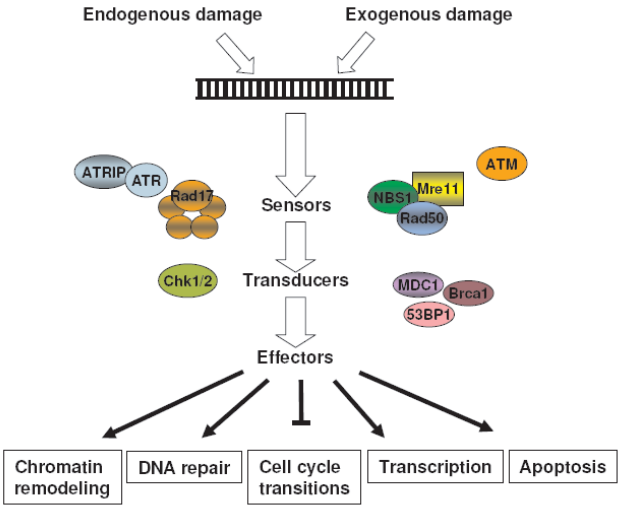

Fig. 1. Simplified representation of the DNA damage response. DNA damage is first detected by sensor proteins which in turn activate transducers in the signal cascade. These transducers then mediate the activation or inhibition of downstream effectors which can arrest the cell cycle, initiate DNA repair or induce apoptosis.

response to DNA damage or replication stress. These regulatory pathways govern the order and timing of cell cycle transitions ensuring completion of one cellular event prior to commencement of another which is essential for maintaining genomic integrity (Abraham, 2001; Bartek and Lukas, 2007). The delay in cell cycle progression enables cells to repair their DNA damage before the damage is passed on to daughter cells thereby ensuring faithful transmission of genetic information (Reinhardt and Yaffe, 2009; Bartek and Lukas, 2007). Mutations that inactivate DNA damage checkpoint genes result in disruption of the DNA damage signaling pathways and differences in the response of cells to DNA damage. DNA checkpoint control can occur in G1, S phase, or at the G2-M transition (Berkovich et al., 2007). The DNA damage response involves first sensing a signal and then transducing it to downstream effectors that regulate the activity of several downstream targets, including factors that regulate cell-cycle progression and DNA repair processes (Sancar et al., 2004). Sensors recognize the lesions themselves or remodelled chromatin structure that follows DNA damage (Downs et al., 2007). Transducers initiate a signal transduction cascade that propogate and amplify the signal and include ATM and ATR as well as p38 MAPK. Effector kinases such as ChK1 and ChK2 execute the specific cellular response (Bartek and Lukas, 2003).

The central components of the signal transduction response are the phosphoinositol-3-kinase-like protein kinases ATM (Ataxia telangiectasia), ATR (Ataxia telangiectasia and Rad3-related) and DNA-PK (Abraham, 2001). These transducers coordinate the initiation, amplification, and activation of the checkpoint through phosphorylation of many different targets. ATM is activated by DNA double strand breaks (DSBs) whilst ATR is activated by single stranded regions of DNA (ssDNA) and RPA exposed for example during UV damage repair and DNA replication fork stalling (Abraham, 2001; Zou and Elledge, 2003). Both kinases phosphorylate many of the same substrates (e.g. p53, Brca1, FANC-D2) and have distinct but overlapping signal transduction pathway functions. An important substrate

of ATM, ATR and DNA-PK in the vicinity of the damaged site is the histone H2AX, which represents a sub-family of histone H2A. Histone H2AX is an adaptor molecule of the DNA repair machinery which is phosphorylated at its C-terminus (designated γ-H2AX) thereby being a marker of DNA lesions (Fernandez-Capetillo et al., 2004). Additionally, γH2AX mediates DNA repair by promoting the maintenance of repair factors close to the DNA lesion (Bonner et al., 2008). ATM is recruited to DSBs via a sensor complex composed of Mre11-Rad50-Nbs1 (MRN complex). ATM phosphorylates histone H2AX, flanking the sites of DNA damage. MDC1, 53BP1 and BRCA1 accumulate at phosphorylated H2AX resulting in Chk2 activation (Canman, 2003). Chk2 which plays a role in controlling the activity of the Cdc25 phosphatases, a family of proteins intimately involved in the activation of various Cdk-Cyclin complexes (Bartek and Lukas, 2003). ATR exists as a heterodimer with ATRIP (ATR interacting protein) and is recruited to ssDNA via the Replication Protein A heterotrimer (RPA). The ATR/ATRIP complex is only activated and functional when the sensor Rad17 and 9-1-1 (Rad9, Rad1, and Hus1) complexes are loaded onto the DNA (Zou et al., 2002). Chk1 and Chk2 are checkpoint transducer serine/threonine kinases that function downstream in the DNA-damage checkpoint signaling pathway (Bartek and Lukas, 2007). Although Chk1 and Chk2 have overlapping roles, Chk1 is expressed during S and G2 where its activity is amplified in the presence of different types of DNA damage (Zhao et al., 2002). Chk2 is expressed throughout the cell-cycle and is also activated in the presence of DNA damage (Lukas et al., 2001).

In response to DNA damage, the G1 checkpoint prevents the cell from attempting to replicate damaged DNA by blocking cells from entering the S phase by inhibiting the initiation of replication (Massague, 2004). If damaged DNA or replication errors are sensed during S-phase, the S-phase checkpoint is activated. DNA synthesis is then slowed down in order to provide time for repair (Bartek et al., 2004). DNA damage incurred in G2 phase, or cells that have escaped G1 and S-phase checkpoints despite earlier DNA damage results in activation of the G2/M checkpoint to prevent entry into mitosis with damaged chromosomes (Taylor and Stark, 2001). There is currently no evidence that UV-induced lesions activiate ATM or DNA-PK. However, during S-phase, NER lesions generate stalled replication forks leading to accumulation of NER-related proteins and the generation of single-stranded streches of DNA. RPA, ATRIP and ATR accumulate triggering replication-stress signalling (Abraham, 2001; Ward et al., 2001; Jeggo and Lobrich, 2001). In addition to replication, nucleotide excision repair (NER) has also been implicated in ATR checkpoint activation (reviewed in Rouse and Jackson, 2002). These studies were conducted in yeast arrested in G1, providing evidence that NER is needed to generate a checkpoint-inducing signal in the absence of replication. It has been speculated that this signal is an RPA-coated, ssDNA-gapped structure (O'Driscoll et al., 2003; Giannattasio et al., 2004). Whether or not NER directly contributes to activation of the ATR-dependent checkpoint in higher eukaryotes is unclear. However, Both ATR and its associated protein ATRIP have been shown to translocate to punctate nuclear foci following treatment of cells with UV (Ward et al., 2004), suggesting that there is an ATR-mediated DNA damage response in normal mammalian cells. The MAPKAP kinase-2 (MK2) is a transducer kinase which is downstream of the stress-response p38 MAPK pathway. MK2 is directly involved in

phosphorylating effectors CDC25B and C, and in maintaining G1, S, and G2 checkpoints triggered by UV-induced DNA damage, cisplatin, camptothecin and doxorubicin (Manke et al., 2005).

Together the transducers ATM/Chk2, ATR/Chk1 and MK2 phosphorylate a variety of effector proteins, such as p53 and the CDC25 phosphatases which transmit the DNA damage signal downstream, eventually arresting the cell cycle, initiating DNA repair, or, if necessary, apoptosis. More detailed information on the DNA damage checkpoint signaling pathways at the molecular level can be found in recent reviews by Reinhardt and Yaffe (2009) and Lazzaro et al., (2009).

DNA REPAIR

Several different DNA repair pathways have been identified which each focus on a specific category of DNA lesions. These repair systems also differ in the way that the damage is recognized which is dependent on the severity of DNA damage (Hoeijmakers, 2001). To date, more than 130 DNA repair enzymes have been identified which ensure genomic integrity (Hoeijmakers, 2007; Schumacher et al., 2008). Once damage is repaired and chromatin is restored, cells are able to proceed through the cell cycle. The main repair pathways in mammals are nucleotide excision repair (NER), Base excision repair (BER), Homologous recombination (HR), Non-homologous End-joining (NHEJ) and Mismatch repair (MMR) (Friedberg, 2003; Hoeijmakers, 2001).

Nucleotide Excision Repair

Nucleotide excision repair (NER) is one of the most versatile DNA repair systems in humans. NER eliminates bulky DNA damage that leads to distortions of the DNA helix. In fact, this repair pathway is essential for the repair of UV-induced lesions, such as CPDs and 6-4PPs. NER is a multi-step process which requires around 30 different proteins and removes DNA damage by dual incision of the damaged strand. NER is divided into two sub-pathways which allow either efficient removal of lesions which block ongoing transcription, known as transcription-coupled repair (TCR), or removal of lesions at any other position in the genome which is known as global genome repair (GGR) (Tornaletti and Hanawalt, 1999). Since my work has focused on a better understanding of the NER pathway, the subsequent chapters in this thesis describe NER in greater detail.

Base Excision Repair and Single-strand break repair

Base excision repair (BER) is a major pathway for the repair of a wide variety of DNA base damages which include oxidative DNA damage (8-oxoguanine, thymine glycols) alkylation products, and single strand breaks (Beckman and Ames, 1997; Fortini et al., 2003). BER is classified into 2 sub-pathways: short-patch and long patch repair. Short-patch BER is a mechanism whereby only 1 nucleotide is replaced, whereas in long patch BER, 2-13 nucleotides are replaced. Various DNA glycosylases have been identified, each with specific affinity for a subset of lesions. These gycosylases first recognize specific damaged bases and excise them from the genome, initiating both long-patch and short-patch BER.

Removing the damaged base, followed be nuclease mediated strand incision results in an abasic site (AP site) (reviewed in Dodson and Lloyd, 2002) which is subsequently refilled by means of DNA synthesis by DNA polymerase pol β. Finally either the XRCC1-DNA Ligase III complex or DNA Ligase I allows efficient ligation restoring the integrity of the helix (Wilson, III and Bohr, 2007; Almeida and Sobol, 2007).

Double-strand break repair

Double-stand breaks (DSBs) are among the most lethal of all DNA lesions since their inefficient or inaccurate repair results in genetic rearrangements that can lead to cancer or cell death. Efficient repair of DSBs is therefore essential for genome stability (Rich et al., 2000). DSBs are caused by a variety of sources including ionizing radiation, certain genotoxic chemicals, endogenously generated ROS and replication of single-stranded DNA breaks (Hoeijmakers, 2001). V(D)J recombination also generates DSBs during rearrangement of genes encoding B cell immunoglobulins and T cell receptors. DSBs differ from most other types of DNA lesions in that they affect both strands of the DNA duplex and therefore prevent use of the complementary strand as a template for repair as seen for example in NER. DSBs are repaired by 2 major mechanisms: homologous recombination (HR) and non-homologous end-joining (NHEJ). The choice of which pathway to utilize appears to be largely influenced by stage within the cell cycle at the time of damage acquisition (reviewed in Delacote and Lopez, 2008; Shrivastav et al., 2008). The NHEJ pathway simply repairs DSBs by re-ligating their 2 ends together, but bases may be added or lost as it occurs making it an inaccurate process. The core NHEJ machinery is known to be composed of 3 core complexes: MRN, Ku and the DNA ligase complex although the order of action of these complexes has not yet been fully established. Nevertheless, it is thought that MRN and Ku complexes bind DSBs shortly after DSB formation, inhibiting their degradation. In addition to bridging the DSB ends together, Ku and MRN also play crucial roles in recruiting, stabilizing and stimulating the ligase complex (Scott and Pandita, 2006; Weterings and Chen, 2008). After replication, HR acts by initiating a series of complex DNA transactions between the identical sister chromatid to properly align the broken ends inserting missing information. If the DNA template used for repair is not identical to the original DNA sequence present at the DSB, HR can cause mutations or more severe genome rearrangements (Szostak et al., 1983). (Reviewed in Natarajan and Palitti, 2008).

Mismatch repair

The mismatch repair (MMR) pathway is a multi-step process which plays an essential role in the correction of mismatches such as those due to replication errors. Such base-base mismatches and insertion/deletion loops result from DNA polymerase mis-incorporation of nucleotides and template slippage (Hsieh and Yamane, 2008). Failure of mismatch correction by cellular repair systems or misdirected repair to the strand containing the original and correct DNA sequence will ultimately lead to genetic mutations (Modrich, 2006). MMR recognizes and corrects all base-base mismatches and small insertions/

deletions and therefore increases the fidelity of DNA replication by up to 1000 fold. Cells harboring mutations in MMR proteins suffer from an increased frequency of spontaneous mutations emphasising the importance of this repair pathway (Kunkel and Erie, 2005). MMR is described in the review by Hsieh and Yamane (2008).

TRANSLESION SYNTHESIS

If DNA damage still persists despite the above mentioned repair processes replication blockage can occur. One way to circumvent this block is by "damage avoidance", using recombinational mechanisms to copy genetic information from the undamaged sister duplex. The alternative is to incorporate nucleotides opposite the damage, a process-designated as translesion synthesis (TLS) (Jansen et al., 2007). TLS makes use of special DNA polymerases that are able to bypass specific types of DNA damage in order to avoid replication blockage.TLS polymerases η, ι, κ and Rev1 (Y-family polymerases), as well as polymerase ζ (B-family) have been identified, each with their own specificities. These polymerases take over synthesis in order to bypass the injured DNA lesion. TLS is reviewed by Green and Lehmann (2005) and Jansen et al., (2007).

3

NUCLEOTIDE EXCISION REPAIR

NER is activated by a wide-range of helix-distorting DNA lesions, including UV-induced photoproducts (CPDs and 6-4PPs) (see Fig.2), bulky chemical adducts and certain oxidative lesions. In human cells, the NER reaction requires around 30 core factors and comprises of the following steps: 1) DNA damage recognition, 2) DNA unwinding and lesion verification, 3) dual incision around the lesion, 4) excision of the single-stranded DNA fragment containing the DNA lesion and 5) repair synthesis and ligation of the nick. NER can be subdivided into 2 distinct subpathways, global genome repair (GGR) and transcription-coupled repair (TCR) which differ with respect to lesion detection and some of the factors involved. The GGR factors UV-DDB and XPC-HR23B are responsible for detecting lesions throughout the genome. Various TCR-specific factors such as CSA and CSB are involved in the repair of lesions in transcribed regions (Tornaletti and Hanawalt, 1999). Following recognition of the damaged DNA, common NER factors are recruited in both sub-pathways of NER to complete the repair process. A detailed description of damage recognition and the subsequent NER processes will be described later in this chapter.

THE CONSEQUENCES OF A DEFECT IN NER

Skin cancer is the most common form of cancer in the United States, more than 1 million skin cancers are diagnosed annually (National Cancer Institute, 2007 SEER Database) and 1 in 3 Caucasians are likely to develop skin cancer in the course of his or her lifetime (American Cancer Society 2007). NER represents the only repair pathway which removes mutagenic photolesions induced by sunlight. A defect in NER therefore leads to extreme sun sensitivity and an elevated risk of developing skin cancer. The average age of a patient with skin cancer is approximately 60 years yet the age of onset is reduced by approximately 50 years in individuals with a defective NER pathway. Defects in NER are associated with 3 major autosomal recessive disorders, namely xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (Fig. 3). At the clinical level XP

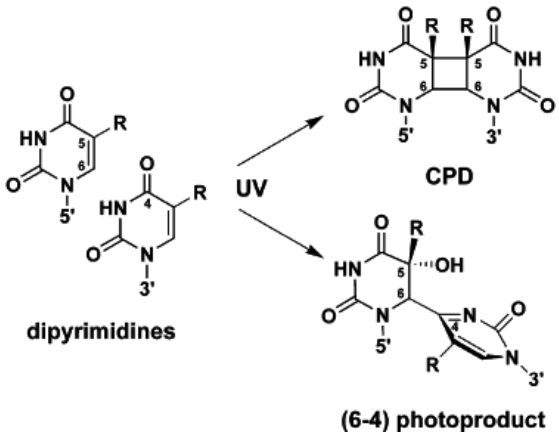

Fig. 2. UV-induced photoproducts. The UV component of sunlight can cause bulky lesions in DNA as a result of dimers forming between thymine bases. The two main types are cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts (Adpated from Li et al., 2006).

Fig 3. Diseases associated with mutations in NER genes.

Disease	Symptoms	Malignant (skin) lesions	Affected genes
Xeroderma Pigmentosum (XP)	Sunburn, hyper- and hypopigmentation, atrophy in sun-exposed regions, risk of internal cancers, neurological symptoms	Extremely high incidence of skin cancer. Basal cell carcinomas, melanomas (UV-induced skin tumours) in childhood	XPA XPB XPC XPD XPE XPF XPG XPV
Cockayne Syndrome (CS) (XP/CS combined effects of XP and CS)	Sunburn, growth failure, hyperpigmentation, physical and mental retardation, bird-like face, cachexia, neuronal degeneration, loss of retinal cells	No increased (skin) cancer risk	CSA CSB XPB XPD XPG
UV-sensitive syndrome ($UV^{S}S$)	Photosensitivity, mild skin abnormalities	No increased (skin) cancer risk	CSB CSA unknown genes
Cerebro-Oculo-Facio-Skeletal syndrome (COFS)	Symptoms similar to CS	No increased (skin) cancer risk	CSB XPD XPG ERCC1
Trichothiodystrophy (TTD)	Sunburn, erythema, ichthyosis, characteristic brittle nails and short, brittle sulfur-deficient hair, neurologic and skeletal degeneration, cachexia	No increased (skin) cancer risk	TTD-A XPB XPD

is characterized by a highly increased incidence of tumors in sun-exposed areas of the skin (reviewed in Cleaver et al., 2009). In contrast, CS and TTD are disorders without enhanced cancer susceptibility characterized by developmental and neurological abnormalities and premature aging. (reviewed in Lehmann, 2003). (Fig. 3)

Xeroderma Pigmentosum (XP)

NER defects cause mutagenic damage to persist across the entire genome giving rise to the cancer prone syndrome xeroderma pigmentosum (XP). Patients who suffer from XP exhibit severe sun-sensitivity, photophobia, freckling and a 1000-fold elevated sun-induced skin cancer risk and accelerated skin aging (Kraemer and Slor, 1985; Berneburg and Lehmann, 2001). Apart from UV-induced skin cancers, XP-patients have a 10-20 fold increased risk to develop internal cancers before the age of 20 years (Kraemer et al., 1984). An XP-phenotype can result from defects in any one of seven genes involved in the NER cascade (XP-A through to XP-G) (Keeney et al., 1994; Legerski and Peterson, 1992; Masutani et al., 1994; Flejter et al., 1992; Scherly et al., 1993; Sijbers et al., 1996; Weeda et al., 1990).

Cells from all these complementation groups are deficient in GGR and with the exception of XPC and XPE also in TCR (Venema et al., 1991; Hwang et al., 1999). Recent findings with XPE, XPC and XPG suggest that they may also be involved in other processes such as the repair of oxidative damage (Lee et al., 2006; D'Errico et al., 2006; Shimizu et al., 2003; Schärer, 2008). XP patients belonging to complementation groups XP-A, XP-B, XP-D and XP-G, exhibit progressive neurological abnormalities in addition to their XP symptoms. These neurological symptoms include reduced tendon reflexes, deafness, and speaking and walking disability because of primary neuronal degeneration (Mimaki et al., 1986; Robbins et al., 1991). The observation that individuals with XPC and XPE abnormalities are virtually free of neurological defects suggests that TCR of lesions in the transcribed strand of genes is important for the development of neurological symptoms and that functional TCR is sufficient to prevent these symptoms from occurring. Cells from XP-V (XP variant) individuals are NER proficient but lack Polymerase eta which is able to bypass CPDs in an error free manner. More error-prone DNA polymerases take over in XP-V cells, resulting in the accumulation of mutations during DNA replication (Kannouche and Stary, 2003). The first indications of XP are a strong increase in sunsensitivity freckling in sun-exposed areas, followed by other pigmentation changes, loss of elasticity and multiple skin cancers including basal and squamous cell carcinomas as well as malignant melanomas. However, if protected from sunlight at an early age, individuals can remain completely free of skin lesions which accordingly increase their lifespan.

Cockayne Syndrome (CS)

Cockayne syndrome (CS) is a rare, autosomal, recessive disease characterized by severe and diverse clinical symptoms such as mental abnormalities, brain degeneration and pigmentary degeneration of the retina followed by pronounced cachexia leading to early death (Nance and Berry, 1992). In CS patients, growth is retarded and a prominent feature is a bird-like face, characterized by sunken eyes, a beaked nose and a narrow mouth. Patients have reduced subcutaneous fat and skeletal abnormalities such as kyphosis (Cleaver et al., 2009). As seen for XP, CS patients are UV-sensitive and show atrophy of sun-exposed skin. Despite a DNA repair defect, CS is not associated with an increased skin cancer risk (Nance and Berry, 1992). Classic CS is caused by mutations in *CSA* or *CSB* (Cockayne syndrome complementation groups A and B respectively). CSA and CSB are essential for the function of the transcription machinery and defects in either CSA or CSB cause a disruption in the recovery of DNA damage inhibited transcription (Mayne and Lehmann, 1982; Venema et al., 1990). In addition, XP-B patients and certain patients belonging to XP-D or XP-G show features of CS in addition to symptoms of XP (XP-B/CS, XP-D/CS and XP-G/CS). XP-CS cells display both impaired GGR and TCR together with the inability to recover RNA synthesis following DNA damage (van Hoffen et al., 1999). Both CSA and CSB primary skin cells were found to be hypersensitive to the lethal effects of oxidizing agents suggesting that the CSA and CSB proteins have additional functions beyond their roles in TC-NER (D'Errico M, et al. 2007). The involvement of CS proteins in the removal of oxidative damage may explain the neurological and aging features typical of CS. This notion has been supported by several independent observations (reviewed in

Cleaver et al., 2009; Bohr et al., 2007; Kyng et al., 2005) as well as a recent study of eye pathology in CS mouse models, implicating accumulation of endogenous oxidative DNA lesions in the retina in pigmentary retinopathy, a feature of CS-specific premature aging (Gorgels et al., 2007). The mean age of death of CS patients is 12.5 years and the most common cause of death is pneumonia as a result of atrophy and cachexia.

UV-sensitive syndrome (UV^{S}S)

A few years ago a new photosensitive disorder was identified called UV-sensitive syndrome (UV^{S}S). UV^{S}S was found to be distinct from XP (including XP-V) and CS since patients show only mild clinical manifestations (Itoh et al., 1994; Itoh et al., 1995). Cells derived from individuals with UV^{s}S showed UV sensitivity and a failure of recovery of RNA synthesis after UV irradiation, in spite of having a normal level of unscheduled DNA synthesis. Two complementation groups have been identified among UV^{S}S patients, defined by mutations in an as-yet-unidentified gene in 4 cases and in the *CSB* gene in 2 individuals (reviewed by Spikak, 2005). Recently, a UV^{S}S patient with a novel mutation in the CSA gene has been identified which represents the third complementation group of UV^{S}S (Nardo et al., 2009). The expression of the mutant CSA cDNA was found to increase the resistance of cells from a CSA patient to oxidative stress, but did not correct their photosensitivity. These observations imply that some mutations in the *CSA* gene may interfere with the TC-NER-dependent removal of UV-induced damage without affecting its role in the oxidative stress response. The differential sensitivity toward oxidative stress might explain the difference between the range and severity of symptoms in CS and the mild manifestations in UV^{s}S patients that are limited to skin photosensitivity without precocious aging or neurodegeneration (Nardo et al., 2009).

Cerebro-Oculo-Facio-Skeletal Syndrome (COFS)

Similar to CS, Cerebro-Oculo-Facio-Skeletal syndrome (COFS) is a recessive disease with symptoms including growth retardation, severe mental retardation, facial features such as deep-set eyes, prominent nose and large ears, cataracts, joint contractures and microcephaly (Longman et al., 2004; Meira et al., 2000; Del Bigio et al., 1997). Since COFS shares several features also associated with CS it is difficult to distinguish between these syndromes. However, COFS patients generally only live up to the age of 3 years and is therefore often described as an early infantile form of CS (Laugel et al., 2008). Similar to CS, mutations have been found in the CSB, XPD and XPG genes (Meira et al., 2000; Graham, Jr. et al., 2001; Hamel et al., 1996; Laugel et al., 2008). The first case of human inherited ERCC1 deficiency has been recently reported. The observed clinical features were very severe and compatible with a diagnosis of COFS (Jaspers et al., 2007).

Trichothiodystrophy (TTD)

Trichiothiodystrophy (TTD) patients include features such as shortened lifespan, small stature, mental retardation, decreased fertility, skeletal abnormalities and skin photosensitivity without decreased pigmentation or increased cancer sucessptibility and immunodeficiency (Cleaver, 2005; Stefanini et al., 1993). Although some TTD patients

show photosensitivity, they do not have cutaneous lesions and a skin cancer predisposition. The disease is however distinct from CS due to the typical appearance of sulfur-deficient brittle hair which has characteristic "tiger-tail" banding when examined in polarized light. This is due to a reduction of cysteine-rich matrix proteins that normally provide strength to the hair shaft by cross-linking the keratin filaments (Itin and Pittelkow, 1990; Stefanini et al., 1993). Since, most of the symptoms are quite similar to that of CS, TTD has likewise been classified as a segmental premature aging syndrome (de Boer and Hoeijmakers, 2000). Three complementation groups have been identified XPB, XPD and TTDA. In TTD patients, basal transcription is altered leading to decreased transcription of specific genes (Lehmann, 2003; Bohr et al., 2005). In fact, certain XPD point mutations cause TFIIH instability, interfering with a number of processes including the completion of terminal differentiation of hair and nails (Botta et al., 2002; Vermeulen et al., 2000) which explains the brittle hair and nails characteristics of TTD patients.

NUCLEOTIDE EXCISION REPAIR

Transcripton-coupled repair

An arrested RNA polymerase at a site of a lesion represents a strong signal for apoptosis (Ljungman, 2005; Ljungan and Zhang, 1996). To counteract apoptosis and to resume transcription, cells possess the capacity to remove transcription blocking lesions by a specialized sub-pathway of NER termed transcription coupled repair (TCR). TCR is initiated by a bulky lesion blocking the elongating RNA polymerase (RNAPIIo) located on the transcribed strand of an active gene (Brueckner and Cramer, 2007). CSB interacts loosely with the elongating polymerase (Selby and Sancar, 1997) but becomes more tightly bound following transcription arrest (van Gool et al., 1997). The CSB gene encodes a 168kDa protein which is related to the SWI/SNF family of ATP-dependent chromatin remodeling factors. CSB displays DNA-dependent ATPase and DNA binding activity, but not helicase activity (Citterio et al., 2000). Furthermore, when added to an RNAPII arrested at a CPD, CSB can stimulate transcription elongation by addition of one nucleotide to the nascent transcript in vitro (Selby et al., 1997). In vitro transcription assays using purified RNA polymerase and initiation factors determined the order of assembly of NER factors at arrested RNA pol IIo complexes. The CSB/RNAPII complex is able to interact with subunits of the TFIIH complex (Tantin, 1998) and may therefore have a role in recruiting TFIIH to the repair complex. In support of this model, it was shown that CSB and XPG can bind in a cooperative manner to RNAPII arrested at a cisplatin lesion *in vitro* (Sarker et al., 2005). Others have also shown XPG recruitment to the stalled RNAPIIo (Tantin et al., 1997; van den Boom, et al., 2004). ChIP analysis of chromatin-bound RNAPIIo isolated from in vivo crosslinked cells provided direct evidence for the interaction of CSB with RNAPIIo in the absence of damage (Fousteri et al., 2006). Upon UV-irradiation, the interaction of CSB with chromatin-bound RNAPIIo complex was enhanced. The fate of the polymerase remains unclear. In vitro results suggest that the RNAPIIo is released on the arrival of XPF with CSB being required for incision activity (Laine and Egly, 2006a; Laine and Egly, 2006b). However, recent findings suggest that the polymerase may stay stalled at the site

of damage while repair of the lesions occurs since XPG is able to interact with RNAPIIo allowing incision without the removal of the elongating polymerase (Sarker et al., 2005). Furthermore, *in vivo* evidence was presented that the stalled RNAPIIo and the NER specific endonucleases can be isolated as a single repair complex (Fousteri et al., 2006; van den Boom et al., 2004). In vivo studies also revealed a CSB-dependent recruitement of CSA to the RNAPIIo complex consistent with previous findings (Fousteri et al., 2006; Kamiuchi et al., 2002). CSA is part of a multiprotein complex with E3 ubiquitin-ligase activity and the RNAPIIo/CSB complex was shown to facilitate the assembly of not just CSA alone but the CSA-DDB1 E3-ubiquitin ligase/CSN complex (Fousteri et al., 2006) which then allowed further recruitment of HMGN1, HAT p300, XAB2 and TFIIS proteins (Fousteri et al., 2006). Recruitment of p300 required CSB alone whereas HMGN1, XAB2 and TFIIS required both CSB and CSA for binding at the stalled polymerase (Fousteri et al., 2006). Conformational changes of the RNAPIIo may be required to allow accessibility to repair proteins. Backtracking of the RNAPIIo may be one of the key mechanisms to allow repair and/or transcription restart. It was shown that backtracking could proceed far enough that a small DNA repair enzyme photolyase could bind and directly reverse the CPD (Tornaletti et al., 2001). Following backtracking, TFIIS enables cleavage of the protruding 3′ mRNA by RNAPIIo so that transcripton can restart upon lesion removal (Kalogeraki

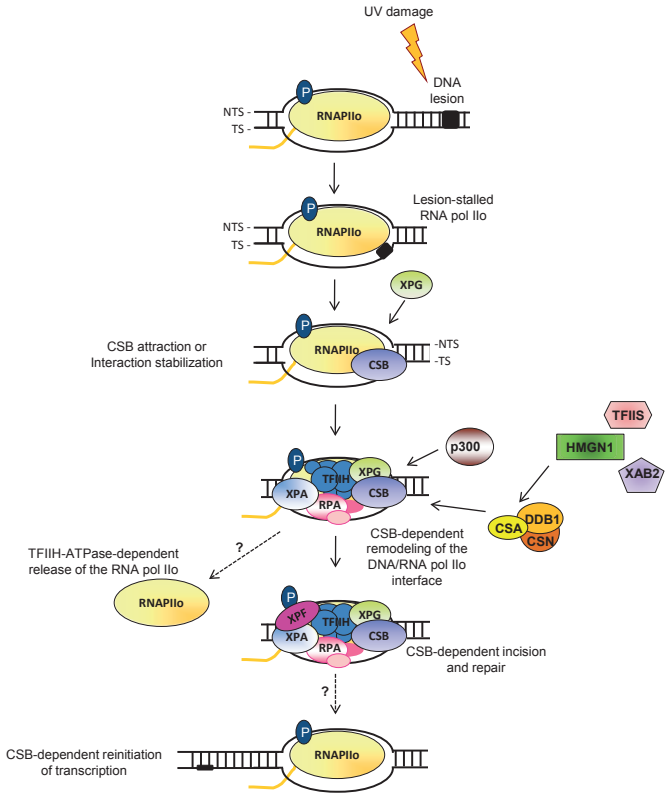

Fig. 4. Transcription-coupled repair. Transcription is arrested when the RNAPII encounters a lesion. CSB becomes tightly bound and facilitates the recruitment of TCR factors. XPG, TFIIH, XPA and ERCC1-XPF localize to the arrested elongation complex with RPA arriving once single-stranded DNA is formed. The chromatin remodeling factors p300 and HMGN1 loosen the nucleosome structure behind the polymerase. The RNAPII reverses direction, backtracking from the lesion allowing cleavage of the 3′ mRNA which is activated by TFIIS. After backtracking, TFIIH extends the denatured region around the lesion in order to allow subsequent incision by XPG and ERCC1-XPF. (Fig adapted from Fousteri et al., 2006; Sarasin and Stary, 2007)

et al., 2005; Fousteri et al., 2006). The backtracking process might require chromatin remodeling events, presently involving the high mobility group protein HMGN1 (Hanawalt and Spivak, 2008). *Hmgn1*-knockout mice exhibit reduced repair of CPDs in active genes implying a role in TCR (Birger et al., 2003). The assembly of HMGN1 may function to help displace nucleosomes that have become re-established behind the translocating transcription complex, so that the RNAPII can regress from the lesion. p300 is also known to have a role in chromatin remodeling during both transcription and DNA repair (Hasan et al., 2001; Cazzalini et al., 2008). XAB2 may have a pivotal role in TCR as well as in transcription. XAB2 is known to interact with XPA which suggests that it may be a key protein at the juncture of both GGR and TCR for which all subsequent steps are shared (Kuraoka et al., 2007) Once TFIIH and other factors have been recruited, the TCR pathway is identical to that of GGR (Bowman et al., 1997; M. Fousteri, unpublished results) and the corresponding repair patch sizes are similar (Bowman et al., 1997).

Global Genome Repair

In the past, numerous models have been proposed to describe the assembly and disassembly of the various NER proteins at the site of damage. One theory proposed that all of the core NER proteins (XPC-hHR23B, TFIIH, XPA, RPA, XPG and ERCC1-XPF) were together in a pre-assembled complex capable of carrying out the excision step. A series of studies showed that the core NER factors were associated in a repair holocomplex in *S. cerevisiae* (Svejstrup et al., 1995; Rodriguez et al., 1998), and in human cells (He and Ingles, 1997). However, several studies provide data against this notion (Araujo and Wood, 1999; Park and Choi, 2006), while others present evidence that the NER factors assemble sequentially at DNA damage (Araujo et al., 2001; Riedl et al., 2003; Mone et al., 2004; Volker et al., 2001). This model is now widely accepted and Chapter 8 describes the assembly and disassembly of NER factors at DNA lesions in detail.

DNA DAMAGE RECOGNITION

UV-DDB and XPC

In GGR, DNA damage recognition proteins are able to distinguish the difference between damaged and undamaged DNA by identifying DNA that has undergone helical distortions. The initial damage recognition factors involved in NER have been the subject of some debate in the past. Some in vitro studies suggested that XPA or a RPA-XPA complex binds first to the damage, subsequently recruiting XPC-hHR23B and the other core NER factors (Wakasugi and Sancar, 1999; Evans et al., 1997). In contrast, other in vitro findings clearly provide evidence that the XPC-hHR23B complex is the primary damage recognition factor (Sugasawa et al., 1998). Finally, in vivo analysis showed conclusive evidence that XPC-hHR23B is the principal damage recognition complex and initiator of NER (Volker et al., 2001). XPC exists in bound form with hHR23B and Centrin 2 in cells (Araki et al., 2001; Shivji et al., 1994). hHR23B protein contains two ubiquitin-associated domains and one ubiquitin-like domain which can stabilize XPC and enhance the binding between XPC and damaged DNA (Sugasawa et al., 2005). Loss of hHR23B results in decreased XPC

levels (Ng et al., 2003). However, recent studies carrying out live cell FRET measurements suggest that hHR23B dissociates when XPC binds to damaged DNA indicating that it is not directly involved in repair (S. Bergink, unpublished data). Centrin 2 is thought to stimulate XPC (Nishi et al., 2005), although its exact function is not yet clear. Although the XPC-hHR23B complex is considered as the primary damage recognition factor, there is considerable evidence for another DNA damage recognition factor i.e., UV-DDB that preceeds XPC-hHR23B.

UV-DDB is a heterodimer of the DDB1 (p127) and DDB2 (p48) proteins. Mutations in DDB2 cause the XPE phenotype. CPD repair is UV-DDB dependent (Fitch et al., 2003b; Tang et al., 2000) which explains why cells from XPE patients are deficient in CPD repair (Nichols et al., 2000; Rapic-Otrin et al., 2003). We also provide data showing that DDB2 significantly stimulates the repair of 6-4PPs especially at low doses (Chapter 6). As already mentioned XPC-hHR23B has a high affinity for DNA lesions such as 6-4PPs, suprisingly it's binding to CPDs is less efficient and in fact repair of CPDs requires DDB2 and XPC-hHR23B (Tang et al., 2000). DDB2 has a binding affinity for a number of different lesions such as mismatches, AP sites and compound lesions (Chu and Chang, 1988; Fujiwara et al., 1999; Payne and Chu, 1994; Wittschieben et al., 2005) but an extremely high affinity for 6-4PPs (Nichols et al., 2000; Reardon et al., 1993; Treiber et al., 1992; Keeney et al., 1993). UV-DDB is not essential for in vitro NER reactions suggesting that it is required for the repair of lesions within chromatin (Aboussekhra et al., 1995; Bessho et al., 1997; Araujo et al., 2000; Kulaksiz et al., 2005). DDB2 is incorporated into a functional CUL4A E3 ubiquitin ligase through its interaction with DDB1 (Groisman et al., 2003; He et al., 2006) and live cell imaging revealed that DDB2, DDB1 and Cul4A are rapidly recruited to UV lesions with similar association kinetics (Chapter 7). DDB2 undergoes ubiquitin mediated proteolysis following UV irradiation and the timing of this degradation determines the arrival of XPC to the damage site (El Mahdy et al., 2006). UV-DDB breakdown increases the binding of XPC to the damaged DNA using a reconstituted NER system (Sugasawa et al., 2005), and interfering with DDB2 proteolysis was shown to compromise the removal of CPDs in vivo (El-Mahdy et al., 2006). These results suggest that the recruitment of UV-DDB to UV damage and the degradation of DDB2 is crucial for XPC recruitment and the repair of photolesions. In line with these observations, we and others found that DDB helps to recruit XPC to both CPDs and 6-4PPs which accelerates the repair of these lesions (Fitch et al., 2003a; Chapters 5 and 6).

It is known that XPC is modified upon UV-irradiation, the modifications include ubiquitylation and sumoylation (Sugasawa et al., 2005). The UV-induced XPC ubiquitylation is regulated by DDB-Cul4A E3 ubiquitin ligase complex comprised of DDB1, DDB2, Cul4A, Roc1 and COP9 signalosome (Groisman et al., 2003). DDB–Cul4A complex can ubiquitylate both DDB2 and XPC, but the fates of ubiquitylated DDB2 and XPC appear to be quite different: ubiquitylated DDB2, but not XPC, is subjected to proteasomal degradation (Sugasawa et al., 2005). XPC is monoubiquitinated after DNA damage which increases the binding of XPC to damaged DNA as well as promoting its interaction with hHR23B (Araki et al., 2001; Ng et al., 2003; Ortolan et al., 2004). The role of DDB2 therefore facilitates prompt recruitment of XPC to UV lesions as well as promoting XPC degradation (Wang et

al., 2007). It has already been reported that during assembly of NER factors, XPC–hHR23B and XPG cannot simultaneously exist in the repair complex and that the entry of XPG into the complex coincides with XPC–hHR23B leaving the complex (Riedl et al., 2003). Although dissociation of XPC from the complex prior to incision has been observed in vitro experiments (Riedl et al., 2003; Wakasugi and Sancar, 1998), recent competition experiments (Chapter 8) in XP-A cells employing a local UV dose that saturates NER, demonstrated that XPC is stably assembled at the initial damage sites in the absence of incision.

HELIX UNWINDING

TFIIH

The later steps of GGR and TCR are conducted by a common mechanism that involves several core repair factors. After DNA damage recognition specific for each subpathway, the DNA duplex must be unwound around the lesion in order to allow access for the subsequent repair proteins. Helix unwinding is accomplished by the basal transcription factor TFIIH. TFIIH is the basal transcription initiation factor, which has an important role in RNA pol I and RNA pol II transcription, promoter escape, and is essential for NER throughout genomic DNA, whether transcriptionally active or not (Coin et al., 1999; Friedberg et al., 2006). In NER, TFIIH unwinds the duplex DNA around the lesion allowing the recruitment of the other core NER components, XPA, RPA, XPG and ERCC1-XPF (Evans et al., 1997). XPC recruits TFIIH to the damage site in GGR (Araujo et al., 2001), whereas CSB mediates the recruitment of TFIIH to TCR complexes (Fousteri et al., 2006). TFIIH is composed of 10 subunits making it the largest factor required for NER. The subunits consist of XPB/p89, XPD/p80, p62, p52, p44, p34, p8/TTDA (Giglia-Mari et al., 2004; Ranish et al., 2004; Coin et al., 2006) and a cdk-activating kinase (CAK) subcomplex; cdk7, cyclin H and MAT 1 (Roy et al., 1994). Certain mutations found in XPD patients disrupt the interaction of XPD with the p44 subunit resulting in dissociation of the CAK subcomplex from the core TFIIH complex (Coin et al., 1998; Dubaele et al., 2003). Both subcomplexes are therefore linked by XPD/p80 which promotes functional transcription (Drapkin et al., 1996; Reardon et al., 1996; Tirode et al., 1999) and DNA damage opening (Coin et al., 1998; Dubaele et al., 2003). The XPB subunit of TFIIH is an ATP-dependent helicase that mediates unwinding of promoter DNA in a 3′ -5′ orientation during transcription initiation (Douziech et al., 2000). The XPD subunit of TFIIH is a 5′ -3′ ATP dependent helicase that is required for strand separation during NER. In NER, XPD requires the ATPase activity of XPB and the helicase activity of p44 (Coin et al., 2007). TTDA/p8 is the smallest subunit of TFIIH which is shown to be essential for open helix formation (Giglia-Mari et al., 2006) and the maintenance of cellular TFIIH concentrations (Coin et al., 2006). TTDA/p8 stimulates the ATPase activity of XPB; this stimulation is probably mediated by the p52 subunit of TFIIH which is known to bind both TTDA/p8 and XPB (Coin et al., 2007). Recent data show that the C-teriminal domain of XPC is required for the recruitment of TFIIH through interactions involving the XPB and p62 subunits (Bernades de Jesus et al., 2008). Once TFIIH associates with XPC/damaged DNA it facilitates structural changes in the damage complex which liberates the

space for damage verification by XPA and the arrival of the other NER factors (Tapias et al., 2004). Recent studies demonstrate that TFIIH is also implicated in the transactivation of genes through a phosphorylation activity resident in a cdk-activating kinase (CAK) subcomplex (Ito et al., 2007).

It was thought that TFIIH remained a stable complex when switching between different cellular processes (Hoogstraten et al., 2002) yet, recent studies have shown that the composition of TFIIH changes. A dynamic dissociation/reassociation of the CAK complex with the core TFIIH was observed, which coincided with the recruitment/release of the NER factors during the NER process (Coin et al., 2008). Release of the CAK from the core TFIIH complex was catalyzed by XPA and coincided with the arrival of the other NER factors promoting incision and excision of the damaged oligonucleotide. The departure of NER factors coincided with the return of the CAK complex to the TFIIH core complex and resulted in transcription (Coin et al., 2008). The C-terminal region of XPA interacts with TFIIH (Park et al., 1995) and is required for the release of the CAK. Similarly, previous data showed that CAK negatively regulates the XPD DNA unwinding activity (Sandrock and Egly, 2001) and that the recruitment of XPA to the XPC/TFIIH pre-incision complex led to full opening of the damaged DNA (Andressoo et al., 2006). C-terminal truncated XPA, which was almost inactive in the presence of a whole TFIIH, was shown to catalyze dual incision in the presence of a TFIIH lacking CAK (Coin et al., 2008). Together, these data show that the release of the CAK complex from the core TFIIH complex is essential for the initiation of the incision/excision step of NER through stimulating TFIIH helicase/ATPase activity allowing enlargement and stabilization of the DNA open structure.

XPA

After unwinding of the DNA duplex by TFIIH, additional proteins are associated forming a complex containing fully opened DNA, called the pre-incision complex. XPA is a 36 kDa scaffold protein which has no enzymatic activity on its own but is nevertheless indispensable for DNA incision. XPA contains a zinc finger domain which is part of the minimal DNA binding domain and consists of zinc complexed to four cysteine residues; substitution of any of the four cysteines leads to severe reduction in NER activity (Miyamoto et al., 1992). Structural studies have revealed that the zinc finger binding motif is important for the protein-protein interaction with RPA (Ikegami et al., 1998). Both XPA and RPA bind to damaged DNA with some specificity (Lao et al., 2000), which is enhanced by the interaction between the two proteins (He et al., 1995; Li et al., 1995). The XPA-RPA complex was originally thought to be responsible for DNA damage recognition, yet substantial in vivo and in vitro evidence shows that recruitment of XPA to DNA lesions occurs later than TFIIH recruitment (Volker et al., 2001; Riedl et al., 2003; Coin et al., 2006) and that the recruitment of XPA is strictly XPC-dependent (Rademakers et al., 2003). XPA has been reported to have binding specificities for certain distorted DNA structures (Missura et al., 2001) which suggests that XPA may recognize certain intermediate conformations of DNA that could emerge during the action of the TFIIH helicase activity. At the same time, the recruitment and assembly of XPA and RPA into the pre-incision complex may occur (Li et al., 1998; Park et al., 1995; You et al.,

2003). As mentioned above, evidence suggests that the detachment of the CAK from the core TFIIH is catalyzed by XPA (Coin et al., 2008) which facilitates enlargement and stabilization of the DNA open structure.

RPA

RPA protein is composed of 3 subunits, 70kDa, 34kDa and 17kDa and has an important role in various processes such as replication, recombination and various DNA repair pathways (Fanning et al., 2006; Wold, 1997). RPA displays an extremely high affinity for single-stranded DNA (ssDNA) which is around 3 orders of magnitude higher than its affinity for double-stranded DNA (Kim et al., 1992). RPA is able to bind to ssDNA through 2 mechanisms; first RPA binds to a region of 8-10 nucleotides which is created by the helicase action of TFIIH, and then elongates to cover a region of around 30 nucleotides which acts as a wedge to separate the DNA strands around the lesion (Blackwell and Borowiec, 1994; de Laat et al., 1998b; Hermanson-Miller and Turchi, 2002). RPA also has an important role in stimulating endonuclease activity by facilitating the correct positioning of the endonucleases (He et al., 1995; Matsunaga et al., 1996) as well as facilitating the open complex formation. RPA interacts with a number of other core NER factors such as XPA (as mentioned above), the endonucleases XPG, ERCC1-XPF and is required for dual incision (Coverley et al., 1992). RPA associates with the undamaged DNA strand and is indispensable for open complex formation. *In vitro* experiments revealed that RPA initially binds 8-10 nucleotides at the 5′ side of the lesion after which RPA stretches along the DNA in the 3′ direction (de Laat et al., 1998a; Kolpashchikov et al., 2001) allowing correct positioning of XPG and ERCC1-XPF. With the 3′-oriented side of RPA facing a duplex ssDNA junction, RPA interacts with and stimulates ERCC1-XPF, whereas the 5′-oriented side of RPA at a DNA junction allows stable binding of XPG (de Laat et al., 1998c).

It was originally thought the XPA and RPA co-exist as a complex in the cell nucleus. However, evidence from GFP-tagged XPA diffusion experiments showed no interaction between XPA and RPA (Rademakers et al., 2003). Furthermore, the binding of RPA to UV lesions was shown to be independent of XPA, suggesting that RPA-XPA interactions only occur on damaged DNA (Rademakers et al., 2003). Recent ChiP results however suggest that XPA and RPA may interact transiently since we found co-precipitation of RPA and XPA in cells containing no DNA damage (Chapter 8).

RPA also has an important function in the latter stages of NER such as DNA repair synthesis and ligation (Shivji et al., 1995). In vitro experiments examining the assembly and disassembly of NER proteins on immobilized DNA damaged templates reveal that RPA remains bound after dual incision, and initiates the assembly of DNA synthesis factors such as PCNA (Riedl et al., 2003). In vivo results support this notion, since the disassembly of RPA after UV irradiation was inhibited in the presence of DNA synthesis inhibitors (Chapter 9). Together, these results support a role for RPA in both pre- and post-incision events in which it may remain localized to ssDNA after excision of the damaged nucleotide and remain bound until DNA synthesis and ligation are completed (Chapter 8). The multi-functional role of RPA in NER is discussed in detail in Chapter 8.

DUAL INCISION

XPG

After the assembly of the pre-incision complex, single-strand breaks are introduced to the damaged DNA strand by XPG and ERCC1-XPF. XPG is a 133kDa structure-specific endonuclease of the FEN-1 family which is indispensable for NER (Friedberg et al., 2006). During NER, XPG functions as a junction-specific endonuclease that specifically incises the damaged single-stranded DNA strand 2-8 nucleotides from the 3′ side of the lesion undergoing repair (O'Donovan et al., 1994a; O'Donovan et al., 1994b). Additionally, XPG is required structurally for formation of the fully opened DNA conformation, since the 5′ incision carried out by ERCC1-XPF endonuclease is dependent on the physical presence of XPG at the damage site (Wakasugi et al., 1997; Constantinou et al., 1999). The XPG endonuclease has been reported to bind to the NER complex in XPA-deficient cells yet was not capable of incision in the absence of XPA (Evans et al., 1997). In contrast, both XPA and XPG are incorporated into the NER complex in XPF-deficient cells (Volker et al., 2001). In this situation, Evans et al (1997) reported that XPG was capable of making the 3′ incision yet other authors have reported that the presence of XPF is required for XPG incision activity (Tapias et al., 2004). Studies using XPA deficient cells show that the DNA binding domain is needed for the action of XPA as a processivity factor and for its ability to enable XPF and XPG to incise damaged nucleosomal DNA (Bartels and Lambert, 2007).

Truncation mutations in the *XPG* gene give rise to individuals with the combined clinical features of XP and Cockayne syndrome (CS), a disorder associated with developmental and neurological abnormalities (Nouspikel et al., 1997). Furthermore, while NER is not essential for the viability of an organism, complete loss of XPG function is incompatible with life in knockout mice (Shiomi et al., 2004) suggesting that XPG is required for additional functions necessary for normal development such as transcription. Recent studies found that certain XPG mutations present in severe XPG and XPG/CS patients were found to disturb the interaction of both CAK and XPD with the core TFIIH complex (Ito et al., 2007). TFIIH-XPG interaction therefore functions to maintain the architecture of TFIIH which also highlights an important role for XPG in transcription. These findings suggest that XPG may be recruited to damaged sites as a pre-assembled complex with TFIIH. However, in vivo (Volker et al., 2001) and in vitro (Riedl et al., 2003) studies propose a sequential assembly model. Moreover, XPG association was shown to be TFIIH dependent (Zotter et al., 2006), suggesting that TFIIH recruits XPG to the NER complex. In addition, live cell imaging studies show that XPG and TFIIH are 2 distinct complexes and only interact on damaged DNA (Hoogstraten et al., 2002; Zotter et al., 2006). Despite these findings, transient interactions between the 2 proteins which would stabilize TFIIH cannot be ruled out.

XPG also contains a PCNA-binding domain which could function to attract PCNA to the repair complex linking the incision and the repair resynthesis steps of NER (Gary et al., 1997). Studies demonstrate that the simultaneous presence of XPG and RPA at the damage site are required for transition between the incision and DNA synthesis steps of NER (Mocquet et al., 2008). Furthermore, recent data found that partial DNA synthesis

was detectable *in vitro* in the presence of catalytically inactive XPG, thereby demonstrating that the incision 5′ to the lesion by ERCC1-XPF is both necessary and sufficient for the initiation of repair synthesis, whereas the 3′ incision by XPG is needed for the completion, but not the initiation of repair synthesis (Staresincic et al., 2009). See section 'DNA repair resynthesis and ligation' for more details regarding the role of XPG in the late stages of NER.

ERCC1-XPF

ERCC1 (33 kDa) and XPF (103 kDa) form a heterodimer which cleaves DNA at the 5′ boundary of the open complex between single-stranded and double-stranded DNA (Matsunaga et al., 1995). ERCC1 is also known to interact with XPA (Bessho et al., 1997; Li et al., 1995). The interaction between ERCC1 and XPA seems to be crucial for its recruitment to the damage site (Li et al., 1994; Park and Sancar, 1994; Saijo et al., 1996). ERCC1-XPF makes an incision in the DNA 15-24 nucleotides away from the 5′ side of the lesion (de Laat et al., 1998c; Matsunaga et al., 1995; Sijbers et al., 1996). Although, XPF contains the nuclease domain which is able to cleave the DNA the ERCC1 subunit is required for nuclease activity (Enzlin and Scharer, 2002). The nuclease domain in ERCC1 is unable to incise DNA (Gaillard and Wood, 2001). The physical presence but not the catalytic activity of XPG is required for ERCC1-XPF incision (Mu et al., 1996; Wakasugi et al., 1997; Constantinou et al., 1999; Tapias et al., 2004; Staresincic et al., 2009).

In theory, both endonucleases are capable of incising both DNA strands of an open complex substrate which highlights the importance of verification of the damaged strand in order to avoid cleavage errors. Damage verification is carried out by TFIIH together with XPA-RPA which is also likely to assemble the pre-incision complex in the correct orientation. As already mentioned RPA binds to the undamaged strand and may guide the endonucleases to their correct positions (de Laat et al., 1998a; Matsunaga et al., 1996). Our studies on the stability of the NER complex suggest that dual incision is the key determinant for the release of the pre-incision proteins (Chapter 8). Similarly, ERCC1-XPF is both necessary and sufficient for the initiation of repair synthesis (Staresincic et al., 2009).

DNA Repair Resynthesis and Ligation

After excision of the damage-containing oligonucleotide, the resulting single-strand gap is filled by DNA synthesis and ligation. In vitro (Shivji et al., 1995) and in vivo (Chapter 9) results show that RPA is involved not only in dual incision but also in subsequent repair synthesis. RPA is likely to bind to the undamaged strand and, upon dual incision, may help recruit PCNA and RFC (Gomes and Burgers, 2001; Yuzhakov et al., 1999). In fact, recent studies demonstrate that the simultaneous presence of XPG and RPA at the damage site are required for transition between the incision and DNA synthesis steps of NER (Mocquet et al., 2008). In vitro experiments revealed that DNA resynthesis is dependent on PCNA (Nichols and Sancar, 1992; Shivji et al., 1995). PCNA forms a homotrimeric clamp (Krishna et al., 1994) which is loaded onto the 3′ end of primers on template strands and supports chain elongation by interacting with replicative DNA polymerases (reviewed in

Maga and Hubscher, 2003). In vitro experiments also show that PCNA loading requires a heteropentameric, DNA-dependent ATPase complex called RFC (Ellison and Stillman, 1998; Tsurimoto and Stillman, 1989). The loading of PCNA by RFC is ATP dependent and occurs at ds- to ssDNA transitions with a 3'-OH end, a structure characteristically formed after 5' incision by ERCC1-XPF (Balajee et al., 1998; Essers et al., 2005). Both endogenous and GFP-tagged RFC and PCNA accumulate at local damage sites after UV irradiation in living cells confirming in vitro observations (R. Overmeer and A. Gourdin, unpublished results; Essers et al., 2005). XPG also contains a PCNA-binding domain which could function to attract PCNA to the repair complex linking the incision and the repair resynthesis steps of NER (Gary et al., 1997). Using XP-A and XP-F cells with mutations that completely abolish UDS but still allow recruitment of XPG (Volker et al, 2001), we found weak accumulation of PCNA (Chapter 9). These results are consistent with a possible assisting role for XPG in the recruitment of PCNA, however it must be noted that XP-G cells (lacking functional XPG) showed similar weak accumulation of PCNA suggesting that XPG is not the only factor involved in recruitment of PCNA prior to incision. Previous work suggests that XPG is recruited together with RFC to the vicinity of the gap (Gary et al., 1999; Miura and Sasaki, 1999). RPA is thought to attract RFC at the same time as XPG recruitment of PCNA (Yuzhakov et al., 1999). Together, loading of RFC and PCNA is associated with the release of the ERCC1-XPF endonuclease (Mocquet et al., 2008). As mentioned above, the incision 5' to the lesion by ERCC1-XPF is both necessary and sufficient for the initiation of repair synthesis, whereas the 3' incision by XPG is needed for the completion, but not the initiation of repair synthesis (Staresincic et al., 2009). Our recent findings suggest that RPA is the only NER protein to remain stably associated at the site of repair following dual incision and remains associated until ligation is complete preventing further strand breaks from accumulating throughout the genome (Chapter 8).

In the past, only in vitro experiments have provided data on the late stages of NER which suggested that polymerase δ and ε are both involved in DNA resynthesis. Similarly, only DNA ligase I was thought to ligate the newly synthesized strand (Aboussekhra et al., 1995; Araujo et al., 2000; Shivji et al., 1995). Our recent in vivo studies show that pol δ is recruited to repair patches in both quiescent and proliferating cells where as pol ε is only recruited to repair patches in proliferating cells after UV irradiation (Chapter 7). We found no accumulation of pol β after UV irradiation which suggests that it plays no or a minor role in NER (Chapter 7). In addition, DNA polymerase κ has been implicated in the repair synthesis step (Ogi and Lehmann, 2006). After resynthesis, the 5' end of the newly synthesized DNA has to be ligated to the original sequence. We found contrary to *in vitro* data that Ligase I alone was not sufficient to rejoin the in repair nicks in chromosomal DNA and only played a role in proliferating cells (Chapter 7). FEN-1 has also been implicated in the ligation process and is thought to function together with Lig1 (Shivji et al., 1995; Araujo et al., 2000; Mocquet., 2008). However, we were unable to identify a role for FEN-1 in our in vivo experiments (J. Moser, unpublished results). We identified the XRCC1-Ligase3 complex as the principle DNA ligase in NER since accumulation of this complex was visualized in both quiescent and proliferating cells (Moser et al., 2007). Repair experiments confirmed the role of XRCC1-Lig3 in NER by demonstrating its requirement for the efficient

removal of lesions and rejoining of nicks in chromosomal DNA (Chapter 7). Together these results show that pol δ and XRCC1-Lig3 are the dominant DNA polymerase/ligase in NER-mediated gap-filling in both quiescent and proliferating cells whereas Pol ε and Lig1 have a role in proliferating cells (Chapter 7). See Chapters 7 and 8 for more details regarding the role of the polymerases and ligases in the late stages of NER.

A MODEL OF GG-NER

Probable sequence of events in mammalian GGR

We propose the following model for global genome repair based on our knowledge of the core NER factors. (See Figure 5)

1. DNA damage recognition

A crucial step in DNA repair is the initial recognition of damaged sites. DNA damage recognition is carried out by UV-DBB and XPC-hHR23B complexes. UV-DDB arrives at the damage site which induces a kink in the DNA allowing recruitment of the XPC-hHR23B complex (Wakasugi et al., 2002; Fitch et al., 2003a). The binding of XPC results in slight opening of the DNA surrounding the lesion (Tapias et al., 2004). DDB2 likely creates a local chromatin environment around lesions that facilitates the assembly of repair complexes (Chapter 5 and 6). UV-DDB physically interacts with XPC and the associated E3 ligase containing cullin 4A and Roc1 which ubiquitylates XPC after UV damage, (Sugasawa et al., 2005) a process that does not cause XPC degradation, but rather enhances its affinity for DNA. DDB2 is also ubiquitylated after UV which leads to rapid degradation after DNA damage (El-Mahdy et al., 2006; Sugasawa et al., 2005). Therefore both ubiquitylated DDB2 and XPC may be important for the efficient transfer of lesions between the two damage recognition factors, switching from tightly-bound UV-DDB, to a lower-affinity XPC-hHR23B complex (Sugasawa et al., 2005).

2. Helix unwinding and damage verification

After DNA damage recognition the DNA duplex is unwound around the lesion, a process that is accomplished by the basal transcription factor TFIIH. The XPC-hHR23B complex recruits TFIIH to the damage site in GGR (Araujo et al., 2001; Yokoi et al., 2000), which allows unwinding of the duplex DNA around the lesion, and promotes the subsequent incision and excision by facilitating the recruitment of the other core NER components, XPA, RPA, XPG and ERCC1-XPF (Evans et al., 1997; Sijbers et al., 1996; O'Donovan et al., 1994b). The detachment of the CAK from the core TFIIH is catalyzed by XPA, in the presence of ATP and is essential for the initiation of the incision/excision step of NER through stimulating TFIIH helicase/ATPase activity allowing enlargement and stabilization of the DNA open structure (Coin et al., 2008). XPA may recognize certain intermediate conformations of DNA that could emerge during the action of the TFIIH helicases. At the same time, the recruitment and assembly of XPA and RPA into the pre-incision complex may occur (Li et al., 1998; Park et al., 1995; You et al., 2003). XPA and TTDA stimulate the

ATPase activity of TFIIH in the presence of lesions, which is required for full opening around the lesion (Coin et al., 2006; Giglia-Mari et al., 2006). XPA also acts as a processivity factor which enables XPF and XPG to incise damaged nucleosomal DNA (Bartels and Lambert, 2007). RPA binds to a region of 8-10 nucleotides which is created by the helicase action of TFIIH, and then elongates to cover a region of around 30 nucleotides which acts as a wedge to separate the DNA strands around the lesion (Blackwell and Borwiec, 1994; Kim et al., 1992; de Laat et al., 1998; Hermanson-Miller and Turichi, 2002). RPA also has an important role in stimulating endonuclease activity by facilitating the correct positioning of the endonucleases (de Laat et al., 1998b; He et al., 1995; Matsunaga et al., 1996; Chapter 8). XPC therefore likely remains associated to the damage site until the complete pre-incision complex is formed or until dual incision is performed.

3. Dual Incision

After the assembly of the pre-incision complex, single-strand breaks are introduced to the damaged DNA strand by endonucleases XPG and ERCC1-XPF complex. The simultaneous presence of XPG and RPA at the damage site is thought to be required for the transition between the incision and DNA synthesis steps of NER (Mocquet et al., 2008). First the 5' cleavage by ERCC1-XPF takes place which is dependent on the physical presence of XPG at the damage site (Wakasugi et al., 1997; Constantinou et al., 1999; Tapias et al., 2004; Staresincic et al., 2009). Recruitment of the repair synthesis factors such as polymerase, clamp loader RFC and PCNA are loaded to the damage site, and repair sythesis is initiated (Staresincic et al., 2009). RPA plays an important role in the transition between pre-incision and post-incision events (Riedl et al., 2003; Chapter 8), by recruiting recruit PCNA and RFC (Riedl et al., 2003; Gomes and Burgers, 2001; Yuzhakov et al., 1999). XPG is likely to recruit PCNA alone or together with RFC to the vicinity of the gap (Gary et al., 1999; Miura, 1999; Chapter 8). RPA is thought to attract RFC at the same time as XPG recruitment of PCNA (Yuzhakov et al., 1999). After incision, all of the pre-incision proteins are free to associate with other sites of damage with the exception of RPA. RPA does not dissociate after incision but likely remains bound to the undamaged strand until ligation is complete (Chapter 8). DNA synthesis is thought to proceed about half way through the repair patch. The stalling of the polymerase at this point might trigger the XPG endonuclease activity, allowing the repair synthesis to be completed (Staresincic et al., 2009). These results suggest that the 5'incision by ERCC1-XPF is both necessary and sufficient for the initiation of repair synthesis, whereas the 3' incision by XPG is needed for the completion of repair synthesis. Late NER factors including PCNA, DNA pol δ and CAF-1 have been found to localise to UV damage sites in cells expressing catalytically inactive XPG, but not in cells expressing catalytically inactive XPF (Staresincic et al., 2009). Therefore, after 5'incision the repair synthesis machinery consisting minimally of polymerase δ, RFC and PCNA and repair synthesis is initiated. XPG specifically incises the damaged single-stranded DNA strand 2-8 nucleotides from the 3' side of the lesion undergoing repair (O'Donovan et al., 1994a; O'Donovan et al., 1994b).

4. DNA repair synthesis and Ligation

With regard to DNA synthesis, we find that DNA pol δ is recruited to repair patches in both quiescent and proliferating cells where as pol ε was only found to be recruited to repair patches in proliferating cells after UV irradiation (Chapter 7). In addition, DNA polymerase κ may also have a role in the repair synthesis step (Ogi and Lehmann, 2006). It is possible that two polymerases, pol δ and pol κ, act at different steps of repair synthesis however further studies are required to elucidate exactly how the various phases in NER are regulated, also with regard to cell cycle status. For years Ligase I was thought to be the ligase responsible for the sealing of NER-mediated gaps. We were the first to provide direct evidence that the XRCC1-Ligase3 complex is the principle DNA ligase in NER. XRCC1-Ligase 3 was found to be functioning in both quiescent and proliferating

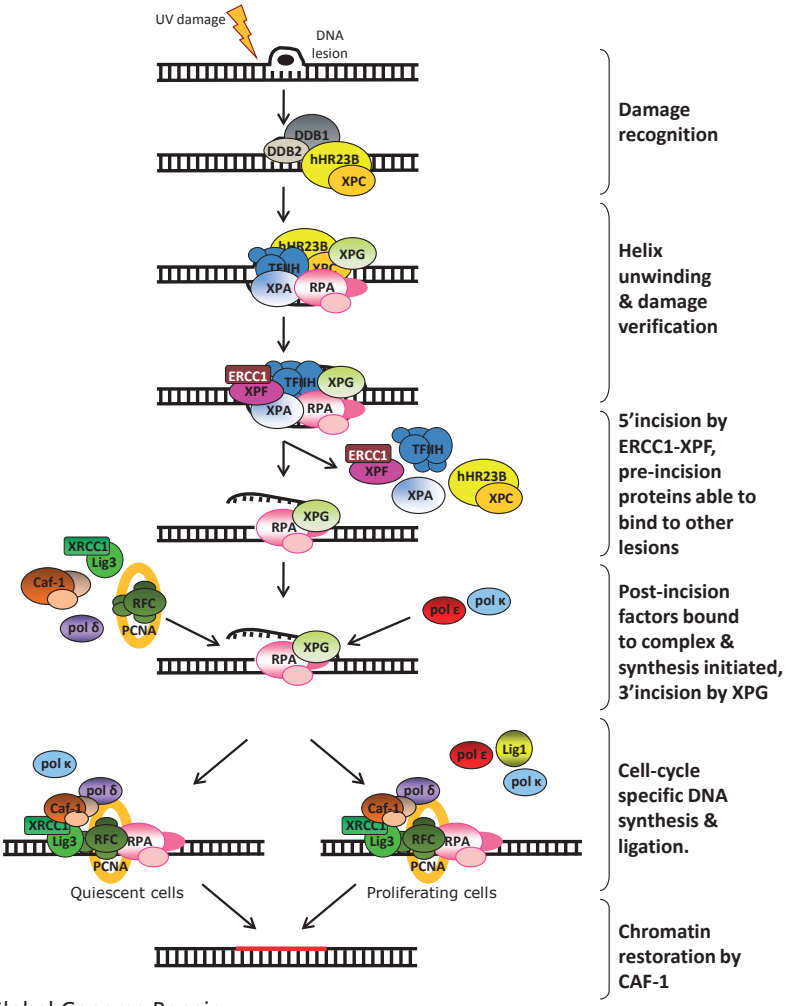

Fig 5. Global Genome Repair

cells and is essential for the efficient removal of lesions and rejoining of chromosomal DNA nicks (Chapter 7). Ligase I was only found to have a role in proliferating cells and partly compensates for a Lig3 deficiency in highly proliferating cells, further supporting a cell-cycle-dependent involvement of Lig1 in NER (Chapter 7). Together these results suggest that pol δ and XRCC1-Lig3 are the dominant DNA polymerase/ligase in NER-mediated gap-filling in both quiescent and proliferating cells whereas Pol ε and Lig1 may only have a role in proliferating cells (Chapter 7).

5. Chromatin restoration

Following successful completion of NER the repaired region needs to be to restored to the pre-existing chromatin structure, and checkpoint signaling switched off, as described in the Access-Repair-Restore model (Smerdon, 1991). Our own in vivo studies as well as other show that CAF-1 is recruited to damage sites within 30 min after UV (Chapter 8; Green and Almouzni., 2003; Staresincic et al., 2009) and also in the presence of DNA synthesis inhibitors HU and AraC (Chapter 8). Similarly, CAF-1 has also recently been found to localise to UV damage sites in cells expressing catalytically inactive XPG, but not in cells expressing catalytically inactive XPF (Staresincic et al., 2009) suggesting that CAF-1 accumulates at UV-induced lesions before the 3'incision by XPG. Together, these results suggest that CAF-1 is recruited to UV damage, presumably via PCNA, prior to completion of DNA synthesis and ligation and therefore cannot carry out nucleosome reassembly when it is first bound (Chapter 8).

4

DNA REPAIR AND CHROMATIN

DNA REPAIR IN CHROMATIN

Genetic information is packaged with histones and other accessory proteins into chromatin. In order to overcome the chromatin barrier, the transcriptional machinery is assisted by factors called chromatin remodeling complexes that increase the plasticity of nucleosomal DNA (Huertas et al., 2009). Chromatin is organized as a string of repeating nucleosome core particles which consists of 164 DNA base pairs organized around an octomer consisting of two copies of each highly conserved histone protein – H2A, H2B, H3 and H4 (Luger et al., 1997). Chromatin remodeling is essential for key cellular processes such as the regulation of gene expression, apoptosis, DNA replication and DNA repair processes (Downs et al., 2007).

Since DNA repair occurs in a chromatin context, repair factors must overcome the restricted access to DNA in chromatin, particularly in nucleosome cores. As already mention, the first step in DNA repair is lesion recognition, and although proteins that recognize specific lesions in naked DNA have been identified for each of the repair pathways, evidence suggests that changes in chromatin may also play a role in lesion recognition (Huertas et al., 2009; Nag and Smerdon, 2009). Structural changes in the chromatin due to lesions may initiate damage recognition. Alternatively, enzymatic modification and repositioning of histones and nucleosomes may occur to facilitate damage recognition. Chromatin remodeling in NER has been examined using in vitro models such as reconstituted nucleosomes with short DNA fragments containing lesions (Wang et al., 1991; Sugasawa et al., 1993).

Several studies have demonstrated that NER is less efficient in chromatin than in naked DNA, suggesting that DNA repair proteins have limited access to DNA damage buried in nucleosome core regions (Evans and Linn, 1984; Wang et al., 1991). In line with these observations, NER was shown to be more rapid in linker DNA between nucleosomes than in nucleosome cores (Gong et al., 2005). Although UV lesions induce extensive bending of duplex DNA, in vitro studies show that it does not alter the way that DNA wraps around nucleosomes, and for lesions in the nucleosome core, there was no repair specificity for lesions facing either inward or outward from the nucleosome surface (Liu and Smerdon, 2000; Liu et al., 2005; Svedruzic et al., 2005). The rate of repair is therefore dependent on the packaging of DNA in nucleosomes since they restrict the access of repair proteins to DNA strands as well as the lesion itself. It is known that access to bulky lesions such as UV-induced lesions in the chromatin is increased when chromatin is relaxed by histone acetyltransferases, or when nucleosomes are altered or moved by ATP-dependent processes (Peterson and Cote, 2004; Thoma, 2005).

Histone modifications and NER

Multiple modifications of core histones have been identified which are linked to gene activity and repair. These modifications include acetylation, methylation, phosphorylation and ubiquitylation (Jenuwein and Allis, 2001). Acetylation and methylation are important chemical signatures that can alter chromatin activity by recruiting proteins that specifically act on chromatin or on DNA.

Acetylation

Inhibition of histone deactylases has been shown to increase the level of actylation as well as the repair in hyperactylated nucleosomes (Ramanathan and Smerdon, 1989). Histones H3 and H4 were also shown to be acetylated after UV (Brand et al., 2001), further suggesting that UV-induced acetylation of histones is a cellular response which may be required to facilitate repair. Histone acetyltransferases (HATs) such as p300/CBP and GCN5 mediate the acetylation of N-terminal histone tails. The primary modifications associated with histone acetylation may stabilize higher order structures by creating a more open and active chromatin structure (Wolffe and Hansen, 2001). HAT p300/CBP is known to interact with both DDB1, DDB2 (Rapic-Otrin et al., 2002; Datta et al., 2001), CSB (Fousteri et al., 2006), PCNA (Hasan et al., 2001) and p53 (Rubbi and Milner, 2003) which may target p300 to UV damage. However, to date, no direct evidence has shown that p300 is required for efficient NER. TFTC and STAGA are HAT-containing complexes which display an affinity for UV-damaged DNA (Martinez et al., 2001). Both the TFTC and STAGA complexes contain SAP130, a protein which shares high homology to DDB1. SAP130 is also known to interact with DDB1 whereas STAGA has been shown to interact with DDB2 (Brand et al., 2001; Martinez et al., 2001).

Methylation

Histone methylation modifications are carried out by histone methyltransferases which covalently modify the lysine and arginine residues of histones. A combination of histone methylation and acetylation modifications has been shown to influence transcription (Kouzarides, 2007), yet not much is known about their role in DNA repair. However, histone methylation levels have been shown affect cell cycle checkpoints through interactions with checkpoint components such as 53BP1 (Huyen et al., 2004).

Phosphorylation

Phosphoylation of serine residue 139 of H2AX by ATM is known to be a marker for DSBs. Phosphorylated H2AX (γ-H2AX) generates a chromosomal microenvironment that promotes recruitment of repair proteins (Fernandez-Capetillo O et al., 2004) and facilitates DNA repair to reduce the risk of mutations (Bartek & Lukas, 2007). Similarly, H2AX has also been shown to be phosphorylated after UV irradiation (Hanasoge and Ljungman, 2007). ATR kinase was shown to mediate the phosphorylation of H2AX in response to UV damage (Mari et al., 2006). Recently, Xiao et al (2009) and Cook et al (2009) discovered that tyrosine residue 142 (Y142) of H2AX is also phosphorylated but in unstressed cells. Y142 became dephosphorylated after X-ray DNA damage which appears to be a prerequisite for γ-H2AX phosphorylation and the recruitment of MDC1 and the other repair factors (Cook et al., 2009). Nevertheless, if Y142-phosphorylated H2AX persists, protein kinase JNK1 was found to bind, triggering apoptosis (Cook et al., 2009). These results suggest that Y142 phosphorylation may determine whether a cell can be repaired, initiating DNA repair processes or whether the DNA damage is too severe and activating apoptosis in order to get rid of damaged genomes. (Reviewed in Stucki et al., 2009)

Ubiquitination (Ubiquitylation)

The covalent attachment of one or more ubiquitin monomers is called ubiquitination. Ubiquitin is a small 8.5kDa peptide that can label proteins for protosomal degradation. Alternatively, ubiquitination can also alter the function, stability and intracellular localization of a wide range of proteins (Herrmann et al., 2007). Modification of proteins by ubiquitination consists of a series of steps which involves the E1 activating enzyme, E2 conjugating enzyme and the specificity-conferring E3 ubiquitin ligase. E3 ubiquitin ligases are known to work together with several E2 conjugating enzymes which catalyze ubiquitin conjugation via different lysine residues. H2A, H3 and H4 are known to be ubiquitinated in response to UV (Wang et al., 2005; Houtsmuller et al., 2006). Mono-ubiquitination of H2A is mediated by Ring2 ubiquitin ligase which was shown to be dependent on NER and ATR but not ATM (Bergink et al., 2006). UV-DDB is part of an E3 ubiquitin ligase that ubiquinates histones H3 and H4 following UV irradiation. Consequently, the interaction between histones and DNA is relaxed which facilitates the assembly of NER proteins (Wang et al., 2006; Chapter 6). Both XPC and DDB2 are ubiquitinated (Nag et al., 2001; Chen et al., 2001; Matsuda et al., 2005; Sugasawa et al., 2005). Yet, whereas the polyubiquitination of DDB2 triggers its destruction by the 26S proteasome (Fitch et al., 2003a; Rapic-Otrin et al., 2002), polyubiquitinated XPC is not degraded and was even shown to have a higher affinity for both damaged and undamaged DNA *in vitro* (Sugasawa et al., 2005).

ATP-dependent chromatin remodeling and NER

Chromatin remodeling complexes which utilize ATP to mobilize nucleosomes along DNA, evict histones off DNA, or promote the exchange of histone varients. These alterations modulate DNA accessibility and alter nucleosomal structures (Gangaraju and Bartholomew, 2007). The ATP-dependent chromatin remodeling enzymes can be divided into four groups based on distinct domain structures: The SWI/SNF (switching defective/sucrose fermenting) family, the ISWI (imitation SWI) family, the NuRD (nucleosome remodeling and deacetylation)/Mi-2/CHD (chromodomain, helicase DNA binding) family and the INO80 (inositol requiring 80) family. ACF (ATP-utilizing chromatin assembly and remodeling factor) is part of the ISWI family of chromatin-remodeling complexes. ISWI-family complexes are generally involved in regulation of transcription, often in transcriptional repression. Human ACF contains the ATPase subunit SNF2h and a noncatalytic subunit, ACF1 (Ito et al., 1999) and has been shown to facilitate the excision of 6-4PP lesions by the NER core factors, in particular those situated in linker DNA (Ura et al., 2001). In addition to ACF1, SWI/SNF was shown to modulate the accessibility of UV-damaged mono-nucleosomes in vitro (Gaillard et al., 2003; Hara and Sancar, 2002; Hara and Sancar, 2003). Further evidence providing a functional link between chromatin remodeling and repair is demonstrated by the identification of CSB as a DNA-dependent ATPase (Citterio et al., 2000). CSB may therefore have a direct role coupling transcription to repair amoung others via remodeling chromatin at damage sites. It is currently unknown whether CSB directly remodels chromatin *in vivo*. However, CSB has been shown to recruit both p300/CBP and HMGN1 mediating chromatin remodeling in TCR (Fousteri et al., 2006). The ING

(inhibitor of growth) tumor suppressor proteins are known to regulate cellular responses to UV, including cell proliferation, p53-dependent apoptosis and DNA repair. Recently, ING2 was shown to cooperate with p53 to induce histone H4 acetylation, chromatin relaxation, and recruitment of the indispensable damage-recognition XPA protein to photolesion site (Wang et al., 2006). ING1b was similarly shown to promote repair in vivo (Cheung, Jr. et al., 2001) by histone H4 acetylation and chromatin relaxation (Kuo et al., 2004).

Non-chromatin remodelers and NER

Several non-chromosomal proteins have the ability to modify chromatin or to bind to specific histone modifications. For example, GADD45 has a specific affinity for UV-damaged and hyperacetylated DNA and is able to modulate the accessibility of DNA-nucleosome complexes (Carrier et al., 1999). Several studies have shown that GADD45 is required for efficient repair of UV-induced lesions (Smith et al., 1996; Smith et al., 2000; Maeda et al., 2002) suggesting a role of GADD45 in facilitating NER in chromatin. HMGN (high mobility group N) proteins are able to alter higher order chromatin structures by targeting two main elements known to compact chromatin, histone H1 and H3 (Ju et al., 2006; Brown et al., 2006; Catez et al., 2004). Cells deficient in HMGN1 showed diminished repair of CPDs in actively transcribed genes (Birger et al., 2003). In line with these observations, HMGN1 was shown to be recruited to TCR complexes through an interaction with CSB (Fousteri et al., 2006).

Chromatin restoration after NER

As mentioned above, packaging of DNA into chromatin presents a challenge for DNA-repair processes since lesions first have to be made accessible to the repair machinery. Following successful completion of DNA repair the repaired region needs to gain its pre-existing chromatin structure, and checkpoint signaling to switch off the DDR, as described in the Access-Repair-Restore model (Smerdon 1991; Green and Almouzni, 2003). Restoration of the original epigenetic state is essential to maintaining a functional genome and histone chaperones are likely to be involved such as chromatin assembly factor 1 (CAF-1). CAF-1, a complex consisting of p150, p60 and p48 subunits is highly conserved from yeast to human and is known to deposit nucleosomes on newly-synthesized DNA during replication, as well as DNA that has been repaired by NER (Mello and Almouzni, 2001; Gaillard et al., 1996). CAF-1 is recruited to UV-damaged DNA both in vitro and in vivo (Moggs et al., 2000) but dependent on functional NER and possibly by binding via PCNA (Green and Almouzni, 2003). In line with these observations, it is now clear that histones H3 and H4 are incorporated during the repair of UV lesions. Stable de novo incorporation of Histone H3.1 occurs at UV repair sites, dependent on functional NER and CAF-1, providing further evidence for a role of CAF-1 in the chromatin restoration step following repair (Polo et al., 2006). In vitro observations suggest that CAF-1 arrives later than the other post-incision factors and only after ligation is complete (Mocquet et al., 2008). We also observed accumulation of CAF-1 after local UV in wild-type cells as previously described (Chapter 9; Green and Almouzni, 2003). However, CAF-1 accumulation was also found 30 minutes after irradiation in HU and AraC treated cells where DNA synthesis and ligation

is almost completely inhibited (Chapter 8). We therefore conclude that in contrast to in vitro observations by Mocquet et al (2008), CAF-1 is recruited to UV damage prior to completion of DNA synthesis and ligation and therefore cannot carry out nucleosome reassembly when it is first bound (Chapter 8). In line with out observation, Staresincic et al., (2009) found accumulation of late NER factors such as PCNA, DNA pol δ and CAF-1 in cells expressing catalytically inactive XPG, but not in cells expressing catalytically inactive XPF. Hence, 5' incision initiates DNA repair synthesis as well as attracting CAF-1. However, the regulation of CAF-1 seems more complicated, as persistent CAF-1 accumulation was no longer visible 20 hours after irradiation in the presence of DNA synthesis inhibitors, despite the rest of the repair factors still being present (Chapter 8). Asf1, a highly conserved histone H3-H4 chaperone can synergize with CAF-1 in repair and replication-coupled nucleosome assembly *in vitro* (Mello et al., 2002; Tyler et al., 1999), although it is unable to promote DNA-synthesis-dependent histone deposition on its own. Exactly how Asf1 can contribute to DNA-damage responses or if it functions in access, restoration or both processes is currently unknown. Future DNA repair studies in histone structural or modification mutants may improve our understanding of how the histone components and their post-translational modifications influence essential cellular functions such as NER.

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5

THE UV-DAMAGED DNA BINDING PROTEIN MEDIATES EFFICIENT TARGETING OF THE NUCLEOTIDE EXCISION REPAIR COMPLEX TO UV-INDUCED PHOTOLESIONS

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SUMMARY

Previous studies point to the XPC-hHR23B complex as the principal initiator of global genome nucleotide excision repair (NER) pathway, responsible for the repair of UV-induced cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) in human cells. However, the UV-damaged DNA binding protein (UV-DDB) has also been proposed as a damage recognition factor involved in repair of UV-photoproducts, especially CPD. Here, we show in human XP-E cells (UV-DDB deficient) that the incision complex formation at UV-induced lesions was severely diminished in locally damaged nuclear spots. Repair kinetics of CPD and 6-4PP in locally and globally UV-irradiated normal human and XP-E cells demonstrate that UV-DDB can mediate efficient targeting of XPC-hHR23B and other NER factors to 6-4PP. The data is consistent with a mechanism in which UV-DDB forms a stable complex when bound to a 6-4PP, allowing subsequent repair proteins – starting with XPC-hHR23B – to accumulate, and verify the lesion, resulting in efficient 6-4PP repair. These findings suggest that (i) UV-DDB accelerates repair of 6-4PP, and at later time points also CPD, (ii) the fraction of 6-4PP that can be bound by UV-DDB is limited due to its low cellular quantity and fast UV dependent degradation, and (iii) in the absence of UV-DDB a slow XPC-hHR23B dependent pathway is capable to repair 6-4PP, and to some extent also CPD.

INTRODUCTION

The nucleotide excision repair (NER) system is a highly versatile repair pathway capable of removing a wide variety of helix-distorting lesions from genomic DNA. Cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP), the main products of short-wave ultraviolet (UV) radiation in DNA, are among the lesions processed by NER, and human cells depend solely on NER to repair these UV-photolesions. Defects in genes encoding NER proteins give rise to UV-sensitive disorders, most notably xeroderma pigmentosum (XP), which comprises seven complementation groups (XP-A–XP-G) (Bootsma et al., 1998). XP patients are photosensitive and display a strongly elevated risk of developing skin cancers in sunlight-exposed parts of the body. Biochemically, all XP patients (except XP variants) are found to be defective in global genome repair (GGR), the subpathway of NER capable of removing lesions from the entire genome. In GGR, a total of 30 proteins work together to consecutively execute the basic steps that comprise NER: recognition of the DNA damage, unwinding the DNA around the lesion, excision of the oligonucleotide containing the damage and finally resynthesis of the strand using the undamaged template and ligation of the nick (de Laat et al., 1999). Several specialized proteins have been proposed to act in the initial step of damage recognition, including the UV-damaged DNA binding protein (UV-DDB) (Chu and Chang, 1988) and (Tang et al., 2000), the complex of XPA and replication protein A (RPA) (Asahina et al., 1994; Li et al., 1995) and the XPC-hHR23B heterodimer (Reardon et al., 1996). Most recent studies point to the XPC-hHR23B complex as the principle initiator of NER (Sugasawa et al., 1998; Volker et al., 2001). However, those studies did not exclude the possibility that in processing certain lesions, e.g. UV-induced photoproducts, the action of XPC-hHR23B in the formation of the incision complex can be preceded by other proteins such as UV-DDB.

UV-DDB is a heterodimer of the p48 and p127 proteins (Keeney et al., 1993), products of the *DDB*2 and *DDB*1 genes, respectively. Microinjection of purified UV-DDB was originally found to restore the repair defect of XP-E cells as measured by unscheduled DNA synthesis (Keeney et al., 1994). Recently, evidence has accumulated implying that the XP group E phenotype is caused exclusively by mutations in the *DDB*2 gene, implicating that the *DDB*2 gene corresponds to the *XPE* gene, and p48 to the XPE protein (Itoh et al., 2000; Itoh et al., 2001; Rapic-Otrin et al., 2003). In vitro binding studies have revealed that the UV-DDB protein complex exhibits a high affinity for UV-induced DNA lesions and a moderate affinity for several other types of DNA lesions (Chu and Chang, 1988; Payne and Chu, 1994; Batty et al., 2000). Moreover, UV-DDB has an equally high specificity for 6-4PP when compared to the XPC protein, but a much higher affinity for DNA in general (Chu and Chang, 1988; Batty et al., 2000). A high affinity for DNA damage would be consistent with a role for UV-DDB in the recognition of these lesions directly after their infliction. Results of in vitro repair experiments to assess a role of UV-DDB in NER are inconclusive. Extracts from XP-E cells lacking UV-DDB did not show reduced repair of UV-irradiated DNA nor of an oligonucleotide substrate harboring a single cisplatin lesion when compared to normal human cells (Rapic-Otrin et al., 1998). Also, the addition of UV-DDB to a reconstituted system stimulated repair only moderately and even led to

inhibition of the reaction at high concentrations (Aboussekahra et al., 1995). In contrast to the above-mentioned studies, a recent study by Wakasugi et al. revealed that in their reconstituted repair system using purified factors, the repair efficiency of CPD and 6-4PP was enhanced by UV-DDB, particularly in the case of CPD (Wakasugi et al., 2001). This result implies that the role of UV-DDB is not limited to repair inside a chromatin context but instead UV-DDB can stimulate repair through direct interaction with the DNA lesion.

Several studies reported that efficient global genome repair of UV damage in vivo did require UV-DDB (Tang et al., 2000; Keeney et al., 1994; Hwang et al., 1999), suggesting that the protein might be involved in the repair of lesions within a chromatin context. Indeed, p48 shares homology with chromatin reorganizing proteins (Hwang et al., 1998) and UV-DDB interacts with the CBP/p300 histone acetyl transferase (Datta et al., 2001; Rapic-Otrin et al., 2002), consistent with a function in remodelling of chromatin to allow efficient repair in the vicinity of the lesion. In vivo studies have shown that p48 and p127 relocalise rapidly to sites containing UV-damaged DNA immediately after irradiation even in the absence of functional XPC protein (Wakasugi et al., 2001) and (Fitch et al., 2003). This observation is consistent with a role for UV-DDB in UV damage recognition, even before XPC is involved. However, the impact of the rapid recruitment of UV-DDB to UV damages on the efficiency of NER remains unclear.

Rodent cells efficiently repair 6-4PP, but in marked contrast to humans, show almost no repair of CPD in the genome overall. This marked difference in CPD repair phenotype between human and rodent cells has been attributed to the absence of UV-DDB activity in rodent cells (Hwang et al., 1999). The situation in rodent cells has led to the well-established notion that UV-DDB is essential for repair of CPD by GGR. However, since the affinity of UV-DDB for 6-4PP is considerable higher than that for CPD (Batty et al., 2000), UV-DDB might contribute also to GGR of 6-4PP in human cells, the rate of which greatly exceeds that of CPD. Indeed, an XP-E patient with defective repair of 6-4PP was reported (Itoh et al., 1999). In contrast, in other reports repair of 6-4PP in the absence of UV-DDB was either not affected or only delayed (Tang et al., 2000; Hwang et al., 1999), indicating that XPC-hHR23B by itself is capable of efficiently recognizing and targeting 6-4PP for repair.

In this study, we assessed the NER incision complex formation and repair of 6-4PP in local spots of UV-damage, in cells proficient or deficient for UV-DDB. We find that both the accumulation of NER complexes and 6-4PP repair are greatly enhanced in the presence of UV-DDB. Moreover, we present evidence that the cellular quantity of UV-DDB is the limiting factor for this enhanced 6-4PP repair. We propose a mechanism in which UV-DDB accelerates GGR of 6-4PP by binding to the lesion, facilitating the recruitment of XPC and subsequent factors. In the absence of UV-DDB, 6-4PP are repaired by the GGR pathway dependent solely on recognition by XPC-hHR23B, leading to slower but nevertheless complete repair of 6-4PP.

RESULTS

Repair of 6–4 photoproducts in normal human and XP-E cells after global UV-irradiation

Repair of UV-induced cyclobutane pyrimidine dimers in human cells is dependent on functional p48 based on the results of biochemical and immunochemical analysis of photolesions in normal and XP-E cells (Hwang et al., 1999). Data on repair of 6-4PP in XP-E cells are only available from immunoslotblot analysis and the results are less consistent than for CPD. Most studies failed to detect major differences in rates of 6-4PP repair between p48-proficient and -deficient cells after global irradiation (Tang et al., 2000; Hwang et al., 1999), whereas a partial repair defect was reported by Itoh et al. (1999). We examined the repair of 6-4PP in globally UV exposed normal human and XP-E cells by two different approaches: immunofluorescence and biochemical (gene specific) analysis. Gene specific analysis requires a relative high UV dose (30 J/m^2) to induce a single 6-4PP per gene fragment (van Hoffen et al., 1995); hence, to allow comparison, both assays were performed with cells exposed to 30 J/m^2. In previous studies, we determined repair of 6-4PP at the gene level with purified UvrABC excinuclease complex to cut at sites of DNA damage (van Hoffen et al., 1995). Since this report presents the first instance of UVDE endonuclease to replace UvrABC in the DNA cutting reaction, we validated the use of UVDE. First, the initial frequency of 6-4PP observed using UVDE is 0.0196-4PP per 10 kb fragment per J/m^2, which is virtually equal to the frequency found with UvrABC (van Hoffen et al., 1995). Second, we find indistinguishable repair kinetics of 6-4PP in normal human cells when assayed by UVDE or UvrABC (Fig. 1).

We assessed the kinetics of 6-4PP repair in normal human fibroblasts (VH25 and VH10hTert) and in human XP-E fibroblasts (XP23PV and GM01389hTert) exposed to 30 J/m^2 of UV light. Autoradiograms of representative experiments and graphs derived from scanning of these autoradiograms are shown in Fig. 2. In normal human cells, 6-4PP are removed fast from both strands of the EcoR1 fragment of the ADA gene: approximately 70–80% of the 6-4PP are repaired within 4 h and repair is virtually complete after 8 h. This is in agreement with results obtained in our previous studies (van Hoffen et al., 1995). Furthermore, there is no difference in the kinetics of removal of 6-4PP between normal and XP-E cells. Also, 6-4PP repair at 4 h observed in primary human fibroblasts (VH25) and

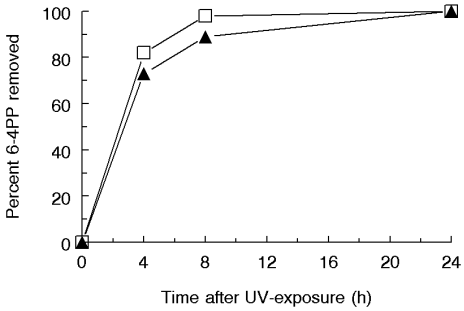

Fig. 1. Similar kinetics of 6-4PP repair assayed by UvrABC or UVDE endonuclease. Normal human cells (VH25) were exposed to 30 J/m^2 of global UV. Removal of 6-4PP at various times after irradiation was measured in EcoRI restriction fragments of the active ADA gene (18.5 kb) using a probe recognising both strands. After photoreactivation of CPD, the DNA was cut using either (□) UvrABC excinuclease or (▲) UVDE endonuclease.

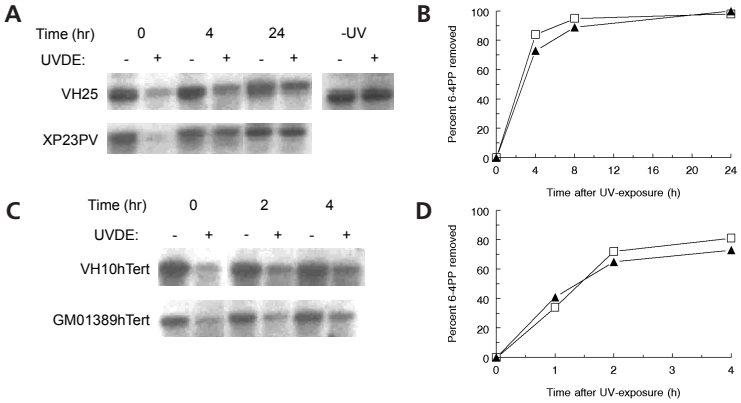

Fig. 2. Normal human and XP-E cells repair 6-4PP with similar kinetics after global exposure to 30 J/m² UV-radiation. Removal of 6-4PP at various times after irradiation was measured in EcoRI restriction fragments of the active ADA gene using a probe recognizing both strands. After in vitro photoreactivation of CPD, the DNA was cut using the UVDE endonuclease. (A) Autoradiogram and (B) line graph showing removal of 6-4PP over a period of 24 h in (▲) primary normal human (VH25) fibroblasts and (□) primary XP-E (XP23PV) fibroblasts. (C) Autoradiogram and (D) line graph showing removal of 6-4PP over a period of 4 h in (▲) normal human hTert-immortalised fibroblasts (VH10hTert) and (□) XP-E hTert-immortalised cells (GM01389hTert).

in hTert immortalized human fibroblasts (VH10hTert) were not different, which is in line with the observation made by Ouelette et al. (2000) that hTert immortalized cells (normal as well as XP-E) show the same UV-survival as primary cells.

Consistent with the findings in these biochemical experiments, immunofluorescence measurements demonstrate similar repair kinetics for 6-4PP in human XP-E fibroblasts and normal human cells after an UV-dose of 30 J/m² (Fig. 3B and D). We note here that the results concerning repair of 6-4PP after global irradiation obtained by the two methodologies are qualitatively similar, i.e. repair in normal and XP-E cells is the same, but that quantitatively the absolute level of repair after 24 h is rather different as measured by the two methods.

Distribution of NER proteins in normal human and XP-E cells after local UV-irradiation of the nucleus

The absence of clear differences in 6-4PP repair kinetics between normal human and XP-E cells suggests equally efficient incision complex formation in normal and XP-E cells. To test this hypothesis, we applied local UV-irradiation to confluent human fibroblasts as described previously (Volker et al., 2001; Moné et al., 2001) taking into account that incision complex formation during the first 2 h after UV is predominantly due to repair of 6-4PP (Volker et al., 2001; Fitch et al., 2003; Wakasugi et al., 2002) (see also, Fig. 4). The distribution pattern of XPC and RPA in normal human and XP-E cells was analyzed before and at various times after local irradiation (30 J/m²). Without UV exposure, both proteins show a homogeneous distribution and strictly nuclear localization (not shown) as observed previously for NER proteins (Volker et al., 2001). In normal human cells, both

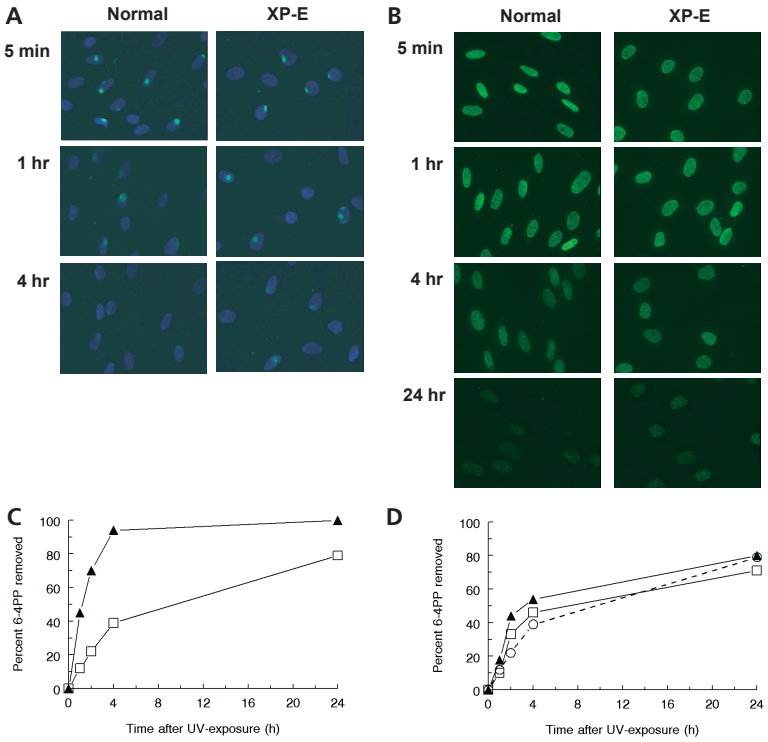

Fig. 3. Accelerated 6-4PP repair in normal human fibroblasts (VH10hTert) compared to XP-E fibroblasts (GM01389hTert) after local UV-irradiation, but not after global UV-irradiation. (A) Fluorescent immunostaining of 6-4PP (green) at various times after local UV-irradiation (30 J/m^2) through an 8 µm pore size filter. Images were taken with equal exposure times and merged with DAPI nuclear DNA staining (blue). (B) Fluorescent immunostaining of 6-4PP (green) at various times after global UV-irradiation (30 J/m^2). Images were taken with equal exposure times. (C and D) Graphs presenting the percentage of 6-4PP removed determined from multiple (>20) fluorescent images for each timepoint. (C) Removal of 6-4PP in (▲) normal human and (□) XP-E cells following exposure to 30 J/m^2 of local UV-irradiation through an 8 µm pore size filter. (D) Removal of 6-4PP in (▲) normal human and (□) XP-E cells following exposure to 30 J/m^2 of global UV. The dotted line is taken from C showing removal of 6-4PP in XP-E cells which were locally UV-irradiated with 30 J/m^2 and is depicted as a reference. Signal intensities were determined by measuring the total fluorescence intensity of a spot or a nucleus, and dividing by the surface area of the measured spot or nucleus, respectively.

XPC and RPA strongly accumulate in the UV-irradiated areas of the nucleus as fast as 5 min after UV exposure. This accumulation persists at a constant level of intensity up to 1 h after UV before slowly returning to the pre-UV pattern (Fig. 5).

Since the changes in spot intensities of RPA with time are essentially similar to those of XPC in all performed experiments, only results of XPC protein are shown. In strong contrast to normal human cells, XP-E cells only display a moderate accumulation of NER proteins at damage spots 5 min after UV, while at later stages (30 min and more) after UV, hardly any accumulation is observed (Fig. 5). This indicates that in the absence of UV-DDB, formation of NER incision complexes is strongly reduced. Other NER proteins XPA, XPB, XPG and

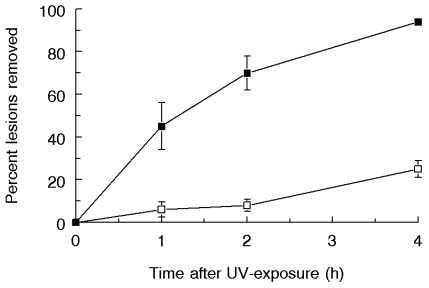

Fig. 4. 6-4PP and CPD repair in normal human fibroblasts (VH10hTert) after local UV-irradiation through an 8 µm pore size filter with 30 J/m². Cells were immunofluorescently labelled for 6-4PP or CPD at various times following irradiation as described in Section 2. Graphs representing percent repair of 6-4PP (■) or CPD (□) were derived from multiple fluorescent images.

ERCC1 also accumulate only marginally in spots of UV-damage after local irradiation of XP-E cells (data not shown), confirming that assembly of the total NER complex is strongly reduced in quantity in the absence of UV-DDB. Hence, our observations indicate that the complete NER machinery is able to bind to DNA photolesions in the absence of UV-DDB, but that UV-DDB positively affects the kinetics of NER complex formation.

Effect of UV-DDB on repair of 6-4PP after local UV-irradiation of cells

The poor incision complex formation in the absence of UV-DDB conflicts the rather efficient repair of 6-4PP in globally irradiated XP-E cells described in this study and observed by other investigators (Tang et al., 2000; Hwang et al., 1999). We reasoned that if the level of NER complex accumulation in local UV-spots corresponds with the efficiency of 6-4PP repair, the strongly decreased complex formation in XP-E cells implies reduced repair of 6-4PP. Hence, we compared the repair rates of 6-4PP in locally irradiated normal human and XP-E cells at the single cell level using specific antibodies against 6-4PP. To allow direct comparison between globally and locally UV-irradiated cells and to limit experimental variations due to processing for immunofluorescence, about half of the cells on a glass slide were globally exposed whereas the remaining cells were locally irradiated and subsequently processed simultaneously, as described in Section 2. Consistent with the profound difference in incision complex formation we find that repair of 6-4PP in local spots of UV-damage differs greatly between the two cell types. From the data (Fig. 3A and C), it can be deduced that the fluorescent signal for 6-4PP in normal human cells drops rapidly in time, virtually disappearing within 4 h. In striking contrast, 4 h after UV the amount of 6-4PP in XP-E cells is only 50% reduced compared to the initial levels. We note here that the initial levels of 6-4PP in normal and XP-E cells indicated by the immunofluorescence signal immediately after UV exposure were virtually the same.

From these findings, we speculated that UV-DDB can mediate the rapid processing of only a limited number of 6-4 photolesions.

To test this hypothesis, we irradiated normal human and XP-E cells both locally and globally with a low dose of UV, i.e. 5 J/m² and assayed repair of 6-4PP at various times following UV using immunofluorescent labeling of 6-4PP. As shown in Fig. 6, we find that following this low-dose repair kinetics of 6-4PP are more rapid in normal human than in XP-E cells both after local and global UV-irradiation. Furthermore, 6-4PP repair in normal

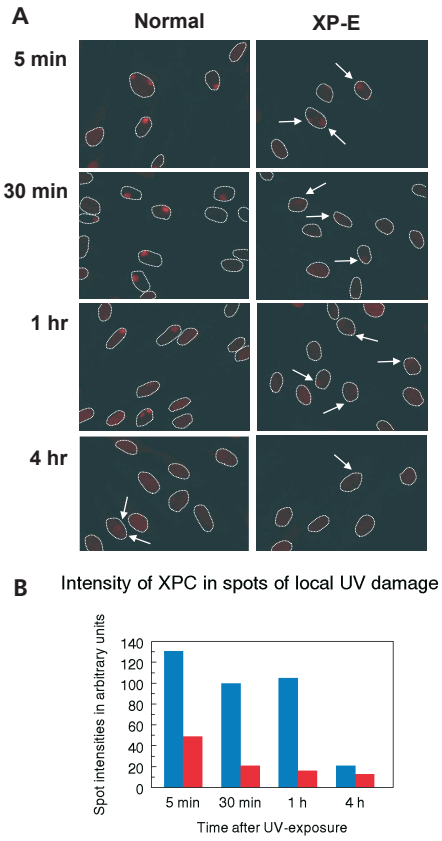

Fig. 5. XPC accumulates differentially in normal human fibroblasts (VH10hTert) and XP-E fibroblasts (GM01389hTert) after local UV-irradiation. Cells were UV-irradiated with 20 J/m^2 through an 8 µm pore size filter. (A) Fluorescent immunostaining of XPC (red) at various times after irradiation. Arrows indicate protein accumulations in UV-damaged spots that are poorly visible. Dotted lines outline the nuclei as determined from DAPI nuclear counterstaining (images not shown). All fluorescent images were taken with equal exposure times. (B) Bar graph showing average intensities of XPC spots at various time points after irradiation (blue bars: normal cells; red bars: XP-E cells). Spot intensities were determined by measuring the total fluorescence intensity of a spot, divided by its surface area, and corrected for background levels of fluorescence in the nucleus. For each time point, at least 20 cells were measured. The fluorescent signal measured 30 min after UV inThe kinetics of accumulation of NER proteins thus coincide with the rate of repair of 6-4PP in local UV spots, strongly supporting the notion that NER complex formation shortly after UV primarily represents recruitment of NER proteins to 6-4PP. This is further supported by the finding that during the first 2 h after UV-irradiation repair of CPD is strongly lagging behind that of 6-4PP and actually does not amount more than 8% removal for CPD, compared to 70% for 6-4PP (Fig. 4).

human cells is more rapid after local irradiation than after global irradiation, whereas in XP-E cells there is no significant difference between 6-4PP repair kinetics following local or global UV. These results corroborate the idea that UV-DDB does stimulate repair of 6-4PP, but that there is a limit to the number of 6-4PP that can be rapidly repaired through UV-DDB. Interestingly, at this low UV-dose, repair of 6-4PP is more rapid in normal human cells after global irradiation than in XP-E cells exposed to local irradiation, despite the fact that in the former situation more lesions are introduced (Fig. 6).

Distribution of p48 in normal human cells after local UV-irradiation of the nucleus

To show that UV-DDB is part of the NER complex that is formed after UV-irradiation and, thus, that accumulation of UV-DDB at locally induced UV-damage spots follows the same kinetics as other NER proteins, we constructed a human cell line (MRC5) stably expressing p48-YFP. A similar approach has been successfully applied by Fitch et al. (Fitch et al., 2003b; Fitch et al., 2003) to investigate the recruitment of p48 to local damage. The resulting cell line termed MRC5-p48-YFP, expresses p48-YFP in >75% of the cells at a moderate to high level (data not shown). Since p127 is present in excess (Nichols

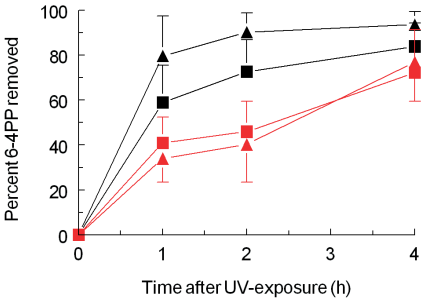

Fig. 6. 6-4PP repair in normal human fibroblasts (VH10hTert) and XP-E fibroblasts (GM01389hTert) after local and global UV-irradiation with 5 J/m². Cells were immunofluorescently labelled for 6-4PP at various times following irradiation as described in Section 2. Graphs representing percent 6-4PP removed were derived from multiple fluorescent images. Removal of 6-4PP in (black, ■) normal human and (red, ■) XP-E cells following exposure to global UV. Removal of 6-4PP in (black, ▲) normal human and (red, ▲) XP-E cells following exposure to UV through an 8 µm pore size filter.

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et al., 2000), we expect that (following UV-irradiation) most if not all p48-YFP to be part of UV-DDB in these cells. The distribution pattern of p48-YFP in unirradiated cells is predominantly nuclear and homogenous, as observed for other NER proteins. After local UV, p48-YFP transiently accumulates at damage spots as observed for other NER proteins such as XPC: intense spots of p48-YFP visible at 30 min after UV, become much weaker in signal 2 h post-UV, and no spots can be detected 4 h after UV (Fig. 7A). At 4 h post-UV, also 6-4PP have become undetectable whereas CPD are clearly detectable in locally irradiated spots (Fig. 7B), indicating that the observed accumulation of p48-YFP in local UV spots mainly takes place at 6-4PP.

To address this point in more detail, we utilized XP-A cells expressing either a CPD or a 6-4PP specific photolyase (referred to as XP-A(CPDpl) and XP-A(6-4pl) cells, respectively). As shown in Fig. 8A, CPD or 6-4PP can be specifically removed by the respective photoreactivating enzyme (Fitch et al., 2003a; Fitch et al., 2003b) and hence, this system provides an adequate tool to study the relative contribution of either type of photoproduct to the observed accumulation of NER proteins at damage spots. Fig. 8B shows typical results obtained for XPC after photoreactivation of either 6-4PP or CPD in XP-A cells.

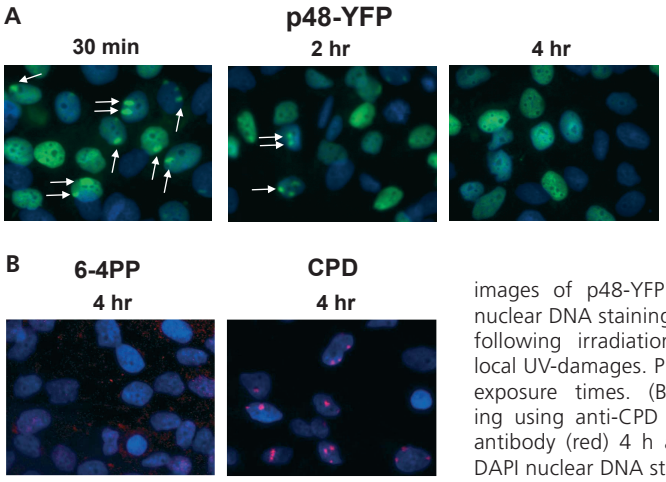

Fig. 7. Formation of p48 spots after induction of local UV-damage coincides with 6-4PP repair. Normal human cells (MRC5) stably expressing p48-YFP were exposed to 30 J/m² of UV through a 5 µm pore size filter. (A) Fluorescent images of p48-YFP (green) merged with DAPI nuclear DNA staining (blue) taken at various times following irradiation. Arrows indicate sites of local UV-damages. Pictures were taken with equal exposure times. (B) Fluorescent immunostaining using anti-CPD antibody (red) or anti-6-4PP antibody (red) 4 h after exposure, merged with DAPI nuclear DNA staining (blue).

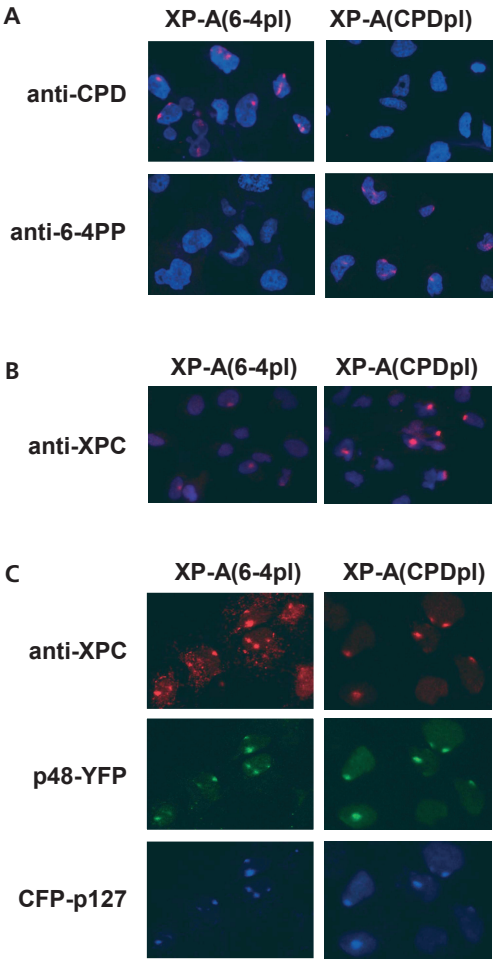

Fig. 8. Accumulation of XPC, p48 and p127 in local UV-damaged spots after removal of one of the two types of UV-photolesion by photoreactivation. XP-A cells stably expressing 6-4PP photolyase [XP-A(6-4)phl)] or CPD photolyase [XP-A(CPDphl)], respectively, were irradiated with 30 J/m^2 of UV through a 5 µm pore size filter, and exposed to photoreactivating light for 1 h. (A) Fluorescent immunostaining of CPD (red) and 6-4PP (red), merged with DAPI nuclear DNA staining. (B) Fluorescent immunostaining of XPC after photoreactivation of 6-4PP (left) or CPD (right) in XP-A photolyase expressing cells. Pictures taken with equal exposure times and merged with DAPI nuclear counterstaining (blue). (C) XP-A(6-4phl) or XP-A(CPDphl) cells transfected with p48-YFP and CFP-p127 expression constructs 24 h prior to local UV-exposure and 1 h photoreactivation. Fluorescent images of XPC (using anti-XPC antibody, red), p48-YFP (green) and CFP-p127 (blue), taken with exposure times optimised for image clarity.

To estimate the relative affinity of XPC for 6-4PP and CPD in vivo, we utilized images that were captured with equal exposure times. A clearly reduced intensity of XPC spots can be seen when 6-4PP are selectively removed by photoreactivation compared to CPD removal in spite of the fact that CPD are present in three-fold excess over 6-4PP. Similarly, fluorescent staining of p89, the XPB component of TFIIH, also showed a clear signal upon photoreactivation of CPD whereas the signal was virtually absent upon photoreactivation of 6-4PP (data not shown). These observations confirm that 6-4PP are a better substrate than CPD for XPC and UV-DDB to bind to, as previously suggested by Fitch et al. (2003). In order to visualize the accumulation of p48 and p127 at damage spots, XP-A(CPDpl) and XP-A(6-4pl) cells transiently transfected with both p48-YFP and CFP-p127 were locally UV-irradiated with 30 J/m^2. After removing CPD or 6-4PP by photoreactivation, cells were examined for the distribution of p48-YFP and CFP-p127 as well as immunofluorescently labelled for XPC (Fig. 8C). Under these conditions, accumulations of p48-YFP and

CFP-p127 as well as XPC, are observed 1 h after UV when either all CPD or 6-4PP have been removed, suggesting that UV-DDB and XPC accumulate on 6-4PP as well as on CPD under conditions where DDB is overproduced and repair is absent (XPA).

DISCUSSION

NER complex formation at early hours after UV occurs predominantly on 6-4PP

In normal human cells, the signal intensity of p48-YFP in local UV-damage spots closely follows the kinetics of 6-4PP repair, in spite of the fact that UV-DDB is essential for repair of CPD in rodent and human cells. This observation suggests that 6-4PP are a stronger recognition signal for UV-DDB than CPD. Indeed, we find shortly (1 h) after irradiation that p48-YFP and CFP-p127, as well as XPC show a pronounced accumulation at 6-4PP in XP-A cells equipped with a photoreactivating enzyme for CPD. In addition, p48-YFP as well as XPC accumulate at UV-damage spots in XP-A cells in which 6-4PP were removed by a 6-4PP photolyase. However, in control cells only containing low levels of endogenous p48, the signal of XPC at CPD was much weaker, while the signal of XPB was virtually absent. Fitch et al. (2003), employing the same cell system, also observed a higher affinity of UV-DDB for 6-4PP than for CPD in living cells. Furthermore, they observed accumulation of XPC on damage spots in only a minority of cells analysed upon removal of 6-4PP by a photolyase, while ectopically expressed p48 was efficiently recruited to all CPD containing spots. This led them to conclude that p48 is a more efficient recognition factor for CPD than XPC. Although we agree with this statement, we conclude, based on immunostaining of the endogenous XPC protein as well as on the repair kinetics in normal human cells, that 6-4PP are a better substrate than CPD for both XPC and UV-DDB to bind to. Accumulation of both p48 and XPC at CPD in XPA cells can be observed due to the build up of incomplete repair complexes. A similar phenomenon is observed in normal human cells in the presence of inhibitors of repair synthesis (unpublished results). Together, these data provide evidence that UV-DDB stimulates excision repair by direct binding to the lesion as indicated by experiments with a non-chromatin template in vitro (Wakasugi et al., 2002) and that the accumulation of UV-DDB and other NER proteins observed shortly after local UV predominantly takes place at 6-4PP indicating a functional role of UV-DDB in 6-4PP repair, although UV-DDB also plays a crucial role in repair of CPD.

UV-DDB mediates accelerated repair of a limited number of 6-4PP

Cells proficient for UV-DDB show a more pronounced accumulation of NER proteins in spots of local UV-damage, compared to cells lacking UV-DDB activity, and this enhanced complex formation coincides with an increased repair rate of 6-4PP in local UV-spots. Also, after global irradiation with a low dose of UV (5 J/m^2), 6-4PP repair is more rapid in normal human than in XP-E cells. In contrast, after global exposure to a high UV-dose (30 J/m^2), we find no significant difference between normal human cells and XP-E cells in the repair kinetics of 6-4PP, i.e. in this situation the accelerating effect of UV-DDB on repair is lost.

We propose the following mechanism to account for these observations. Since we applied equal UV doses in local and global irradiation experiments, the photolesion

frequency per unit of DNA is the same in the irradiated areas, and hence the major difference between the two approaches is the total number of lesions introduced per cell. The results imply that the amount of cellular UV-DDB is sufficient to allow enhanced 6-4PP repair after the induction of a (relatively) low number of lesions, i.e. after irradiating locally with 30 J/m^2 or globally with 5 J/m^2, but insufficient to allow rapid repair of the majority of 6-4PP introduced after global irradiation with a high-UV dose. The following calculation corroborates this notion. A dose of 30 J/m^2 of UV-C light introduces approximately 2 × 10^6 CPD (van Hoffen et al., 1995) in the (diploid) human genome (6 × 10^9 nucleotides). 6-4PP are introduced at one-third of the frequency of CPD, i.e. 7 × 10^5 in total. The typical volume irradiated after local UV through an 8 µm filter encompasses about one sixth of the whole nucleus of a human fibroblast (diameter approximately 20 µm), hence, this volume contains 10^5 6-4PP. A comparison of the numbers of 6-4PP and the number of UV-DDB molecules, estimated to be ~10^5 (Keeney et al., 1993), shows that after global irradiation with 30 J/m^2 of UV the number of 6-4PP is about seven-fold larger than the number of UV-DDB molecules; whereas, after 30 J/m^2 of local UV, this number is roughly equal. A similar calculation for global UV-irradiation with only 5 J/m^2 shows that in that situation too, the number of 6-4PP is approximately the same (~8 × 10^4) as the number of UV-DDB molecules. The most plausible picture of the function of UV-DDB we envisage is that UV-DDB is present in the cell in rate-limiting amounts and is largely consumed during repair of 6-4PP. Moreover, the data suggest that UV-DDB can only function once in the NER reaction and that after recognition of a DNA lesion it becomes inactivated. Such a mechanism is supported by a recent report of Rapic-Otrin et al., (2002) who find virtually complete degradation of p48 in primary human fibroblasts in the first 3 h following 15 J/m^2 of UV. Other studies (Wakasugi et al., 2001) reported similar results. As a consequence of this breakdown, most 6-4PP induced after high doses of UV are not repaired with enhanced kinetics through UV-DDB. Yet, the complete removal of 6-4PP from the genome of XP-E cells after global irradiation indicates that these lesions can also be repaired, albeit at reduced rate, through the GGR pathway in which recognition of 6-4PP solely depends on the XPC-hHR23B complex. In contrast, functional UV-DDB is essential for GGR of CPD.

Summarizing, we hypothesize that in vivo (i) 6-4PP can be repaired through a basal mechanism depending only on recognition by XPC-hHR23B; (ii) binding of UV-DDB to 6-4PP will however accelerate their repair; (iii) after binding to a 6-4PP, UV-DDB is degraded and thus cannot assist in subsequent repair of remaining 6-4PP (and CPD). This implies that if 6-4PP are introduced in molar excess to UV-DDB, most 6-4PP will be repaired through direct recognition by XPC-hHR23B, which is a relatively slow process compared to the UV-DDB-stimulated repair mechanism.

Mechanisms of action of UV-DDB

Several mechanisms might account for a stimulatory effect of UV-DDB on repair of 6-4PP. Binding of UV-DDB to a 6-4PP may result in a structure that facilitates recognition by, and stabilizes binding of subsequent repair components. NER is driven by random diffusion of single components rather than by a pre-assembled repairosome (Houtsmuller et al., 1999; Hoogstraten et al., 2002; Rademakers et al., 2003), i.e. a NER complex will be

formed by the consecutive recruitment of proteins. Generally, XPC-hHR23B is assumed to be the crucial factor for initiation of the GGR process (Sugasawa et al., 1998; Volker et al., 2001). However, in the absence of UV-DDB the interaction of XPC-hHR23B with a 6-4PP might be relatively weak and frequently subject to dissociation before binding of subsequent factors such as TFIIH, XPA and RPA can stabilize the complex as it is being formed. Consequently, only a few NER incision complexes will be formed to completion.

Two observations provide evidence for this notion. Firstly, the poor accumulation of NER proteins in local UV-damage spots in XP-E cells indicates a low steady-state level of NER pre-incision complexes. Secondly, repair of 6-4PP in XP-E cells is equally fast, expressed as percent removed per time unit, after local and global UV-irradiation although in the latter situation substantially more lesions are induced. These results are consistent with a model in which both the general affinity of XPC-hHR23B for DNA and its specific affinity for 6-4PP compared to undamaged DNA is relatively low in vivo. Furthermore, several findings indicate that UV-DDB, in contrast to XPC-hHR23B, is capable of forming a stable complex when bound to a 6-4PP. The general affinity of UV-DDB for DNA is much higher (100–1000-fold) than that of XPC-hHR23B, while the specific affinity for 6-4PP is comparable (Chu and Chang, 1988; Batty et al., 2000). As a result of this high affinity, UV-DDB is the only factor in human whole cell extracts that binds readily to UV-damaged DNA (Chu and Chang, 1988). Also, it has been shown that in vivo UV-DDB can bind to photolesions independent of XPC (Wakasugi et al., 2002). Finally, in UV-DDB-proficient normal human cells we find strong accumulations of NER proteins in local UV-damaged spots and fast repair of 6-4PP. All these data are consistent with the idea that UV-DDB, by virtue of its high affinity for DNA and 6-4PP forms a stable complex when bound to a 6-4PP. This stable state provides subsequent repair proteins – starting with XPC-hHR23B – time to detect and verify the lesion, resulting in efficient 6-4PP repair. The described scenario, in which UV-DDB directly stimulates 6-4PP repair is in agreement with the reported stimulation of 6-4PP repair by UV-DDB in an in vitro repair system employing DNA damage in naked DNA as substrate (Wakasugi et al., 2001).

Stimulation of 6-4PP repair by UV-DDB might also involve UV-DDB-mediated local chromatin remodelling at sites of photolesions, increasing their accessibility for recognition by XPC-hHR23B. In accordance with a role in chromatin remodelling, p48 has recently been reported to interact with p300/CBP (Datta et al., 2001; Rapic-Otrin et al., 2002), while p48 itself bears homology to chromatin-reorganizing proteins (Hwang et al., 1998). Indications that remodelling of chromatin can enhance repair come from recent studies showing that chromatin interacting proteins such as SWI/SNF (Hara and Sancar, 2002) and HMGN1 (Birger et al., 2003) enhance NER specifically in the context of chromatin. Moreover, a recent report showed the presence of p48 and p127 together with cullin 4A in a multi-protein complex (the COP 9 signalosome) that possesses ubiquitin ligase activity (Groisman et al., 2003). Not only is this complex a possible candidate to assist in chromatin remodelling by virtue of its combined ubiquitin ligase/UV-damaged chromatin binding activities (Birger et al., 2003), but also, cullin 4A has been shown to target p48 for ubiquitination and degradation following UV (Nag et al., 2001).

Finally, UV-DDB might enhance GGR of various types of other lesions for which it displays affinity, such as CPD, *cis*-diamminedichloroplatinum(II) adducts, and nitrogen mustard, among others (Chu and Chang, 1988; Payne and Chu, 1994) by the same mechanism that accelerates 6-4PP repair.

MATERIALS AND METHODS

Cell culture. Primary diploid human fibroblasts used for immunofluorescence studies, derived from a normal individual (VH25) and xeroderma pigmentosum group E patients (XP2RO and XP23PV), were grown in Ham's F10 medium from which hypoxanthie and thymidine were omitted, and supplemented with 15% fetal calf serum and antibiotics at 37 °C in a 2.5% CO_2 atmosphere. XP23PV cells were kindly provided by Dr. M. Stefanini (Instituto di Genetica Molecolare, Pavia, Italy). Simian virus 40 (SV40)-immortalized human fibroblasts, wildtype (MRC5) and XP-A (XP12RO), and wildtype Chinese hamster cells (V79B), were cultured under similar conditions in a 5% CO_2 atmosphere. XP12RO cells stably expressing CPD- or 6-4PP photolyase (S. Nakajima and A. Yasui, manuscript in preparation) were kindly provided by Dr. A. Yasui (Nagoya University, Nagoya, Japan). We immortalized fibroblasts derived from a normal individual by telomerase transfection (VH10hTert) (Ouellette et al., 2000). Immortalized cells from a XP-E patient (GM01389hTert) were kindly provided by Dr. E.C. Friedberg (University of Texas, Dallas, TX). Both immortalized cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics in a 5% CO_2 atmosphere. VH10hTert cells and XP12RO cells expressing photolyases were grown in the presence of 25 µg/ml and 0.5 mg/ml G418, respectively.

Construction of vectors expressing fusion proteins. Full-length murine *DDB*1 cDNA was amplified by PCR using Hot Start GoldStar DNA polymerase (Eurogentec) with the primer set 5'-CCG-GAA-TTC-C-GGA-GGC-ATG-TCG-TAC-AAC-TAC-GTC-GT-3' (forward) and 5'-TCC-CCG-CGG-CTA-ATG-GAT-CCG-AGT-TAG-CT-3' (reverse) and digested with *Eco*RI (forward) and *Sac*II (reverse) endonucleases. The cDNA fragments were cloned in-frame into the *Eco*RI and *Sac*II site of pECFP-C1 (Clontech), resulting in a vector expressing a CFP-p127 fusion protein. Murine *DDB*2 cDNA was amplified similarly, using the primers 5'-CCG-GAA-TTC-CTC-TTC-ACC-GAG-TAC-GTC-AT-3' (forward) and 5'-CGC-GGA-TCC-CC-GCC-TCC-TAG-TCT-TTC-ATG-ATC-TTT-CT-3' (reverse). To create a vector expressing p48-YFP fusion protein, after digestion of the cDNA with *Eco*RI and *Bam*HI, the cDNA fragments were cloned in-frame into the *Eco*RI and *Bam*HI site of pEYFP-N1 (Clontech). A vector expressing a p48-FLAG fusion protein was created after PCR amplification of murine *DDB*2 cDNA using the primer set 5'-AT-CCC-AAG-CTT-CTC-TTC-ACC-GAG-TAC-GTC-AT-3' (forward) and 5'-AAG-GAA-AAA-AGC-GGC-CGC-GCT-GCC-ACT-CCT-CAC-AGA-AT-3' (reverse) and digestion with *Hin*dIII and *Not*I. Resulting cDNA fragments were cloned in-frame into p3XFLAG-CMV (Sigma) at the *Hin*dIII and *Not*I sites to form the p48-FLAG vector, expressing the FLAG polypeptide fused to the C terminus of p48. All vectors contain a *NEO* gene, and a cytomegalovirus promoter to control expression of the fusion genes. After construction of the vectors, all fusion genes were sequenced to ensure that no mutations had been introduced during PCR a

Transient transfection of human cells. Immortalised XPA-deficient human fibroblasts stably expressing either CPD- or 6-4PP photolyases were sub-cultured 24 h before transfection, and subsequently grown in the *absence* of G418 to improve transfection efficiency. Cells were transfected with plasmids p48-YFP and CFP-p127 using the FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. After transfection, cells were cultured for an additional 24 h to allow expression of the fusion proteins before experiments were carried out.

Global and local UV-irradiation. Confluent cells were washed with phosphate-buffered saline (PBS), irradiated with a Philips TUV lamp (predominantly, 254 nm) at a dose rate of 0.2 W/m^2 as described previously (Venema et al., 1991). For local UV-irradiation, cells on coverslips were washed once with PBS, covered with an isopore polycarbonate filter with pore sizes of 5 or 8 µm (Millipore, Bradford, MA) and UV-irradiated (Moné et al., 2001). Only half of a coverslip was covered with the filter for experiments with simultaneous local and global UV-irradiation. After irradiation, the filter was removed, and cells were returned to culture conditions for times indicated. Photoproducts were removed from the genome of photolyase-expressing cells by exposing the cells for 1 h to light from a far-blue TL tube.

Antibodies. The following primary antibodies were employed: affinity-purified rabbit IgG polyclonals anti-XPC and anti-ERCC1, kindly provided by Hanny Odijk and Dr. Wim Vermeulen (Erasmus MC, Rotterdam, The Netherlands); affinity-purified mouse IgG monoclonal anti-RPA70, a gift from Dr. H.P. Nasheuer (National University of Ireland, Galway, Ireland); mouse IgG monoclonals anti-6-4PP and anti-CPD, gifts from Dr. O. Nikaido (Kanazawa University, Kanazawa, Japan); mouse IgG monoclonal anti-XPB, a gift from Dr. J.-M. Egly (IGMC, Illkirch, France); affinity-purified mouse monoclonals anti-XPA and anti-XPG, gifts from Dr. Rick Wood (UPCI, Pittsburg, PA). Secondary antibodies that were utilized are: FITC-conjugated donkey anti-mouse and donkey anti-rabbit IgG; Cy2-conjugated goat anti-mouse IgG and goat anti-rabbit IgG; Cy3-conjugated goat anti-rabbit IgG and goat anti-mouse IgG + IgM (Jackson Laboratories, Westgrove, PA). Alexa Fluor 488-conjugated goat anti-mouse IgG was obtained from Molecular Probes (Leiden, The Netherlands). All secondary antibodies were used according to the manufacturer's instructions.

Fluorescent labelling. The fluorescent labelling was performed essentially as described (Volker et al., 2001). Briefly, cells were washed twice with cold PBS, fixed and lysed in PBS with 2% formaldehyde and 0.2% Triton X-100 for 15 min on ice and washed again twice with cold PBS. Cells were then incubated with 3% bovine albumin in PBS for 30 min at room temperature. To visualize CPD or 6-4PP, the cellular DNA was denatured with 0.1 M HCl for 10 min at 37 °C. Primary and secondary antibodies were incubated for 2 h and 1 h, respectively, at room temperature in washing buffer (WB: PBS, 0.5% bovine albumin, 0.05% Tween-20). After each antibody incubation, cells were washed three times for 5 min with WB. Cells were mounted in Vectashield mounting medium containing DAPI (1.5 µg/ml) (Vector Laboratories, Burlingame, CA) or in Aqua/polymount (Polysciences Inc., Warrington, PA) containing DAPI (1.5 µg/ml) that gave identical results.

Microscopy and quantification of fluorescent signal. To capture fluorescence images, a Zeiss Axioplan 2 epifluorescence microscope fitted with appropriate filters coupled to an AttoArc HBO 100 W adjustable mercury arc lamp, and a Hamamatsu C5935 cooled CCD camera, were used. The pictures were captured and processed with Metasystems (Altlussheim, Germany) ISIS software. Using the ISIS software package, the total fluorescence intensity of the area to be quantified (whole nuclei or local UV-spots) was measured and divided by the surface area, resulting in a specific fluorescence intensity expressed in arbitrary units.

Gene specific determination of 6-4PP. Cells were grown to confluency in petridishes to ensure that no cells were in S-phase during the experiments. Cells were UV-irradiated with 30 J/m^2, and subsequently incubated in culture medium, and lysed. High-molecular-weight DNA was isolated and purified as described (van Hoffen et al., 1995). The DNA was restricted with *Eco*RI, purified and CPD were removed from the DNA by in vitro photoreactivation employing the photolyase derived from *Anacystis nidulans*, kindly provided by Dr. A. Eker (Erasmus University, Rotterdam, The Netherlands) (van Hoffen et al., 1995). Photoreactivation was checked for completeness by treatment of DNA samples with T4 endonuclease V and subsequent Southern analysis. Equal amounts of DNA (5 µg) were either treated or mock treated with UvrABC excinuclease (2 pmol of each subunit per µg DNA) or Δ228-UVDE (ultraviolet DNA endonuclease; [31]) endonuclease (5 pmol/µg DNA). UvrABC and Δ228-UVDE were kindly provided by M. de Ruiter (Leiden University, Leiden, The Netherlands). After incubation, 10 mM EDTA and 0.1% SDS were added and the DNA was purified by phenol and chloroform extraction, precipitated with ethanol and dissolved in TE. The samples were electrophoresed in 0.6% alkaline agarose gels. The DNA was transferred to Hybond N+ membranes by vacuum Southern blotting and hybridised with a ^{32}P-labeled gene-specific probe recognizing both strands of an 18.5 kb *Eco*RI fragment of the human *ADA* gene (exon 12). Filters were scanned using the Instant Imager (Packard Instrument Company). The number of 6-4PP per restriction fragment was calculated from the relative band densities of full size restriction fragments in the lanes containing DNA treated or not treated with either UvrABC excinuclease or UVDE endonuclease, using the Poisson expression. The use of UVDE, a DNA repair enzyme of *Schizosaccharomyces pombe* that cuts at sites of DNA photolesions (Fitch et al., 2003) was introduced in the latter assay to circumvent background cutting in DNA from mock-treated cells, a known property of UvrABC (van Hoffen et al., 1995). Fig. 2 shows that UVDE does not exhibit non-specific cutting and hence, UVDE provides an important improvement over UvrABC.

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DYNAMIC IN VIVO INTERACTION OF DDB2 E3 UBIQUITIN LIGASE WITH UV-DAMAGED DNA IS INDEPENDENT OF DAMAGE-RECOGNITION PROTEIN XPC

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SUMMARY

Damage DNA binding protein 2 (DDB2) has a high affinity for UV-damaged DNA and has been implicated in the initial steps of global genome nucleotide excision repair (NER) in mammals. DDB2 binds to CUL4A and forms an E3 ubiquitin ligase. In this study, we have analyzed the properties of DDB2 and CUL4A in vivo. The majority of DDB2 and CUL4A diffuse in the nucleus with a diffusion rate consistent with a high molecular mass complex. Essentially all DDB2 binds to UV-induced DNA damage, where each molecule resides for 2 minutes. After the induction of DNA damage, DDB2 is proteolytically degraded with a half-life that is two orders of magnitude larger than its residence time on a DNA lesion. This indicates that binding to damaged DNA is not the primary trigger for DDB2 breakdown. The bulk of DDB2 binds to and dissociates from DNA lesions independently of damage-recognition protein XPC. Moreover, the DDB2-containing E3 ubiquitin ligase is bound to many more damaged sites than XPC, suggesting that there is little physical interaction between the two proteins. We propose a scenario in which DDB2 prepares UV-damaged chromatin for assembly of the NER complex.

INTRODUCTION

Genome integrity is continuously challenged by various sources that potentially damage DNA. Short-wavelength UV light introduces DNA injuries, such as 6-4 photoproducts (6-4 PPs) and cyclobutane pyrimidine dimers (CPDs), both of which can interfere with transcription and replication. The mammalian cell utilizes nucleotide excision repair (NER) to remove UV-induced DNA damage and various other bulky DNA lesions from the genome (de Laat et al., 1999; Hoeijmakers, 2001) Two different modes of NER exist: transcription-coupled NER (TC-NER) and global genome NER (GG-NER). TC-NER removes lesions from the transcribed strand of active genes, whereas GG-NER repairs damage at any other position in the genome (de Laat et al., 1999). More than 20 gene products are involved in mammalian NER (Aboussekhra et al., 1995) that employs a dual incision mechanism to remove lesions. A crucial step in the initiation of GG-NER is the detection of DNA lesions. Although the mechanism of damage detection in chromatin is not well understood, various studies have identified the XPC-RAD23B-CEN2 complex as the principal initiator of GG-NER (Araki et al., 2001; Sugasawa et al., 1998; Sugasawa et al., 2001; Volker et al., 2001). Detection of CPDs by damage-recognition protein XPC is inefficient and it has been suggested that the UV-damaged DNA binding (UV-DDB) complex plays a crucial role in CPD detection and repair. UV-DDB consists of DDB1 (p127) and DDB2 (p48) (Dualan et al., 1995; Takao et al., 1993), and mutations in the DDB2 subunit are responsible for the XP-E phenotype (Nichols et al., 2000; Rapic-Otrin et al., 2003). DDB2-deficient cells such as rodent and human XP-E cells, have a deficiency in the removal of CPDs and delayed removal of 6-4 PPs, particularly at low UV doses (Hwang et al., 1999; Moser et al., 2005; Tang et al., 2000). Transfection of DDB2 cDNA in hamster cells restores the binding activity of UV-DDB to damaged DNA, showing that DDB2 rather than DDB1 is responsible for binding to damaged DNA (Hwang et al., 1998; Li et al., 2006; Tang et al., 2000). The complete inactivation of DDB2 expression in mice results in a significant increase in the formation of malignant tumors (Yoon et al., 2005), whereas overexpression of DDB2 protects mice from the carcinogenic effects of UV-B irradiation (Alekseev et al., 2005), illustrating the tumor-preventing property of DDB2.

Recently, UV-DDB was implicated in 6-4 PPs repair in vivo (Alekseev et al., 2005; Moser et al., 2005), which supports results of several in vitro assays that demonstrated a higher affinity of UV-DDB for 6-4 PPs compared with CPDs (Fujiwara et al., 1999; Reardon et al., 1993; Treiber et al., 1992). UV-DDB has a 500,000-fold preference for damaged over non-damaged DNA (Hwang and Chu, 1993), which is significantly higher than the selectivity of XPC for damaged DNA (3,000-fold preference) (Sugasawa et al., 1998). UV-DDB appears to bind to DNA lesions in the absence of functional XPC protein (Fitch et al., 2003; Wakasugi et al., 2001), in contrast to the NER components XPA, TFIIH, XPG and ERCC1-XPF, which all require XPC for binding to damaged DNA (Volker et al., 2001). Although much less efficiently, XPC binds to 6-4 PPs in XP-E cells, indicating that UV-DDB is not an absolute prerequisite for XPC binding to this particular type of lesion (Moser et al., 2005). DDB2 is crucial for the recognition and repair of CPDs and overexpression of DDB2 leads to enhanced recruitment of XPC to CPDs (Fitch et al., 2003). These observations strengthen

the hypothesis that UV-DDB facilitates and/or stabilizes the binding of XPC to UV-induced photoproducts.

Biochemical evidence suggests that UV-DDB is part of an E3 ub ligase complex that contains cullin 4A (CUL4A) (Shiyanov et al., 1999b) and the COP9 signalosome, which is a negative regulator of the ub ligase activity of the E3 DDB2 complex (Groisman et al., 2003). The ub ligase activity of the E3 DDB2 complex is transiently activated by UV irradiation and several substrates for ubiquitylation have been identified, including DDB2 itself, XPC and histones H2A, H3 and H4 (Groisman et al., 2003; Kapetanaki et al., 2006; Sugasawa et al., 2005; Wang et al., 2006). It is currently believed that DDB2 recruits XPC to lesions, after which both DDB2 and XPC are ubiquitylated (Sugasawa et al., 2005). Ubiquitylated DDB2 is proteolytically degraded (Chen et al., 2001; Nag et al., 2001; Rapic-Otrin et al., 2002) whereas the reversible ubiquitylation of XPC increases its affinity for DNA (Sugasawa et al., 2005). To investigate the interplay between DDB2 and XPC in vivo, we have analyzed the properties of fluorescently labeled DDB2 in wild-type and XPC-deficient cells. Our results indicate that the bulk of DDB2 interacts with lesions independently of XPC and that there is little interaction between the two recognition proteins on damaged DNA.

RESULTS

Generation of cell-lines expressing YFP-tagged DDB2 and GFP-tagged CUL4A

To study the cellular distribution and dynamics of DDB2 we tagged the protein with enhanced yellow fluorescent protein (EYFP), which was fused to the C terminus of murine DDB2 (Fig. 1B) resulting in a DDB2-EYFP fusion protein that was stably expressed in human fibroblasts (MRC5-SV) (Moser et al., 2005). EYFP was also fused to the N terminus of DDB2 and stably expressed in the same cells. Both fusion proteins were found predominantly in the nucleus (Fig. 1A). Western blot analysis using anti-DDB2 antibodies showed that DDB2-EYFP migrates in SDS-PAGE with a mobility corresponding to the expected size of the full-length fusion protein (75 kDa; Fig. 1C) (Dualan et al., 1995). The fluorescently tagged DDB2 protein binds to UV-damaged DNA (Moser et al., 2005) and is proteolytically degraded after UV irradiation as shown by western blot analysis of whole cell extracts (Fig. 1C). This indicates that the fusion protein exhibits wild-type behavior. To quantify the expression level of DDB2-EYFP, we compared the fluorescence of a solution of EYFP protein of a known concentration with the fluorescence of cells expressing DDB2-EYFP. The average concentration of DDB2-EYFP in living cells was 0.7±0.1 μM (data not shown), which corresponds to 1.2×10^5 molecules in a 300 μm^3 nucleus. The number of endogenous DDB2 molecules per cell is 1×10^5, indicating that DDB2-EYFP is expressed at levels comparable to endogenous DDB2 (Keeney et al., 1993). Moreover, western blot analysis using a polyclonal antibody against DDB2 (Wang et al., 2005b) demonstrated that DDB2-EYFP is expressed at about the same level as endogenous DDB2 in the same cells (Fig. 1C). DDB2-EYFP was also expressed in XPC-deficient and XPA-deficient human fibroblasts (XP20MA-SV and XP12RO-SV, respectively) and displayed a similar cellular distribution as observed in wild-type cells (data not shown). In addition to DDB2, CUL4A

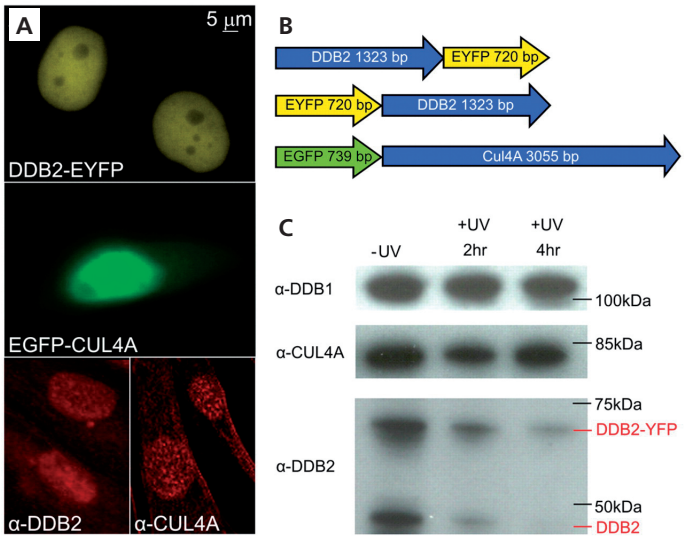

Fig. 1. Expression of DDB2-EYFP and EGFP-CUL4A. (A) Localization of DDB2 and CUL4A. The upper panel shows the distribution of DDB2-EYFP in an MRC5 cell, the middle image shows the distribution of EGFP-CUL4A in a HeLa cell and the lower images show the localization of endogenous DDB2 and CUL4A detected with specific antibodies in primary human cells (VH10). (B) Representation of the DNA constructs encoding fluorescently tagged DDB2 and CUL4A. (C) Western blot analysis of MRC5-SV40 cells expressing DDB2-EYFP. Whole cell extracts (10 μg) of non-UV-irradiated cells and UV-irradiated cells (2 and 4 hours after irradiation with 20 J/m^2), were probed with antibodies against DDB2, DDB1 and CUL4A.

was fused to EGFP and expressed in HeLa cells, where it was found predominantly in the nucleus (Fig. 1A, B). The cellular distribution of endogenous CUL4A and DDB2 was mainly nuclear (Fig. 1A), showing that the localization of the fluorescent fusion proteins reflects that of the endogenous proteins.

DDB2 and CUL4A have the same mobility in undamaged cells

Analysis of purified proteins from insect cells and whole cell lysates suggested that DDB2 exists in at least four different assembly states in vitro: monomeric DDB2, heterodimeric DDB1-DDB2 and as a protein complex containing either ROC1/CUL4A or ROC1/CUL4A with COP9 (Kulaksiz et al., 2005). However, DDB2 isolated from cells is co-purified with DDB1, CUL4A and all subunits of COP9 (Groisman et al., 2003). To investigate whether DDB2 is part of different protein complexes in vivo we employed fluorescence recovery after photobleaching (FRAP) (Houtsmuller and Vermeulen, 2001; Rabut and Ellenberg, 2005), in which a strip (2 μm width) spanning the nucleus was bleached and the fluorescence recovery in the strip was monitored (Fig. 2A).

Most DDB2-EYFP was mobile in non-UV-irradiated cells monitored at 37°C (Fig. 2B) and a similar mobility was measured at 27°C (Fig. 2C). Curves generated by Monte Carlo simulations (Farla et al., 2004) were fitted to experimental FRAP curves, yielding an effective diffusion constant (D_{eff}) for DDB2 in undamaged cells of 2.4±0.4 μm^2/second

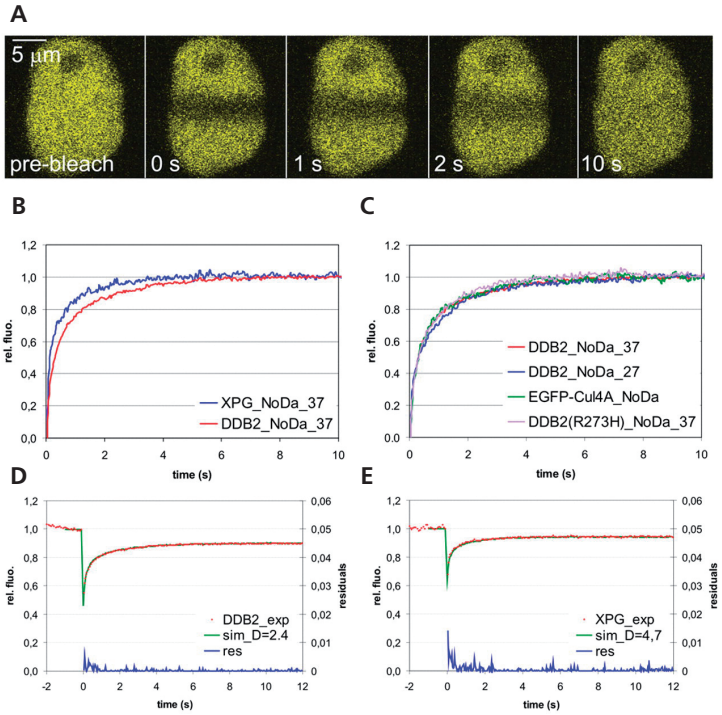

Fig. 2. FRAP analysis of DDB2 mobility in non-damaged cells (NoDa). (A) Example of a DDB2-EYFP cell in which a strip (2 µm) spanning the nucleus was bleached. (B) Recovery plots of DDB2-EYFP (n=14) and XPG-EGFP (n=23), normalized between 0 and post-bleach intensity after full equilibration. (C) Normalized recovery plots of DDB2-EYFP at 37°C and 27°C (n=12), DDB2(R273H)-EYFP (n=13) at 37°C and EGFP-CUL4A (n=11) at 37°C. (D) Recovery of DDB2-EYFP normalized to pre-bleach intensity (red line). Monte Carlo simulation assuming a diffusion constant of 2.4 µm²/second (green line) and the residuals (blue lines at the bottom) that are a measure for the quality of the simulation. (E) Recovery of XPG-EGFP normalized to pre-beach intensity (red line), Monte Carlo simulation assuming a diffusion constant of 4.7 µm²/second (green line) and the residuals (blue lines).

(Fig. 2D). To compare the mobility of DDB2 to that of other NER proteins we performed FRAP on cells expressing NER endonuclease XPG-EGFP (Zotter et al., 2006). The rationale to investigate XPG is that the molecular mass of this protein (180 kDa) (O'Donovan et al., 1994) is about the same as the molecular mass of heterodimeric DDB1-DDB2 (175 kDa). The recovery of XPG-EGFP (Fig. 2B, E), D_{eff}=4.7±1.0 µm²/second) was significantly faster than the recovery of DDB2-EYFP. In addition to DDB2, we have tagged the CUL4A protein with EGFP (Fig. 1B) and measured its mobility in non-UV-irradiated cells. The FRAP curves of EGFP-CUL4A and DDB2-EYFP at 37°C were similar, showing that both proteins move through the nucleus with the same mobility (Fig. 2C). To estimate the size of the protein complexes in which DDB2 resides, we compared the mobility of DDB2 and CUL4A with that of other GFP-tagged NER factors (TFIIH, ERCC1, XPG and XPA) and free EGFP (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003; Zotter et al., 2006). We assumed that the shape of the different NER factors is the same and that the

mobility of these proteins is determined predominantly by their molecular mass. Based on these assumptions, we estimate that the majority of DDB2 and CUL4A has a diffusion rate consistent with high molecular mass complexes of 500-700 kDa (Fig. S1). CUL4A was recently reported to be part of several protein complexes, varying in size between 300 and 700 kDa (He et al., 2006), which is in agreement with our estimate. Immunoprecipitation studies have demonstrated that the naturally occurring DDB2 protein with an R273H substitution in the WD40 motif is unable to interact with the E3 ubiquitin ligase core proteins DDB1 and CUL4A (Chen et al., 2001; Rapic-Otrin et al., 2003; Shiyanov et al., 1999a). In contrast to these studies, purified R273H mutant DDB2 forms a complex with DDB1 in vitro (Wittschieben et al., 2005). We generated a YFP-tagged DDB2 (R273H) protein, which was expressed in human HeLa cells. The mobility of DDB2 (R273H) and wild-type DDB2 was similar (Fig. 2C), suggesting that mutant DDB2 is part of the DDB-CUL4A-ROC complex in vivo. Although DDB2 can exist in different assembly states in vitro (Kulaksiz et al., 2005), our results indicate that the majority of DDB2 in vivo resides in high molecular mass complexes of at least 500 kDa. Such a molecular mass is consistent with the size of the DDB-CUL4A-ROC (280 kDa) complex with the COP9 signalosome (450 kDa).

DDB2 recruitment to lesions is independent of XPC

To investigate how DDB2 associates with damaged DNA in vivo, we analyzed its binding to UV-induced lesions in human fibroblasts that expressed DDB2-EYFP and that were subjected to local UV irradiation (100 J/m^2 through 5 µm pores) with UV-C light (Fig. 3A) (Moné et al., 2004). DDB2-EYFP accumulated at 37°C with a half-time ($t_{1/2}$) of 40 seconds (Fig. 3D), which is significantly faster than other GFP-tagged NER proteins tested so far. Similar results were obtained with DDB2 tagged with the red fluorescent protein mCherry (Shaner et al., 2004) (data not shown). To verify that the fluorescent tag does not affect the binding kinetics of DDB2 we measured accumulation of EYFP-DDB2 (i.e. N-terminally tagged DDB2; Fig. 1B), which displayed binding behavior similar to that of C-terminally tagged DDB2 (Fig. 3D). To monitor the binding of DDB2 and another NER protein simultaneously, we expressed DDB2-mCherry and XPG-EGFP in XPG-deficient rodent cells and measured the binding kinetics of both proteins upon local UV-C irradiation (Zotter et al., 2006). Results show that DDB2 binds considerably faster than XPG ($t_{1/2}$ of 40 seconds and 200 seconds, respectively) and that the binding kinetics of DDB2 in rodent and human cells are similar (data not shown). To investigate the kinetics of CUL4A recruitment to sites of DNA damage we transiently expressed EGFP-CUL4A in human HeLa cells (Fig. 3B) and exposed cells to local UV irradiation. Clear accumulation of GFP-CUL4A at UV-irradiated areas was detected. Since CUL4A alone does not bind to damaged DNA, this indicates that the GFP-CUL4A interacts with endogenous DDB2 (Li et al., 2006). The binding kinetics of EGFP-CUL4A ($t_{1/2}$ of 47 seconds) and DDB2-EYFP ($t_{1/2}$ of 40 seconds) were similar (Fig. 3D), suggesting that they reflect the assembly of the DDB-CUL4A-ROC1 protein complex onto UV-damaged DNA. In agreement, DDB1-mCherry also binds to UV-damaged DNA with similar kinetics (S. Alekseev and W. Vermeulen, unpublished). To investigate whether

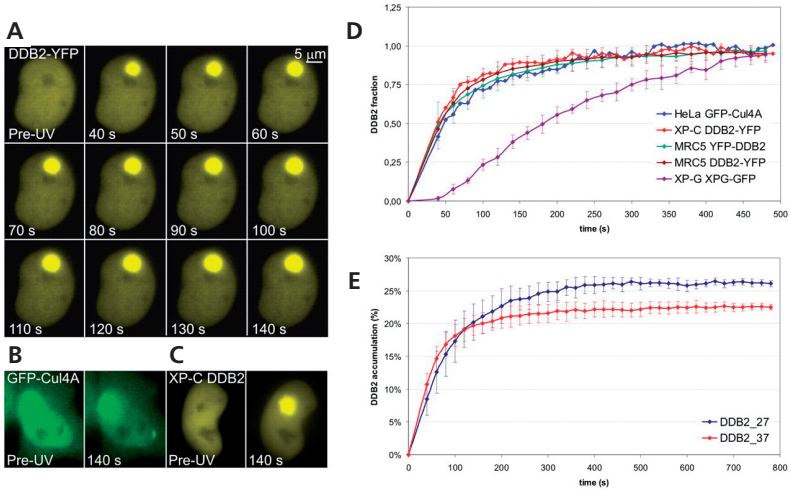

Fig. 3. Binding kinetics of DDB2 after local UV irradiation. (A) Example of a DDB2-EYFP cell that shows local accumulation after local UV-C irradiation with 100 J/m^2 through 5 µm pores at 37°C. (B) Example of a HeLa cell expressing EGFP-CUL4A and (C) an XP-C cell expressing DDB2-EYFP. Both fusion proteins accumulate after local UV-C irradiation through 5 µm pores at 37°C (B,C). (D) Quantification of accumulation kinetics of DDB2-EYFP in MRC5 cells (brown line, n=9), EYFP-DDB2 in MRC5 cells (green line, n=8), DDB2-EYFP in XP-C cells (red line, n=3) and EGFP-CUL4A in HeLa cells (blue line, n=3). Curves were normalized to the plateau value. Time point t=0 is defined as the start of UV irradiation. (E) Quantification of assembly kinetics of DDB2-EYFP at 37°C (red, n=9) and 27°C (blue, n=8). The local accumulation of DDB2-EYFP was measured and plotted as a percentage of the total EYFP fluorescence in the cell nucleus. The error bars represent the standard deviation.

the association of DDB2 with UV-damaged DNA involves a temperature-dependent step, for instance an enzymatic reaction, we analyzed its binding kinetics at 27°C. The initial slope of the assembly curves at 37°C and 27°C are similar (Fig. 3E), indicating that the association of DDB2 with damaged DNA is not temperature sensitive. Previous NER assembly approaches showed that exogenously expressed DDB2 binds to UV-induced DNA lesions in the absence of functional XPC (Fitch et al., 2003; Wakasugi et al., 2002). In this study, we show that endogenous DDB2 accumulates at sites of localized UV damage in cells lacking XPC, XPA, XPG and XPF (Fig. S2), indicating that the binding of DDB2 does not require any of these NER proteins. Although binding of DDB2 can be detected in several XP cell lines, it is possible that the kinetics of binding are different in NER-deficient cells. To investigate this, we expressed DDB2-EYFP in XP-C cells (XP20MA-SV40) (see Rademakers et al., 2003). Similar binding kinetics were measured in XP-C cells and wild-type cells (Fig. 3C, D). These findings show that DDB2 rapidly binds to damaged DNA independently of XPC.

Most DDB2 is bound to chromatin after global DNA damage induction

The distribution of DDB2-EYFP in non-UV-irradiated living fibroblasts is homogeneous although the protein is partially excluded from the nucleoli, possibly due to their very high

macromolecular concentration (Fig. 4A-C). To investigate if UV irradiation results in nuclear redistribution of DDB2 we globally irradiated cells (16 J/m^2), inducing a uniform distribution of damage throughout the nucleus. Confocal microscopy showed that the distribution of DDB2 changed upon UV irradiation, which is not observed for other NER factors except TFIIH (Volker et al., 2001). Irradiated cells show regions of dense and less dense DDB2 fluorescence (Fig. 4F-H), similar to the spatial distribution of chromatin (Kimura and Cook, 2001). In agreement, we observed co-localization between Cerulean-tagged histone H2A and DDB2-EYFP after global UV irradiation (Fig. 4H-J and line-scan Fig. 4L), which was not observed in non-UV-irradiated cells (Fig. 4C-E and line-scan Fig. 4K). This indicates that DDB2 binds to UV-damaged chromatin in interphase nuclei (Rapic-Otrin et al., 1997). Previous studies have shown that XPC readily associates with interphase and mitotic chromatin in the absence of DNA damage (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003) (van der Spek et al., 1996). In contrast to XPC, the distribution of DDB2 in metaphase cells was homogenous and no association with mitotic chromatin could be observed (Fig. 4M). Global irradiation (16 J/m^2) of cells in metaphase resulted in rapid association of DDB2 with the condensed mitotic chromosomes after UV exposure (Fig.

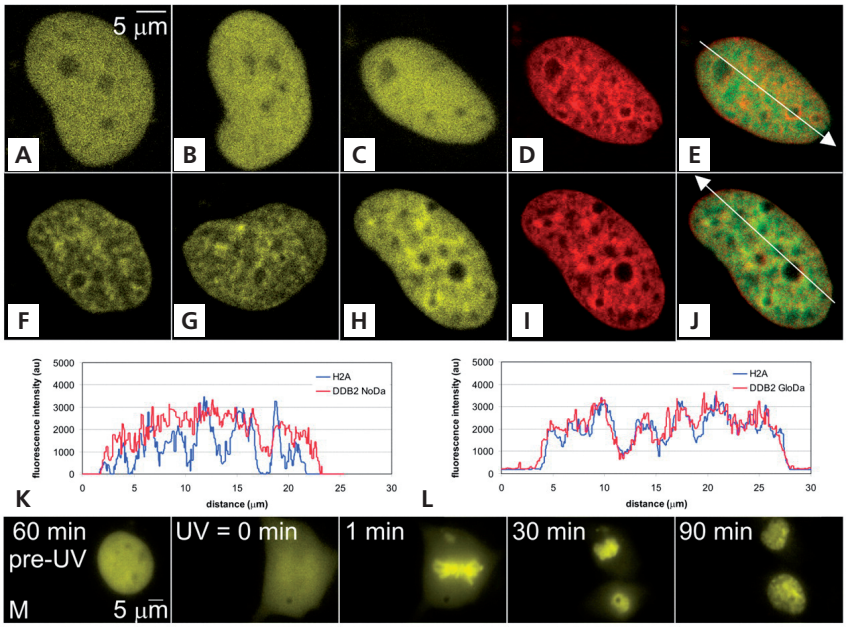

Fig. 4. Nuclear distribution of DDB2 after global UV exposure. (A,B) Representative confocal images of DDB2-EYFP cells (C-E) Example of a DDB2-EYFP cell (C) that was transfected with cerulean-histone H2A (D); the merged images of the two signals is shown in E. (K) Line scan along the arrow in E. (F,G) Representative confocal images of DDB2-EYFP cells that were exposed to global UV-C irradiation (16 J/m^2). (H-J) Globally UV-irradiated DDB2-EYFP expressing cell (H) that was transfected with cerulean-histone H2A (I); the merged images of the two signals is shown in J. (L) Line scan along the arrow in J. (M) Example of a cell expressing DDB2-EYFP, that was globally UV-C irradiated (16 J/m^2) in metaphase and monitored until cell division was completed.

4M). After mitosis, daughter cells displayed the characteristic chromatin localization as observed in irradiated interphase nuclei (Fig. 4M). Binding of GFP-tagged XPG to mitotic chromatin was not observed after global UV-C irradiation (data not shown). It is interesting to note that DDB2 is the only NER protein studied so far that displays this distribution pattern in interphase nuclei upon UV irradiation. Our findings show that DDB2 has the ability to bind to UV-damaged interphase and metaphase chromatin.

The amount of DDB2 that is proteolytically degraded is UV dose dependent

To investigate whether YFP-tagged DDB2 is degraded upon UV irradiation we analyzed whole cell extracts by western blotting using antibodies against DDB2 (Fig. 1C). Our analysis shows that DDB2-EYFP is degraded within 4 hours (Fig. 1C) and that expression of DDB2 is restored after 20 hours (data not shown). The amount of DDB1 and CUL4A remains unchanged after UV irradiation (Fig.1C). Next, we used live-cell imaging to measure the degradation kinetics of DDB2-EYFP in living UV-irradiated cells up to 6 hours. We observed that locally UV-irradiated cells (100 J/m^2) showed a significant decrease in total nuclear fluorescence (within 6 hours) in contrast to cells that were not hit by local UV irradiation (Fig. 5A). Cells that were globally UV irradiated (16 J/m^2) showed a complete loss of nuclear fluorescence with a $t_{1/2}$ of 2 hours (Fig. 5B-D). To unambiguously show that the decrease in nuclear fluorescence was due to proteolytic degradation of DDB2-EYFP we treated cells with proteasome inhibitor MG-132 and observed that DDB2-EYFP levels remained unchanged up to 6 hours after irradiation at 16 J/m^2 (Fig. 5C). Irradiation with UV doses of 8 and 4 J/m^2 resulted in degradation of 75% and 50% of the DDB2 pool, respectively (Fig. 5D). Non-UV-irradiated DDB2-EYFP cells (Fig. 5D) and globally irradiated HeLa cells transfected with EYFP-NLS (8 J/m^2) showed no change in nuclear fluorescence between 0 and 6 hours. Previous experiments showed that DDB2 is degraded in several NER mutant cell lines, including XP-C (Rapic-Otrin et al., 2002). To determine if the rate of degradation is affected by the ongoing NER process, we measured degradation of DDB2-EYFP in XP-C and XP-A cells. The initial breakdown of DDB2 was faster in both XP lines than in wild-type cells irradiated with the same dose of UV (Fig. 5D). The kinetics of degradation in repair-proficient cells resembles the repair kinetics of 6-4 PPs in the UV dose range employed (van Hoffen et al., 1995). We suspect that breakdown of DDB2-EYFP in cells irradiated with UV doses of 4 and 8 J/m^2 is not complete because a significant fraction of 6-4 PPs is removed before all DDB2 is degraded. For example, NER proficient cells remove 50% of their 6-4 PPs in the first hour after a dose of 8 J/m^2 (Moser et al., 2005). It is possible that the breakdown of DDB2 is faster in NER-deficient cells due to the persistence of DNA damage in the genome of these cells. It is unclear why DDB2 is not completely degraded in XP-C and XP-A cells (Fig. 5D). Although a single high dose of UV-C can result in almost complete break-down of DDB2, such a situation, and thus complete break-down of DDB2, is not likely to occur in cells of an organism that is exposed to solar UV irradiation. Together, our data indicate that the amount of DDB2 that is proteolytically degraded is UV-dose dependent and that the rate of DDB2 breakdown depends on ongoing NER.

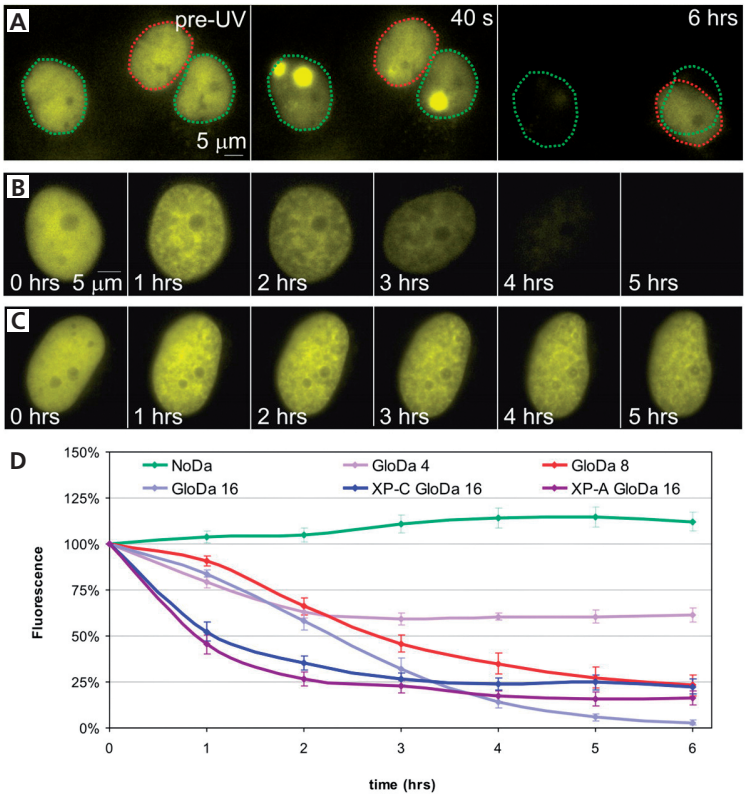

Fig. 5. UV-induced degradation of DDB2. (A) Two DDB2-YFP cells were locally damaged by UV irradiation (nuclei indicated by the green dotted outline), while a third cell was not irradiated (indicated by the red dotted outline). The same three cells are shown shortly after (40 seconds) and 6 hours after UV irradiation. (B) Example of a DDB2-EYFP cell that has been globally UV irradiated (16 J/m^2) and monitored for 5 hours. (C) Example of a globally UV irradiated (16 J/m^2) DDB2-EYFP cell that had been treated with 50 μM MG-132. (D) Quantification of the total nuclear fluorescence in the absence of UV-C irradiation (green line, NoDa, n=8) and after global UV irradiation (GloDa) of MRC5 human cells with 4 (light-purple line, n=10), 8 (red line, n=12) and 16 J/m^2 (light-blue line, n=10), and in XP-C (dark-blue line, n=15) and XP-A (dark-purple line, n=7) cells with 16 J/m^2. All values were corrected for background fluorescence and the initial total nuclear fluorescence intensity was set to 100%. The error bars represent the standard error of the mean.

All DDB2 can bind to lesions in UV-irradiated cells

To examine the properties of bound and unbound DDB2 upon UV irradiation, we applied strip-FRAP on globally irradiated cells (Fig. 6A). The FRAP curves showed incomplete recovery (1 hour after 16 J/m^2 at 37°C) indicative of a significant immobile fraction (Fig. 6C). We observed fast but incomplete recovery (within 2 seconds) of DDB2 in irradiated cells. This initial very fast recovery ($t_{1/2=}$0.1 seconds) is probably due to fluorescence blinking, given that the average lifetime of GFP in a dark non-emissive state is around 2 seconds (Garcia-Parajo et al., 2000). Blinking of GFP is the result of switching between an emissive (anionic) on-state and a non-emissive (neutral) off-state (Dickson et al., 1997).

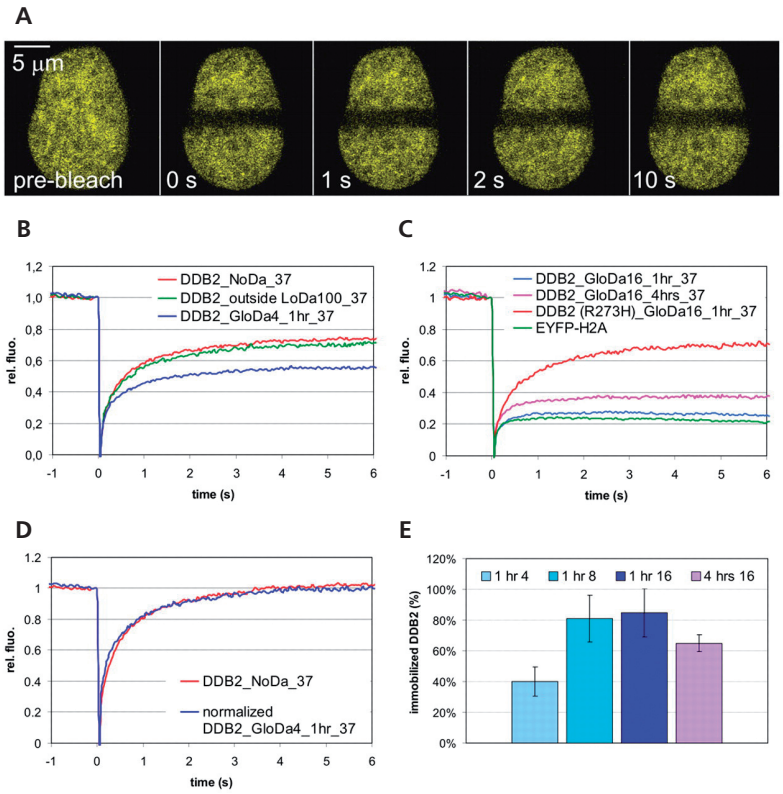

Fig. 6. FRAP analysis of DDB2 mobility in UV-irradiated cells. (A) Example of a globally UV-irradiated DDB2-EYFP cell in which a narrow strip (2 µm) across the nucleus was bleached. (B) Recovery kinetics of DDB2-EYFP in undamaged cells (red line, NoDa, n=14), in locally damaged cells (green line, outside LoDa, n=14) and in globally UV-irradiated cells (4 J/m^2; blue line, GloDa, n=15). The recovery plots are normalized to 0 and to the pre-bleach intensity. (C) The recovery plots of DDB2-EYFP in globally damaged cells 1 hour after (purple line, GloDa, n=16) and 4 hours after (blue line, GloDa, n=15) irradiation with 16 J/m^2. The recovery plots of mutant R273H DDB2 (red line, n=12) and EYFP-histone H2A (green line, n=15) 1 hour after irradiation (16 J/m^2) are also shown. The recovery plots are normalized to 0 and to the pre-bleach intensity. (D) Normalized recovery of DDB2-EYFP in cells irradiated with 4 J/m^2 (blue line, GloDa). The recovery plot is normalized to post-bleach intensity and compared to DDB2-EYFP in undamaged cells (red line, NoDa). (E) UV-immobilized fraction of DDB2-EYFP in MRC5 cells 1 hour after global irradiation with 4, 8 and 16 J/m^2 and 4 hours after 16 J/m^2. The immobilized fractions depicted in E were determined by Monte Carlo simulations. The bound fractions obtained from the ten best simulations that fitted the experimental data were averaged. The error bars represent the standard deviation.

The off-time is independent of excitation intensity whereas the on-time is shortened at high excitation intensities (such as the FRAP bleach pulse), which makes blinking a light-induced process (Garcia-Parajo et al., 2000). Comparable fast but incomplete recovery was measured with EYFP- and EGFP-tagged histone H2A (Fig. 6C and data not shown), which are immobile chromatin proteins on this time-scale (Phair and Misteli, 2000). In contrast to wild-type DDB2, the R273H mutant DDB2 protein was not immobilized after

UV-C irradiation (Fig. 6C). This confirms the inability of DDB2 R273H to bind to damaged DNA (Hwang et al., 1998) and demonstrates that the R273 residue is essential for binding to damaged DNA. Monte Carlo simulations of the experimental FRAP curves, which were corrected for blinking (Farla et al., 2004), yielded an immobile fraction of 85±15% for DDB2, measured 1 hour after UV irradiation at a dose of 8 or 16 J/m^2 (Fig. 6E). Such a UV-C dose produces 3.10^5 6-4 PPs and 1.10^6 CPDs (Perdiz et al., 2000), indicating that there is sufficient damage to bind all DDB2. Even 4 hours after UV irradiation (16 J/m^2) we still observed a significant immobilization of DDB2 (65±5%, %, Fig. 6C,E).

It is likely that this immobilization reflects binding to CPDs because the majority of 6-4PPs is removed within 4-6 hours (Moser et al., 2005; van Hoffen et al., 1995). For comparison, the fraction of immobilized XPC-EGFP decreases to background levels 2 hours after global irradiation at a UV dose of 8 J/m^2 (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003), indicating that NER complexes assembled on slowly repaired CPDs cannot be detected. There is significant degradation of DDB2 between 1 (15% degradation; Fig. 5D) and 4 hours after UV (85% degradation; Fig. 5D). Considering this degradation, we estimate that the number of DDB2 molecules bound to damaged DNA between 1 and 4 hours after UV decreases with a factor of 8. A lower UV dose (4 J/m^2) induced a less pronounced immobilization of DDB2 (40±9%; Fig. 6B, E). Unbound DDB2 in these UV-irradiated cells has the same mobility as DDB2 in unchallenged cells (Fig. 6D). Accordingly, the mobility of DDB2 outside local UV-induced DNA damages (100 J/m^2 through 5 µm pores) was similar to the mobility in undamaged cells (Fig. 6B), indicating that DDB2 molecules that are not bound to a lesion have the same mobility and thus a similar complex composition as DDB2 in non-damaged cells. These findings indicate that DDB2 binds to lesions in a UV-dose-dependent manner up to complete binding of all DDB2 molecules. Moreover, DDB2 that dissociates from lesions has a similar diffusion rate as DDB2 in undamaged cells, suggesting that the composition of the DDB2 complex that dissociates from lesions is similar to that in undamaged cells.

Release of DDB2 from lesions is XPC independent

To determine the dissociation kinetics of DDB2 from UV-damaged DNA, we applied fluorescence loss in photobleaching (FLIP). A region distant from the local UV-induced DNA damage was continuously bleached (Fig. 7A) and the decrease of fluorescence in the locally damaged area was measured (Fig. 7B). The half-time ($t_{1/2}$) of the FLIP curve is indicative for the k_{off} of DDB2 at sites of DNA damage. Directly after bleaching we observed a decrease of fluorescence intensity, which corresponded to the non-bound DDB2 pool. The overall decrease in the locally UV-irradiated area was much slower due to the binding of DDB2 to UV-induced damage. The $t_{1/2}$ of release of DDB2 at sites of UV damage (at 37°C) was 110 seconds (Fig. 7B). Surprisingly, the dissociation kinetics of DDB2 from UV-induced lesions was similar in cells lacking XPC (Fig. 7B), indicating that XPC has no influence on the release of DDB2. The residence time of DDB2 was significantly longer at 27°C ($t_{1/2}$ 220 seconds; Fig. 7B) compared with 37°C ($t_{1/2}$ 110 seconds), indicating that the dissociation of DDB2 from UV-damaged DNA is temperature sensitive.

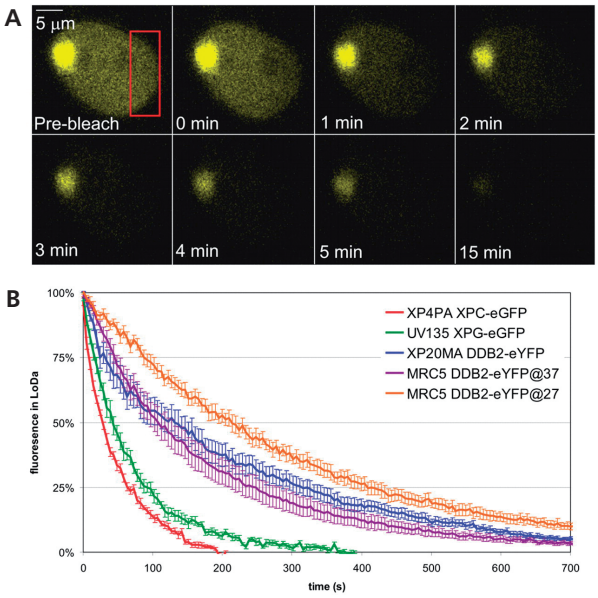

Fig. 7. Dissociation kinetics of DDB2. (A) Example of a DDB2-EYFP cell after local UV irradiation with 100 J/m^2. A strip of about one third of the cell nucleus opposite the local damage was continuously bleached and the fluorescence decrease in the local damage was monitored. (B) Quantification of the decrease in fluorescence signal in the damaged area of the nucleus. The half-time ($t_{1/2}$) of a FLIP curve corresponds to the residence time of a protein molecule in the locally damaged area. FLIP was performed on DDB2-EYFP in MRC5 human fibroblasts at 37°C (purple line, n=10) and at 27°C (orange line, n=10), DDB2-EYFP in XP20MA human XP-C fibroblasts (blue line, n=11), XPF-EGFP in UV135 Chinese hamster ovary XP-G cells (green line, n=8) and XPC-EGFP in XP4PA human XP-C fibroblasts (red line, n=9). The error bars represent the standard error of the mean.

To compare the release kinetics of DDB2 with that of other NER proteins, we have previously applied FLIP on cell lines expressing XPC-EGFP and XPG-EGFP (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003) (Zotter et al., 2006). Residence times of 28 and 45 seconds were measured for XPC-EGFP and XPG-EGFP, respectively ($t_{1/2}$ of the FLIP curves shown in Fig. 7B). Comparison of the residence time of DDB2 with the residence times of XPC-EGFP and XPG-EGFP shows that DDB2 resides significantly longer on UV-damaged DNA than XPC (about four times longer, since $t_{1/2}$ values are 110 seconds and 28 seconds, respectively) and about two times longer than the NER endonuclease XPG.

DISCUSSION

Mammalian nucleotide excision repair is initiated by the XPC complex, which has high affinity for UV-damaged DNA (Sugasawa et al., 1998; Volker et al., 2001). Besides XPC, mammalian cells express a second UV-damage-recognition protein with high affinity for UV-induced lesions: DDB2, which is part of an E3 ubiquitin (ub) ligase containing CUL4A,

ROC1 and DDB1 (Groisman et al., 2003). Currently, it is not clear how these two protein complexes cooperate in the DNA-damage-recognition step of mammalian NER in the living cell. To investigate the interplay between DDB2 and XPC in vivo we used quantitative live cell imaging of fluorescently tagged proteins.

DDB2 resides in a high molecular weight protein complex

We measured the mobility of EYFP-tagged DDB2 and of EGFP-tagged CUL4A in nuclei of living human fibroblasts (Fig. 2) and found that the mobility of both fusion proteins is similar and remarkably low. The apparent diffusion constants of DDB2 and CUL4A (2.5 μm^2/second) correspond to that of globular protein complexes with a molecular mass of between 500 and 700 kDa (see Fig. S1). This suggests that the majority of DDB2 resides in the DDB-CUL4A-ROC1 (280 kDa) E3 ub ligase complex, possibly associated with COP9 (450 kDa). The naturally occurring R273H mutant DDB2 protein displays a similar slow behavior as wild-type DDB2 (Fig. 2), suggesting that this protein is assembled into the DDB-CUL4A-ROC1 complex. In agreement with these results, DDB2 R273H was recently demonstrated to form a complex with DDB1 in vitro (Wittschieben et al., 2005). Although CUL4A and DDB2 have the same mobility in living cells, it should be noted that CUL4A is also part of a number of other protein complexes not containing DDB2 and therefore its mobility is not necessarily directly related to that of DDB2 (He et al., 2006). About 15 proteins containing WD40 repeats were shown to associate with DDB1-CUL4A (He et al., 2006). All of these DDB1-interacting proteins have a molecular mass of 50 kDa, comparable to DDB2. This might explain why the mobility of CUL4A is similar to the mobility of DDB2. Our results show that transiently transfected EGFP-CUL4A, which can be part of all of these DDB1-CUL4A complexes, accumulates at UV-damaged sites in significant quantities. CUL4A does not accumulate at UV-damaged sites unless associated with DDB2 (Li et al., 2006). Therefore, the accumulation of EGFP-CUL4A indicates that a significant fraction of CUL4A complexes contains DDB2. Recently, DDB2 was shown to bind to UV-damaged DNA after knock-down of DDB1 and CUL4A (El-Mahdy et al., 2006; Li et al., 2006). In the absence of DDB1, the proteins CUL4A and DDB2 do not interact (He et al., 2006), showing that DDB2 alone binds to UV-damaged DNA (Li et al., 2006). Our experiments suggest that the majority of DDB2 in living DDB1-containing cells is part of the DDB-CUL4A-ROC1 complex, rather than free DDB2. UV-induced DNA damage triggers the binding of DDB2 and CUL4A to UV-damaged chromatin (Figs 3, 4). Mutant DDB2 with a R273H substation does not become immobilized after UV-C irradiation (Fig. 6), demonstrating that the R273 residue is essential for binding to damaged DNA. DDB2 molecules that dissociate from lesions have the same mobility and thus complex composition as DDB2 in undamaged cells (Fig. 6). This suggests that DDB2 that is released from damaged DNA is bound to COP9, which is the negative regulator of the DDB2 E3 complex. Our findings are consistent with a model in which activation of the DDB2 E3 ub ligase complex (by dissociation of COP9) only occurs on damaged chromatin and in which the bulk of DDB2 that dissociates from lesions is inactivated by binding to COP9. In this way the ub ligase activity is specifically directed to chromatin at DNA damaged sites.

DDB2 binds to DNA lesions independently of XPC

To investigate if the dynamic behavior of DDB2 is dependent on functional NER, we measured the accumulation of endogenous and YFP-tagged DDB2 in NER-deficient cells. Binding of endogenous DDB2 to UV-damaged DNA can be detected in the absence of functional XPC, XPA, XPG or XPF protein (see Fig.). Moreover, the association kinetics of DDB2-EYFP with UV-damaged DNA is not influenced by XPC (Fig. 3). Up to 85% of the cellular DDB2 pool becomes immobilized after UV irradiation (Fig. 6), which is considerably higher than the percentage of the cellular XPC pool (25%) that is immobilized after a similar UV dose (W.V., unpublished data). The number of endogenous DDB2 molecules per cell is $1x10^5$ (Keeney et al., 1993), whereas there are $3x10^4$ XPC molecules per cell (Araujo et al., 2001). Given that the expression level and the damage-bound fraction of XPC is lower than that of DDB2 (Araujo et al., 2001; Keeney et al., 1993), we estimate that shortly after UV irradiation the number of damage-bound DDB2 molecules is 10 times higher than the number of damage-bound XPC molecules (85% of $1x10^5$ versus 25% of $3x10^4$ for DDB2 and XPC, respectively). This comparison is possible since the fluorescently tagged proteins are present in the same amounts as their endogenous counterpart (see Materials and Methods, and the Results section). Immunoprecipitation experiments have indicated that XPC and DDB2 directly interact even in the absence of UV-induced lesions (Sugasawa et al., 2005). It has been suggested that DDB2 recruits XPC to UV-induced lesions resulting in DDB2 displacement (Sugasawa et al., 2005). If each DDB2 protein would recruit an XPC molecule, resulting in the dissociation of DDB2 due to inactivation by ubiquitylation (Sugasawa et al., 2005), the kinetics of DDB2 dissociation should be XPC dependent. In vivo measurements reveal that DDB2 resides about four times longer on UV-damaged DNA than XPC and that the release of DDB2 from UV-induced lesions is not affected by XPC (Fig. 7). These findings, in combination with the difference in the number of damage-bound XPC and DDB2 molecules, suggest that DDB2 does not physically interact with XPC on UV-damaged DNA. It is possible that there is an interaction between the two recognition factors on some (possibly poorly recognized) lesions resulting in the DDB2-mediated ubiquitylation of XPC (Sugasawa et al., 2005), but our results indicate that the bulk of DDB2 binds to lesions independently of XPC.

DDB2 primes chromatin around lesions to be targeted by NER

Based on our findings we propose the following model for DDB2 function in the living cell. DDB2 is part of an inactive E3 ub ligase complex that contains DDB1, CUL4A and the COP9 signalosome (Groisman et al., 2003). Essentially all DDB2 binds to UV-damaged DNA at high UV doses independently of XPC. It is likely that the E3 ub ligase binds to damages as a holocomplex, since DDB2 and CUL4A have the same rate of binding to lesions. A DDB2 molecule that binds to DNA damage is not inactivated immediately, but can dissociate and rebind several times to UV-damaged DNA, since the residence time of DDB2 is 2 minutes, whereas the half-time of breakdown is two orders of magnitude larger (2 hours). This indicates that a DDB2 molecule can bind about 75 times to UV-damaged DNA before it is degraded. The binding-deficient DDB2 mutant K244E is resistant to degradation. It is has

been suggested that binding to damaged DNA is the trigger for DDB2 breakdown (Nichols et al., 1996; Rapic-Otrin et al., 2002). Our findings show that binding of DDB2 to UV-damaged DNA is not the primary trigger for its proteolytic degradation. Other factors are likely to be involved in the regulation of DDB2 breakdown, such as the checkpoint protein Claspin (Praetorius-Ibba et al., 2007). However, the exact trigger for DDB2 breakdown remains to be elucidated.

Our findings show that DDB2 interacts with UV-damaged DNA independently of XPC. It is attractive to speculate that DDB2 marks lesions to be targeted by NER. Several studies have shown that DDB2 interacts with chromatin remodeling complexes, such as p300 histone acetyltransferase (Datta et al., 2001; Rapic-Otrin et al., 2002). The DDB2 E3 ub ligase was recently shown to ubiquitylate histone H2A, H3 and H4 (Kapetanaki et al., 2006; Wang et al., 2006), linking DDB2 to chromatin remodeling. We propose a scenario in which the binding of DDB2 to a lesion and subsequent remodeling creates a local chromatin environment that facilitates the assembly of a NER complex. The ubiquitylation of histone H3 and H4 results in a weakened interaction with DNA (Wang et al., 2006). Thus, DDB2-dependent histone ubiquitylation could result in the dissociation of histones from the DNA. Evidence for this is that histone H3 was recently shown to be incorporated into newly repaired chromatin in a CAF-1-dependent manner (Polo et al., 2006). Alternatively, the DDB2-dependent ubiquitylation of histones might facilitate binding of XPC through the ubiquitin-associated (UBA) domains of RAD23B (Bertolaet et al., 2001; Kapetanaki et al., 2006). After the initial massive binding of DDB2 to UV-induced lesions, thereby marking these sites for NER, the effective DDB2 concentration is gradually lowered by its proteolytic degradation. Breakdown may be important because otherwise potential binding sites for XPC are occupied by DDB2. Evidence for this comes from in vitro studies using purified components, showing that NER is inhibited by the addition of UV-DDB in the absence of ubiquitylation factors (e.g. E1, E2 and ub itself). Inhibition is relieved when ubiquitylation factors are added (Sugasawa, 2006; Sugasawa et al., 2005). The importance of DDB2 degradation in vivo is illustrated by the compromised removal of CPDs upon interference with DDB2 proteolysis (El-Mahdy et al., 2006; Groisman et al., 2003; Wang et al., 2005a).

Together, our results give insight into the early steps of DNA damage detection. DDB2 diffuses in the undamaged nucleus as part of an E3 ubiquitin ligase. In UV-damaged cells most DDB2 binds to DNA lesions. We provide evidence that DDB2 functions independently of the damage-recognition protein XPC. Although DDB2 is not part of the pre-incision complex, it has a central role in NER, probably by preparing chromatin around DNA lesions for assembly of the pre-incision complex.

MATERIALS AND METHODS

Cell lines. The cell lines used in this study were human fibroblasts MRC5-SV expressing DDB2-EYFP and EYFP-DDB2, XPC-deficient XP20MA-SV expressing DDB2-EYFP, XPA-deficient XP12RO-SV, XPC-deficient XP4PA-SV expressing XPC-EGFP (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003), XPA-deficient XP2OS-SV expressing EGFP-XPA (Rademakers et al., 2003), XPG-deficient XPCS1RO-SV expressing XPG-EGFP (Zotter et al., 2006), XPB-deficient XPCS2BA-SV expressing

XPB-EGFP (Hoogstraten et al., 2002), HeLa cells, Chinese hamster ovary cells: UV135 expressing XPG-EGFP (Zotter et al., 2006) and 43-3B expressing ERCC1-GFP (Houtsmuller et al., 1999). The expression level of all GFP-tagged repair proteins is comparable to the level of endogenous proteins as shown by western blot analysis (D. Hoogstraten, PhD thesis, Erasmus University Rotterdam, 2003) (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003; Zotter et al., 2006). MRC5-SV cells were cultured in Ham's F12 medium supplemented with 400 µg/ml G418, XP20MA-SV cells were cultured in DMEM supplemented with 600 µg/ml G418 and HeLa cells were cultured in DMEM. The cell lines expressing GFP-tagged NER proteins were cultured in a 1:1 mixture of DMEM:Ham's F10 medium. All media contained glutamine (Gibco, Breda, The Netherlands) supplemented with antibiotics and 10% FCS and all cells were cultured at 37°C in an atmosphere of 5% CO_2.

DNA constructs. Murine DDB2 cDNA was ligated in-frame into pEYFP-N1 and pEYFP-C1 (Moser et al., 2005) (Clontech Laboratories, CA) and stably expressed in MRC5-SV cells and XP-C cells (XP20MA-SV). DDB2 was also ligated with EGFP and mCherry. CUL4A cDNA was ligated in frame with EGFP and transiently transfected into HeLa cells using Lipofectamine 2000 (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. DDB2-EYFP was transiently transfected in XP12RO-SV cells and monitored 5 days after transfection. PCR-based overlap extension (Ho et al., 1989) was used to generate a DDB2 mutant protein with an R273H substitution. Briefly, two products were amplified from the DDB2 gene using two different primer sets, each containing the same nucleotide substitution (details available on request). The first PCR product covers the first 837 bp of the DDB2 gene, whereas the second PCR product encompasses the last 512 bp of the DDB2 gene. Both products contain the region with the G818A mutation that results in an R273H amino acid substitution. In a subsequent `fusion' reaction, both overlapping products were added together and amplified using PCR. The generated product was subsequently inserted into pEYFP-N1 with *Eco*RI and *Bam*HI and transiently transfected into HeLa cells.

Quantification of DDB2-EYFP expression level. To quantify the expression level of DDB2-EYFP, the cellular fluorescence of living cells was compared to the fluorescence of purified EYFP protein from *E. coli.* The concentration of purified EYFP protein was determined by the extinction coefficient of 72,000 $M^{-1}.cm^{-1}$ (Kremers et al., 2006). Confocal slices of EYFP solutions (1 and 4 µM) and living cells (n=44) were quantified using Metamorph software (Molecular Devices Corporation, Sunnyvale, CA).

Immunoblot analysis. Western blot analysis was performed as described previously (Fousteri et al., 2006). DDB2 was detected with mouse polyclonal anti-DDB2 antibodies (1:2000, a gift from A. A. Wani, The Ohio State University, OH), DDB1 was detected with goat polyclonal anti-DDB1 antibodies (1:600, Abcam, Cambridge, MA) and CUL4A was detected with rabbit polyclonal CUL4A antibodies (1:300, Santa Cruz, CA). Protein bands were visualized via chemiluminescence (ECL-Plus, Amersham Biosciences, Uppsala, Sweden) using horseradish peroxidase (HP)-conjugated secondary antibodies and exposure to ECL-Hyperfilms (Amersham Biosciences, Uppsala, Sweden).

Immunofluorescence. Immunolabeling was carried out as described previously (Moser et al., 2005). Primary antibodies used were: mouse monoclonal antibody against XPB (a gift from J. M. Egly, IGMC, Illkirch, France), rabbit polyclonal antibody against DDB2 (Acris, Hiddenhausen, Germany), rabbit polyclonal against CUL4A (1:100, Santa Cruz, CA) and goat polyclonal against DDB1 (1:300, Abcam, Cambridge, MA). Secondary antibody used were: Alexa Fluor 488-conjugated goat anti-mouse antiserum (Molecular probes/Invitrogen, CA) and Cy3-conjugated goat anti-rabbit antiserum (Jackson ImmunoResearch Laboratories, West Grove, PA). All antibodies were diluted in PBS containing 0.15% glycine and 0.5% bovine serum albumin.

Microscopic analysis. Assembly and degradation kinetics were measured on a Zeiss Axiovert 200M wide field fluorescence microscope, equipped with a 100x Plan-Apochromat (1.4 NA) oil immersion lens (Zeiss, Oberkochen, Germany) and a Cairn Xenon Arc lamp with monochromator (Cairn research, Kent, UK). Images were recorded with a cooled CCD camera (Coolsnap HQ, Roper Scientific). FRAP and FLIP experiments were performed on a Zeiss LSM 510 confocal microscope, equipped with a 63x Plan-A (1.4 NA) oil immersion lens (Zeiss, Oberkochen, Germany) and a 60 mW argon laser (488 and 514 nm). Both microscopes were equipped with an objective heater and cells were examined in microscopy medium (137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM D-glucose and 20 mM Hepes) at 37°C or 27°C.

UV irradiation. For all experiments, cells were irradiated with a UV source containing four UV lamps (Philips TUV 9W PL-S) above the microscope stage. The UV dose rate was measured to be 3 W/m^2 at

254 nm. For induction of global UV damage, cells were rinsed with microscopy medium and irradiated for 1.5 seconds (4 J/m^2), 3 seconds (8 J/m^2), 6 seconds (16 J/m^2), or 12 seconds (32 J/m^2). For induction of local UV damage, cells were UV irradiated through a polycarbonate mask (Millipore Billerica, MA) with pores of 5 µm (Moné et al., 2001) and subsequently irradiated for 39 seconds (100 J/m^2).

Fluorescence recovery after photobleaching (FRAP). FRAP analysis was used to measure the mobility of DDB2-EYFP and EGFP-CUL4A as described by Houtsmuller and co-workers (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Rademakers et al., 2003; Zotter et al., 2006). The data were normalized to pre-bleach intensity or to post-bleach intensity after full equilibration using the second data point for normalization to 0. Normalization to post-bleach intensity was used to compare the shape of different FRAP curves.

Fluorescence loss in photobleaching (FLIP). FLIP analysis was performed by continuously bleaching a third of a locally UV-irradiated nucleus as previously described (Essers et al., 2002; Hoogstraten et al., 2002). Fluorescence in the locally damaged area was monitored with low laser intensity. All values were background corrected.

Monte Carlo simulations. For analysis of FRAP data, experimental FRAP curves were normalized to pre-bleach values and corrected for monitor bleaching. FRAP curves were generated by Monte Carlo simulations in which three parameters were varied: diffusion constant (ranging from 0.04 to 36 µm^2/second), immobile fraction (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 99.9%) and time spent in an immobile state (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, seconds) (Farla et al., 2004; Zotter et al., 2006). Fluoresce blinking was also simulated based on FRAP measurements on cells expressing EGFP-H2A and EYFP-H2A (Rabut and Ellenberg, 2005). Simulated curves that fitted the experimental curves were selected based on the method of least squares. The reported error is the standard deviation calculated from ten independent simulations. Monte Carlo simulations were based on a model of random diffusion in an ellipsoid volume and simple binding kinetics representing binding to immobile elements. Diffusion was simulated by deriving novel positions M(x+dx, y+dy, z+dz) for all mobile molecules M(x, y, z) after each time step, where dx=G(r_1), dy=G(r_2) and dz=G(r_3), r_i is a random number (0 r_i 1) chosen from a uniform distribution, and G(r_i) is an inversed cumulative Gaussian distribution with µ=0 and ^{2}=6Dt, where D is the diffusion coefficient and t is time measured in unit time steps. Immobilization was based on simple binding kinetics described by: $k_{on}/k_{off}=F_{imm}/(1-F_{imm})$, where F_{imm} is the relative number of immobile molecules. The chance for each particle to become immobilized was defined as $P_{immobilise}=k_{on}=k_{off}$. $F_{imm}/(1-F_{imm})$, where $k_{off}=1/t_{imm}$, and t_{imm} is the average time a molecule is immobile, measured in unit time steps. The chance for each particle to be released from an immobile state was defined as: $P_{mobilise}=k_{off}=1/t_{imm}$. The FRAP procedure was simulated on the basis of an experimentally derived three-dimensional laser intensity profile providing a chance for each molecule to get bleached during simulation of the bleach pulse based on the three-dimensional position of the molecule.

Assembly kinetics. Cells were grown in glass bottom dishes (MatTek, Ashland, MA) and locally UV-irradiated as described by van Driel and co-workers (Moné et al., 2004; Zotter et al., 2006). Accumulation after local irradiation was quantified with Object-Image software. Time courses were normalized with respect to the plateau level. Start of the UV irradiation was defined as t=0.

Degradation kinetics. To measure the degradation of DDB2-EYFP, cells were globally irradiated with 4, 8, 16 and 32 J/m^2. For the treatment of cells with proteasome inhibitors, MG-132 (Sigma, Zwijndrecht, The Netherlands) was dissolved in DMSO at 10 mM. Three hours prior to UV irradiation, medium was replaced with microscopy medium containing 50 µM MG132 (Pajonk et al., 2005). Cells were monitored for 6 hours and the fluorescence intensities at every time point were quantified.

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SUPPLEMENTARY FIGURES

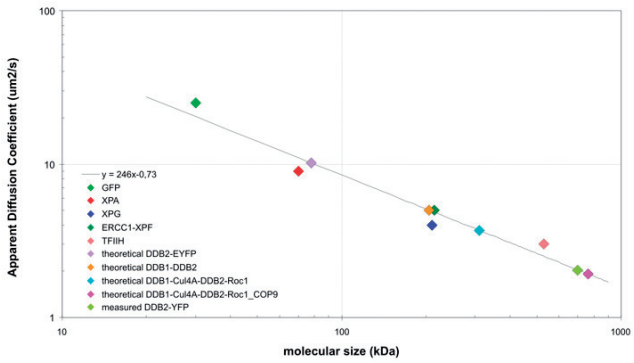

Fig. S1. Relationship between the apparent diffusion constant and the molecular mass of a protein. The mobility of EGFP alone (30 kDa) and of the EGFP-tagged NER proteins XPA (70 kDa), XPG (210 kDa), ERCC1-XPF (215 kDa) and TFIIH (500 kDa) was determined by FRAP and subsequent Monte Carlo simulations, yielding apparent diffusion constants for all fusion proteins and EGFP alone. The data points were fitted with the exponential equation $y=246x^{-0,73}$, where x is the molecular mass in kDa and y the apparent diffusion constant in μm^2/second. Using this equation, diffusion constants were derived for monomeric DDB2 (78 kDa), dimeric UV-DDB (205 kDa), multi-protein complex DDB-CUL4A-ROC1 (310 kDa) and DDB-CUL4A-ROC1 associated with COP9 (760 kDa). The calculated diffusion constants are plotted in the same graph as the measured diffusion constant for DDB2-EYFP that fits well to the expected diffusion constant of DDB-CUL4A-ROC1 associated with COP9. All values include an additional 30 kDa because of the presence of yellow or green fluorescent protein.

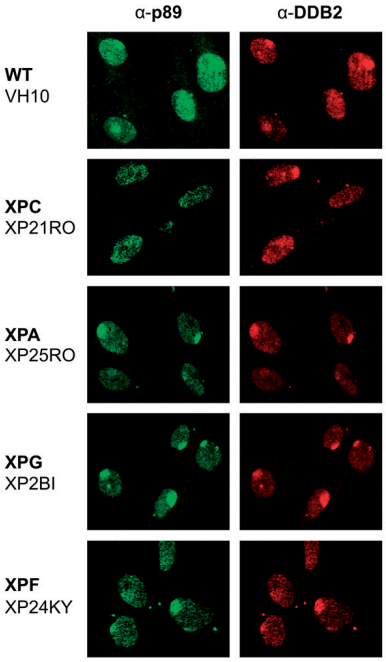

Fig. S2. Accumulation of endogenous DDB2 and TFIIH (p89 subunit) after local UV damage (30 J/m^2) in wild-type human cells (VH10) and several NER mutant cell-lines (XP-C, XP-A, XP-G and XP-F). The left column show the immunofluorescent labeling with anti-XPB antibody (green) and the right column shows labeling with anti-DDB2 antibody (red).

6

7

SEALING OF CHROMOSOMAL DNA NICKS DURING NUCLEOTIDE EXCISION REPAIR REQUIRES XRCC1 AND DNA LIGASE IIIα IN A CELL-CYCLE-SPECIFIC MANNER

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SUMMARY

Impaired gap filling and sealing of chromosomal DNA in nucleotide excision repair (NER) leads to genome instability. XRCC1-DNA ligase IIIα (XRCC1-Lig3) plays a central role in the repair of DNA single-strand breaks but has never been implicated in NER. Here we show that XRCC1-Lig3 is indispensable for ligation of NER-induced breaks and repair of UV lesions in quiescent cells. Furthermore, our results demonstrate that two distinct complexes differentially carry out gap filling in NER. XRCC1-Lig3 and DNA polymerase δ colocalize and interact with NER components in a UV- and incision-dependent manner throughout the cell cycle. In contrast, DNA ligase I and DNA polymerase are recruited to UV-damage sites only in proliferating cells. This study reveals an unexpected and key role for XRCC1-Lig3 in maintenance of genomic integrity by NER in both dividing and non-dividing cells and provides evidence for cell-cycle regulation of NER-mediated repair synthesis in vivo.

INTRODUCTION

Nucleotide excision repair (NER) is a multistep process that removes a wide variety of helix-distorting DNA lesions by a dual incision mechanism. Global genome repair (GGR) is the NER subpathway that deals with genome-wide repair of lesions. Defects in genes encoding for GGR proteins give rise to the UV-sensitive and cancer-prone genetic disorder xeroderma pigmentosum (XP) (Friedberg, 1996). GGR is subdivided into pre- and postincision stages. The preincision stage has been studied extensively in vivo and in vitro and comprises the step of DNA damage recognition, local unwinding of the DNA and verification of the lesion, and the generation of 3′and 5′ incisions flanking the DNA lesion (reviewed in Gillet and Scharer 2006). The postincision stage of NER involves gap filling by DNA repair synthesis, ligation, and restoration of chromatin structure; however, its regulation and the factors involved in vivo are much less understood. Reconstituted mammalian NER systems demonstrated that DNA repair synthesis could be executed by both replicative DNA polymerases δ and (polδ and pol) in the presence of RPA, RFC, and PCNA. In these studies, DNA ligase I (Lig1) was able to seal the nick in the DNA preferably in combination with pol (Aboussekhra et al., 1995 and Shivji et al., 1995).

Lig1 has an essential role in DNA replication and has been implicated in long-patch base excision repair (BER) and NER (reviewed in Tomkinson and Levin 1997 and Martin and MacNeil 2002). The involvement of Lig1 in NER in vivo was primarily based on the UV sensitivity of cells derived from a patient deficient in Lig1 (46BR Teo et al., 1983). 46BR cells contain normal levels of Lig1 protein but display only 3%-5% DNA joining activity due to mutations in the catalytic domain of the protein (Barnes et al., 1992). Biochemical studies showed that more strand breaks persisted in UV-irradiated 46BR than in normal cells (Nocentini, 1995). However, additional studies with 46BR cells did not show enhanced UV sensitivity (Bentley et al., 2002); moreover, wild-type and *Lig1* mutant mouse fibroblasts displayed no significant differences in sensitivity to UV (Bentley et al., 2002). In addition to Lig1, two other mammalian DNA ligases (III and IV) have been described that target to different repair and replication pathways (Martin and MacNeill, 2002). DNA ligase IV (Lig4) and its partner protein, XRCC4, are involved in repair of DNA double-strand breaks (DSBs) by nonhomologous end joining (NHEJ) and V(D)J recombination (Grawunder et al., 1998). DNA ligase IIIα (in the following referred to as Lig3) and its partner protein, XRCC1, are key components of both single-strand break repair (SSBR) and BER (Caldecott, 2003 and Brem and Hall, 2005). XRCC1 and Lig3 interaction is mediated by their C termini, both of which encode for a BRCT domain (Nash et al., 1997 and Taylor et al., 1998).

Herein we describe the identification of two unanticipated factors required for the ligation step in NER. We show for the first time, to our knowledge, that XRCC1 and Lig3 are essential core components of mammalian NER. Our data demonstrate that downregulation of Lig3 impairs removal of UV lesions and rejoining of UV-induced nicks in chromosomal DNA. Moreover, XRCC1-Lig3 together with polδ interacts and colocalizes with NER components in a UV-specific manner throughout interphase. On the other hand, recruitment of Lig1 and pol to UV-irradiated sites is only observed in proliferating cells, suggesting a differential requirement of DNA ligases and polymerases in NER-mediated

repair synthesis during the cell cycle. Based on the above, we discuss the role of the newly identified components in NER and the likely mechanisms by which NER is regulated in mammalian cells.

RESULTS

Lig1-Deficient Cells Exhibit Efficient Repair of UV Photolesions

To study the regulation of the postincision mechanism of NER in vivo, we initially focused on the ligation of the NER-mediated repair patches. Evidence for a functional role of Lig1 in mammalian NER has been gained from in vitro studies (Aboussekhra et al., 1995 and Araujo et al., 2000) and the UV sensitivity of the Lig1 mutant cell line 46BR (Teo et al., 1983). To delineate the role of Lig1 in vivo, we examined the repair of 6-4PP in UV-exposed confluent primary normal human (VH25) and Lig1-deficient fibroblasts (46BR). As controls, we examined Lig4-deficient (SC2) and NER-deficient XP-A cells. Additionally, we employed UV-irradiated confluent normal human cells treated with hydroxyurea (HU) and cytosine-β-arabinofuranoside (AraC) to inhibit repair-patch synthesis and ligation (Mullenders et al., 1985 and Smith and Okumoto, 1984). Immunofluorescence measurements demonstrated equally efficient levels of repair of 6-4PP in normal human, Lig1-, and Lig4-deficient cells (Figure 1A). In contrast, XP-A and normal cells treated with HU/AraC showed no repair of 6-4PP up to 16 hr after UV irradiation (Figure 1A). We also checked the assembly/disassembly of the NER incision complex in the various cell lines by using local UV irradiation and immunofluorescence (Volker et al., 2001). Normal cells treated with HU/AraC (Figure 1B) displayed retarded disassembly of the NER preincision complex (monitored by XPA or a TFIIH-specific antibody, p89); in contrast, the kinetics of disassembly of the NER preincision complex in confluent Lig1 and two distinct Lig4-deficient cell lines (SC2 and 411BR cells) were similar to normal cells.

We assessed the effect of Lig1 deficiency on repair-patch ligation by using the alkaline comet assay (Figure 1C). We employed normal (VH10/SV) and Lig1-deficient (46BR/SV) SV-40 immortalized cells to allow useful comparison with the siRNA-transfected SV-40 cell lines (Figure 5). In the absence of UV irradiation, the comet tail size of normal cells treated or mock treated with HU/AraC did not differ significantly from the tail of Lig1-deficient cells, suggesting that the inhibitors alone or the absence of Lig1 activity had only marginal effects on the frequency of endogenously induced breaks. In all cells, UV irradiation induced an increase in tail size 30 min after UV irradiation when the majority of lesions are still not repaired. In normal cells treated with HU/AraC, the UV-induced breaks remained open at 4 hr after UV irradiation, whereas normal and Lig1-deficient cells showed efficient sealing of breaks to a level indistinguishable from nonirradiated controls (Figure 1C). Taken together, the above data suggest that Lig1 activity is not essential for the repair of UV photolesions in mammalian cells or sealing the nicks generated during NER.

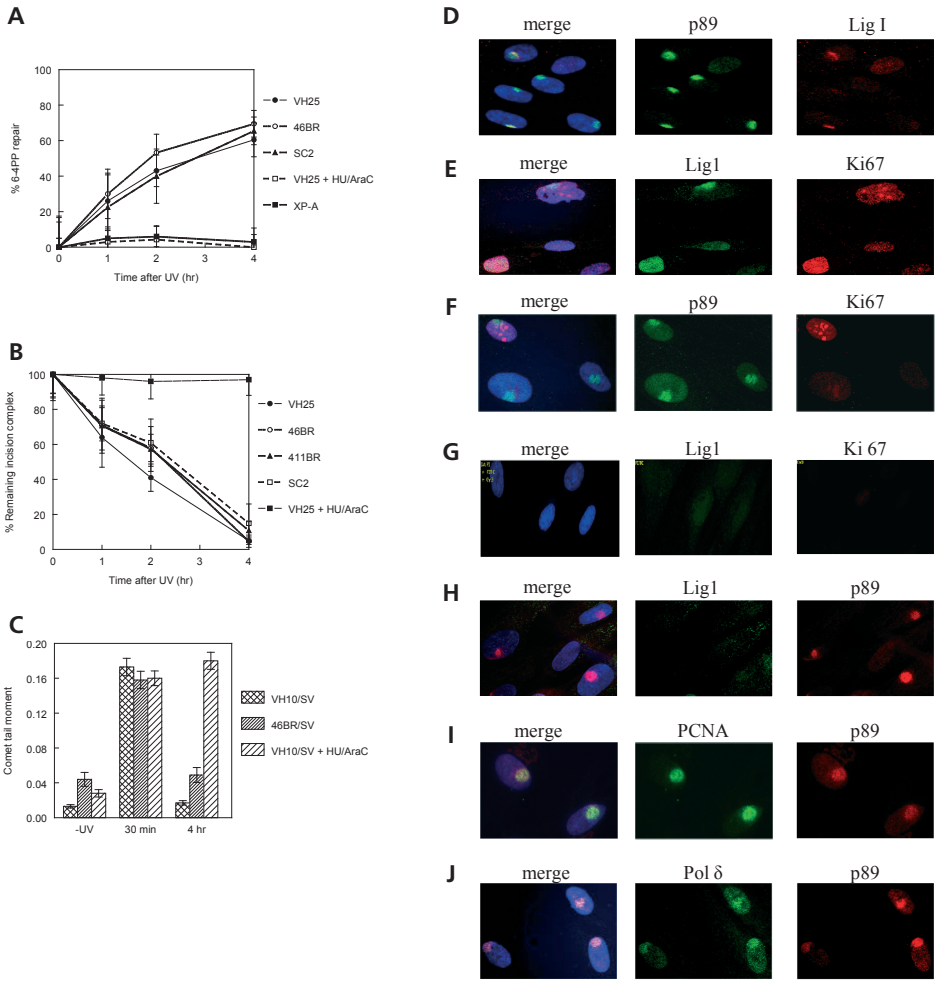

Figure 1. DNA Ligase I-Deficient Cells Exhibit Normal Repair of UV-Induced Lesions. (A) 6-4PP repair as determined by fluorescent immunostaining of 6-4PP at various times after global UV irradiation. Removal (%) of 6-4PP in primary confluent fibroblasts: VH25 (normal), 46BR (Lig1 deficient), and 411BR (Lig4 deficient). Error bars represent the SEM values of 40 nuclei. (B) Disassembly kinetics of the NER preincision complex. Disassembly was monitored by fluorescent immunostaining of p89 spots at various time points after local UV irradiation in primary confluent fibroblasts. Error bars represent the SEM values of 40 nuclei.(C) Strand-break repair of globally UV-irradiated (15 J/m^2) normal and Lig1-deficient cells analyzed by the alkaline comet assay. Breaks were quantified as comet tail moments. Histograms are means ± SEM of at least three independent experiments. Images (D)–(J) were obtained with locally UV-irradiated (30 J/m^2) primary normal cells. "Merge" refers to the combined image of DAPI and the pair of antibodies used for each panel.(D) Immunolocalization of Lig1 (2B1 antibody) in confluent cells. UV spots are visualized by local recruitment of TFIIH (p89). (E) Immunolocalization of Lig1 (5H5 antibody) in proliferating cells as visualized by Ki67 staining. (F) Immunolocalization of p89 in confluent cells. A proliferating cell is visualized by Ki67 staining. (G) Immunolocalization of Lig1 in quiescent cells as visualized by the absence of Ki67 marker.(H) Immunolocalization of Lig1 in quiescent cells. UV spots are visualized by recruitment of p89. (I) Immunolocalization of PCNA and p89 in quiescent cells.(J) Immunolocalization of DNA polδ and p89 in quiescent cells.

Lig1 Colocalizes to Sites of UV-Induced Damage in a Subset of Cells

The efficient repair of UV photolesions in Lig1-deficient human cells and the observation that UV-irradiated wild-type and Lig1 mutant mouse cells display equal levels of clonal survival (Bentley et al., 2002) prompted us to study Lig1 accumulation to UV damage in vivo. We found recruitment of Lig1 to UV spots but surprisingly not in all cells harboring damage: only about 30%-50% of the damaged cells showed Lig1 accumulation (Figure 1D), coinciding with cells that expressed the Ki67 antigen, a marker for proliferation (Figure 1E). The results were identical with various fixation procedures and two different Lig1-specific antibodies, 2B1 and 5H5, recognizing both the unmodified and phosphorylated forms of Lig1 and only the phosphorylated form of Lig1, respectively (Rossi et al., 1999 and Vitolo et al., 2005).

Lig1 is downregulated in serum-starved (Ki67-negative) human fibroblasts (quiescent cells, G0) when compared to growing human fibroblasts that expressed Ki67 (Vitolo et al., 2005). Local UV irradiation of quiescent cells failed to induce accumulation of Lig1 at sites of UV damage (Figures 1G and 1H). In contrast, preincision NER factors such as p89 (TFIIH, Figure 1F), and the postincision NER components PCNA and polδ (also downregulated in quiescent cells) were clearly detectable under these conditions (Figures 1I and 1J). These results show that Lig1 accumulates at UV damage only in proliferating cells.

XRCC4 and Lig4 are not involved in the repair of UV-induced lesions

We speculated that either Lig3 or Lig4, or both, might perform the ligation step of NER in quiescent cells. XRCC4 functions to stabilize and target Lig4 to sites of DNA double strand breaks (Bryanset al., 1999; Grawunder et al., 1998; Modesti et al., 1999). We assessed the recruitment ofXRCC4/Lig4 to locally induced UV-damage in confluent normal human and Lig1 deficient fibroblasts at different time intervals i.e. 20 min or 2 h post UV irradiation using an XRCC4 specific antibody. XRCC4 displayed distinct nuclear staining in both cell lines tested, yet UV-irradiated normal and Lig1 deficient human fibroblasts lacked accumulation of XRCC4 at sites of UV damage (Figure S1). This result is consistent with normal kinetics of 6-4PP repair and assembly/disassembly of the NER-incision complex in Lig4 deficient human cells (Figure 1A, 1B) and provides strong evidence that the XRCC4/Lig4 complex is not involved in the repair of UV-induced lesions. In addition, we noted that the two Lig4 deficient cell lines tested in this study, i.e. SC2 and 411BR, exhibited levels of clonal survival after UV irradiation similar to that of normal cells (W.W. Wiegant and M.Z. Zdzienicka, personal communication).

NER-Dependent Recruitment of XRCC1 and Lig3 to UV-Induced Damage

Lig3 and its partner, XRCC1, play key roles in BER and SSBR (Caldecott, 2003), but to our knowledge, XRCC1-Lig3 has not been previously implicated in NER. Interestingly, immunostaining of XRCC1 and Lig3 in locally UV-irradiated confluent normal cells revealed localization of both proteins at UV spots 20 min after UV irradiation (Figures 2A and 2B). In fact, both XRCC1 and Lig3 accumulated at UV spots as quickly as 5 min after UV irradiation but were also visible 2 and 4 hr after UV treatment (similar to the other

postincision factors, data not shown). In contrast, accumulation of neither XRCC1 nor Lig3 was detected in UV-irradiated confluent XP-A and XP-F cells (Figures 2A and 2B), indicating that recruitment of XRCC1 and Lig3 requires preincision complex assembly. The intensities of XRCC1 and Lig3 at UV spots were 1.7-fold increased in Lig1-deficient cells when compared to normal and Lig4-deficient cells (Figure 2C). Western blot analysis confirmed the enhanced level of Lig3 in Lig1-deficient cells (Figure 5A). Together these data suggest that XRCC1 and Lig3 are recruited to UV damage in normal human cells in an incision-dependent manner. This event is particularly marked in Lig1-deficient cells, suggesting that Lig3 might compensate for Lig1 deficiency. XRCC1 and Lig3 accumulated at UV damage and colocalized with p89 in both cycling and quiescent cells (Figures 2D and

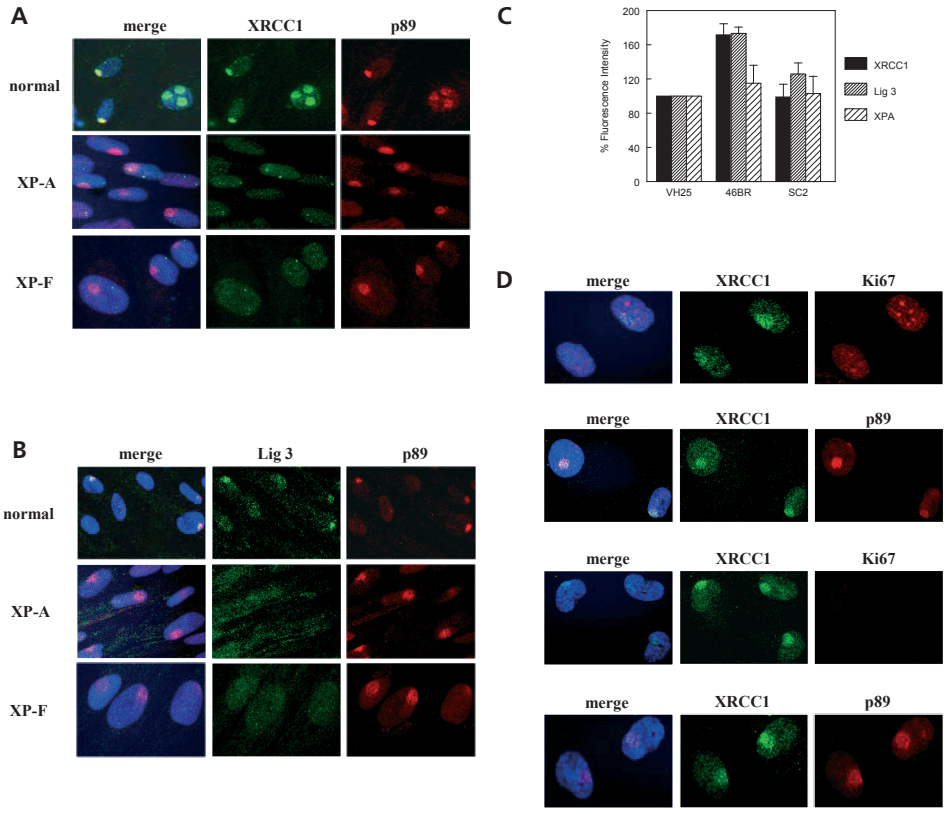

Figure 2. XRCC1 and Lig3 Recruitment to UV-Induced DNA Damage Is Dependent on Functional NER. (A and B) Immunolocalization of XRCC1 (A) and Lig3 (B) in locally UV-irradiated (30 J/m^2) confluent normal, XP-A, and XP-F human fibroblasts as indicated. UV-damage sites are visualized by local accumulation of p89. (C) Relative fluorescence intensity of XRCC1, Lig3, and XPA spots 20 min after local UV irradiation in VH25 (normal), 46BR (Lig1-deficient), and SC2 (Lig4-deficient) cells. Images were taken with equal exposure time. Error bars represent the SEM values of 40 nuclei. (D) Immunolocalization of XRCC1 and p89 in locally UV-irradiated (30 J/m^2) cycling cells as visualized by Ki67 staining. (E) Immunolocalization of XRCC1 and p89 in locally UV-irradiated (30 J/m^2) quiescent cells as visualized by negative Ki67 staining.

2E). These results point to an XRCC1-Lig3-dependent ligation mechanism that functions in mammalian NER in response to UV damage independent of cell cycle. In contrast, a role for Lig1 in NER-mediated ligation appears to be restricted to Ki67-positive S- or late G1/S phase cells (Figures 1D, 1G, and 1H).

Despite the fact that XRCC1 has no known catalytic activity itself, it does interact with several other DNA repair proteins required for BER and SSBR, including poly (ADP-ribose) polymerase 1 (PARP-1) and DNA polymerase β (polβ) (Caldecott, 2003). PARP-1, an enzyme synthesizing poly (ADP-ribose) (PAR) from NAD^+, is essential to recruit XRCC1 and Lig3 at sites of single-strand breaks (SSBs) (Okano et al., 2003) and oxidative damage (El-Khamisy et al., 2003). To disclose a role of PARP-1 in NER and in the recruitment of XRCC1-Lig3 to

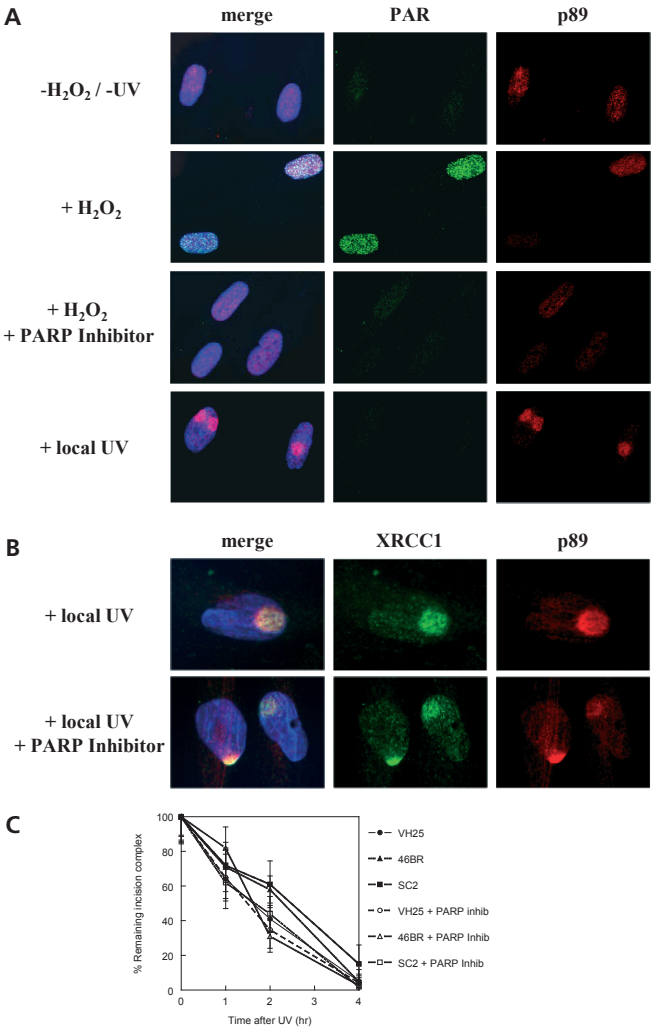

Figure 3. PARP-1-Independent Recruitment of XRCC1-Lig3 to UV Damage Sites. (A) PAR and p89 immunolocalization in normal human cells. Cells were mock treated or treated with 100 μM H_2O_2, or pretreated for 30 min with 10 μM PARP inhibitor (KU0058948) before H_2O_2 treatment. In addition, locally UV-irradiated cells (30 J/m^2) were stained for PAR and p89. (B) Immunolocalization of XRCC1 and p89 after local UV irradiation (30 J/m^2) in confluent mock-treated cells or cells pretreated with KU0058948. (C) Disassembly kinetics of the NER preincision complex from UV spots in the presence of KU0058948. Disassembly was monitored by fluorescent immunostaining of p89 spots at various time points after local UV irradiation (30 J/m^2) in primary human fibroblasts as indicated.

UV damage, we performed immunostaining of PAR in primary quiescent normal human fibroblasts exposed either to 100 μM hydrogen peroxide (H_2O_2) for 20 min or to local UV irradiation (Figure 3A). H_2O_2 exposure induced a strong and homogenous PAR nuclear staining consistent with previous reports. No PAR spots appeared in UV-irradiated quiescent cells (Figure 3A), whereas weak and transient PAR staining occurred in a subset of cells of a UV-irradiated asynchronous population within 30 min after exposure (data not shown). Moreover, the PARP inhibitor KU0058948 (Farmer et al., 2005) had no effect on the recruitment of either p89 or XRCC1 to UV damage in quiescent normal cells (Figure 3B). The activity of KU0058948 was proven by the abolishment of PAR accumulation in H_2O_2-treated cells in the presence of the inhibitor (Figure 3A). Furthermore, the disassembly kinetics of the NER preincision complex did not differ significantly between UV-irradiated primary normal, Lig1-, and Lig4-deficient cells treated or mock treated with the PARP inhibitor (Figure 3C). The above results suggest that the induction of base damage or SSBs by UV irradiation must be negligible and that inhibition of PARP has no effect on the interactions of XRCC1-Lig3 with NER components at UV damage in quiescent cells.

In addition, we were unable to detect PARP-1 accumulation at UV spots in either normal or Lig1-deficient cells, whereas PARP-1 accumulation was evident at Laser (800 nm) induced DNA damage (Mari et al., 2006) (Figure S2A and data not shown). Although a role of polβ has been implicated in NER in the presence of HU/AraC (Smith and Okumoto, 1984), recruitment of polβ was not observed in the presence of HU/AraC (Figure S2B), in agreement with a previous report (Albertella et al., 2005). In summary, the data suggest that XRCC1 and Lig3 function in the postincision step of NER independently of PARP-1 and polβ.

UV-Dependent Association of XRCC1-Lig3 with Core NER Components

The results described so far provide evidence for the involvement of two new factors, XRCC1 and Lig3, in NER. Similarly to other postincision components such as PCNA, polδ/pol, and CAF-1 (Gillet and Scharer, 2006), the recruitment of XRCC1-Lig3 requires dual incision of the damaged DNA (Figures 2A and 2B). To study putative interactions between the XRCC1-Lig3 complex and NER components, we isolated NER-specific complexes by using in vivo protein crosslinking and chromatin immunoprecipitation (ChIP) (Fousteri et al., 2006).

To eliminate coprecipitation of proteins involved in chromosomal DNA replication, we used nondividing cells. Cells were irradiated with a UV dose of 20 J/m^2, which induces approximately four photolesions (i.e., CPD plus 6-4PP) per 10 kb of double-stranded DNA (Van Hoffen et al., 1995). After crosslinking, chromatin fragments (300-600 bp) were precipitated with an XRCC1-specific antibody. Figure 4A shows that equal amounts of Lig3 were coprecipitated with XRCC1 from UV-irradiated and mock-treated cells. However, an association of XRCC1 with the postincision NER components RPA, PCNA, and polδ was observed only in chromatin of UV-irradiated normal human cells. XRCC1-specific ChIP from UV-irradiated noncycling XP-A cells failed to reveal an association between XRCC1 and NER proteins, whereas the interaction with Lig3 was retained (Figure 4A). We did not detect association between XRCC1 and pol under these conditions in any of the cell lines

tested. Performance of the reciprocal ChIP with an RPA-specific antibody showed increased interaction with XRCC1 and Lig3 as well as XPA and polδ in chromatin of UV-irradiated normal cells compared to XP-A or mock-irradiated cells (Figure 4B). Nevertheless, a UV-specific association between RPA and XPG was present in chromatin of both normal and XP-A cells in agreement with previous studies (Volker et al., 2001 and Riedl et al., 2003). These results provide further evidence that recruitment of XRCC1-Lig3 to UV damage is a postincision event in NER. Western blot analysis of XRCC1-specific ChIP did not reveal any interaction between XRCC1 and the preincision NER factors XPC and p89 (TFIIH) in UV-irradiated cells (Figure 4A), in line with results from in vitro studies (Riedl et al., 2003). However, a UV-specific interaction was observed between XRCC1, XPA, and the NER-specific endonucleases XPF and XPG (Figure 4A).

Downregulation of Lig3 in Human Cells Impairs Ligation of NER-Mediated DNA Breaks and Repair of UV Lesions

To test whether XRCC1-Lig3 plays an essential role in the repair of UV photolesions, we employed siRNA double-stranded oligonucleotides to knock down Lig3. To achieve efficient siRNA transfection, we employed normal (VH10/SV) and Lig1-deficient (46BR/SV) SV-40-transformed cells (Figure 5A). Examination of 6-4PP levels in UV-irradiated Lig3si(1)-transfected normal human and Lig1-deficient cells revealed that downregulation of Lig3 significantly impaired repair of UV lesions in normal cells, and even more in Lig1-deficient cells (Figure 5B). No decrease in the repair of 6-4 PP was observed in either cell line transfected with an siRNA against GFP (control siRNA). Furthermore, the alkaline comet assay demonstrated that impaired ligation coincides with the reduced levels of repair

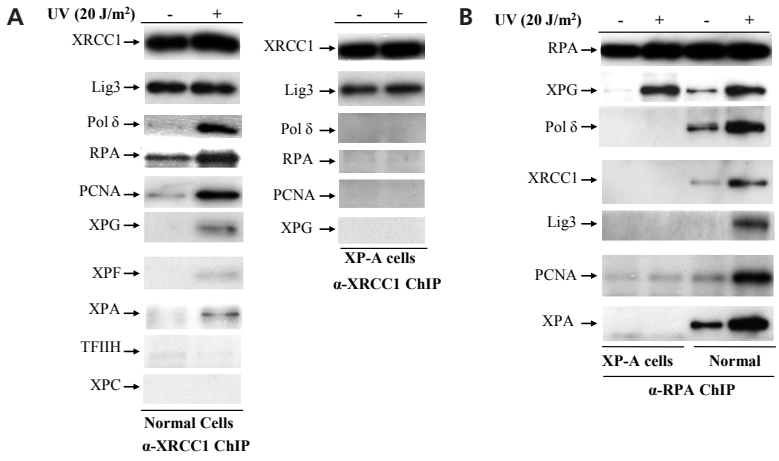

Figure 4. UV-Dependent Association of XRCC1-Lig3 with Core NER Components. The antibodies used for western blot analysis (WBA) are as indicated in the figure. (A) WBA of XRCC1-specific ChIP using chromatin of crosslinked UV- and nonirradiated confluent normal and XP-A cells. (B) WBA of RPA-specific ChIP using chromatin of crosslinked UV- and nonirradiated confluent normal and XP-A cells.

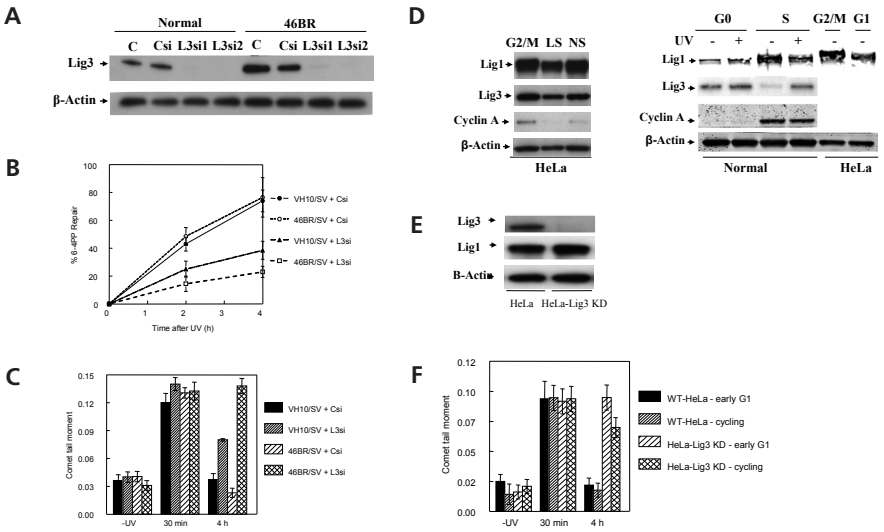

Figure 5. Downregulation of Lig3 Impairs the Repair of UV-Induced Lesions. WBA of normal and Lig1-deficient cells treated with a control siRNA targeted against GFP (Csi) or two different Lig3-specific siRNAs (L3si1 and L3si2). (B) 6-4PP repair as determined by fluorescent immunostaining of 6-4PP at various times after global UV irradiation (15 J/m^2). (C) Strand-break repair of globally UV-irradiated (15 J/m^2) cells transfected with either Csi or a L3si that were analyzed by the alkaline comet assay. Breaks were quantified as comet tail moments. Histograms are means ± SEM of at least three independent experiments. (D) HeLa cells were synchronized in mitosis (G2/M) by nocodazole treatment and shake off. G2/M cells were either lysed directly or replated in low (0.2%, LS) or normal serum medium (10% NS), and whole-cell extracts (WCE) were made and analyzed by WBA 6 hr after release with antibodies as indicated (left panel). The chromatin-bound fraction of UV- (15 J/m^2) or nonirradiated quiescent (G0) or S phase (S) VH10 htert (normal) cells was analyzed for the presence of different proteins as indicated. WCE from HeLa G2/M and G1 phase cells were included for comparison of Lig1 phosphorylation level (right panel). (E) WCE of HeLa cells and HeLa-Lig3 KD were analyzed by western blotting. The antibodies used are as indicated. (F) Mitotic HeLa and HeLa-Lig3 KD cells were released from nocodazole block and replated as described above for 2 hr before global UV irradiation (15 J/m^2). Cells were allowed to repair for 30 min or 4 hr and analyzed by alkaline comet assay. Strand break repair was quantified as comet tail moments. Histograms are means ± SEM of at least three independent experiments.

in Lig3si(1)-transfected normal and Lig3si(1)-transfected Lig1-deficient cells (Figure 5C). These data show that, in immortalized human cells lacking both Lig1 and Lig3, repair of UV damage is severely abolished. However, the residual repair in cells lacking Lig3 only suggests that in highly proliferating cells Lig1 may partly compensate for Lig3 deficiency.

Cell-Cycle-Dependent Regulation of the Gap-Filling/Sealing Step in NER

Lig1 is recruited to UV spots only in S phase and late G1/S cells and might partly compensate for Lig3 deficiency in highly proliferating cells, further supporting a cell-cycle-dependent involvement of Lig1 in NER. To assess this point and to study the role of Lig3 outside S phase, we employed the alkaline comet assay and analyzed the ligation efficiency of UV-induced chromosomal breaks in synchronized HeLa cells and in HeLa

cells with stable Lig3 knockdown (HeLa-Lig3-KD) (Figure 5E). We synchronized the cells in G2/mitosis (G2/M) by treatment with nocodazole and reseeded them in fresh medium containing either low (0.2%) or normal (10%) serum to delay cell-cycle progression or to allow the cells to go through G1, respectively. Two hours after release, the cells were UV irradiated and returned to the same medium to allow repair for 30 min or 4 hr at 37°C (the cell-cycle progression was followed by monitoring the phosphorylation status of Lig1 and cyclin A, Figure 5D) (Rossi et al., 1999). Rejoining activity was virtually absent in HeLa-Lig3-KD cells that were released in low serum (Figure 5F), indicating a complete Lig3-dependent ligation of repair patches. HeLa-Lig3-KD cells that progressed through G1 also showed a significant ligation defect, though to a lesser extent than the noncycling cells. Full restoration of the UV-induced breaks occurred in the parental HeLa cell line, demonstrating that the synchronization procedure did not affect NER and Lig3 activity in the nocodazole-treated UV-irradiated cells (Figure 5F).

Our data support previous observations (Rossi et al., 1999 and Ferrari et al., 2003) that Lig1 is inactive in G2/M phase due to CDK2-dependent hyperphosphorylation and in G0 phase due to its hypophosphorylation status (Figure 5D). UV irradiation of mitotic cells released in low serum even in the absence of a functional Lig3 did not render Lig1 active. Interestingly, Lig3 level in the chromatin of S phase cells was substantially increased in response to UV irradiation (Figure 5D), supporting active involvement of Lig3 in repair synthesis during S phase. Taken together the above observations favor a model in which Lig3 together with XRCC1 is indispensable for the repair of UV photolesions in quiescent or postmitotic cells, whereas both XRCC1-Lig3 and Lig1 are engaged in the sealing step of NER in dividing cells. In vitro studies have implicated pol as one of the major DNA polymerases involved in NER-dependent DNA repair synthesis. Immunolabeling of locally UV-irradiated confluent normal cells showed that pol was recruited to UV damage (Figure 6A).

Using p89- or XPA-specific antibodies to monitor UV spots, we noticed, however, that similar to the picture seen for Lig1 only a subset of irradiated cells showed colocalization of pol with p89, whereas colocalization of other postincision factors such as PCNA, polδ, and XRCC1 with XPA was evident in all cells (Figure 6C). Employing quiescent and cycling cells and immunolabeling with antibodies against pol, p89, and Ki67 (Figures 6A and 6B), we found that, parallel to Lig1, pol colocalized with UV damage only in Ki67-positive cells. Immunolabeling of UV-irradiated quiescent cells (Figure 6D) revealed accumulation of all postincision components tested except pol and Lig1 (Figures 6B and 6D).

Immunolocalization of Lig1 and pol with XRCC1 in UV-irradiated confluent cells showed that accumulation of Lig1 and pol at UV-damage sites does not exclude recruitment of XRCC1 at the same sites (Figure 6E). In summary, in Ki67-positive cells, both polδ and pol as well as XRCC1-Lig3 and Lig1 are involved in NER gap filling and ligation, whereas in quiescent cells, only polδ and XRCC1-Lig3 are required, suggesting that NER-mediated repair synthesis and ligation in mammalian cells is differentially regulated throughout the cell cycle.

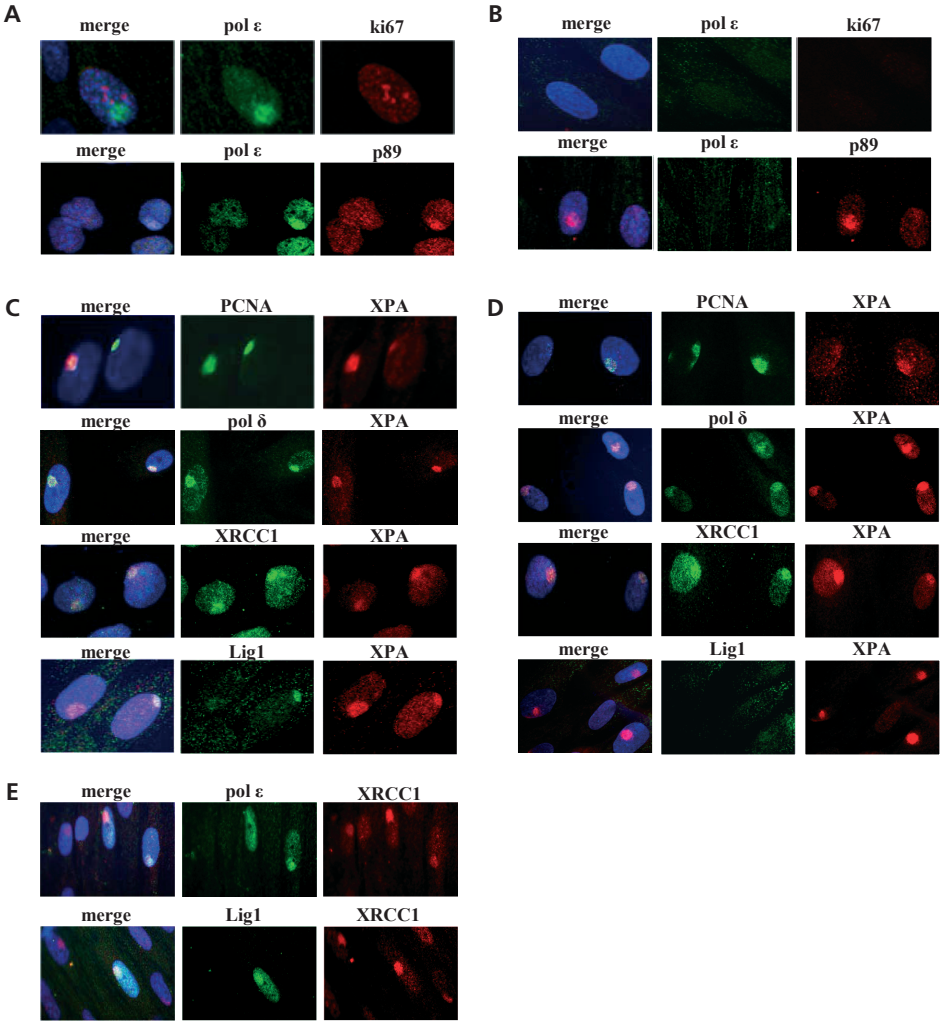

Figure 6. Cell-Cycle-Dependent Regulation of Gap Filling/Sealing in NER. (A–E) Images were obtained with locally irradiated (30 J/m2) primary human fibroblasts. (A) Immunolocalization of pol/ki67 and pol/p89 in cycling cells.(B) Immunolocalization of pol/ki67 and pol/p89 in quiescent cells. (C and D) Immunolocalization of postincision factors (PCNA, polδ, XRCC1, and Lig1) with XPA in either confluent (C) or quiescent (D) cells. UV-damage sites are visualized by local accumulation of XPA.(E) Immunolocalization of pol and Lig1 with XRCC1 in confluent cells.

DISCUSSION

XRCC1 and Lig3 Are Core Components of the NER Machinery *In Vivo*

Efficient sealing of SSBs in chromosomal DNA during DNA replication and repair is vital in maintaining genome integrity and cell viability (Darroudi et al., 1990 and Okano et al., 2000). Lig3 and XRCC1 are both required for repair of SSBs either generated during BER or

induced by agents such as H_20_2 and camptothecin (El-Khamisy et al., 2003 and El-Khamisy et al., 2005). XRCC1 acts as a scaffold protein to recruit, stabilize, or stimulate multiple enzymatic components of BER and SSBR, including Lig3 (Caldecott, 2003).

In this study, we describe a so far unanticipated role of XRCC1-Lig3 in repair of UV-induced DNA photolesions and sealing of the chromosomal breaks in mammalian cells. In UV-irradiated cells either cycling or arrested, XRCC1 and Lig3 are recruited to sites of DNA damage. Using in vivo crosslinking and ChIP, we demonstrated that the XRCC1-Lig3 complex associates with core NER factors following UV. A number of postincision NER factors were detected, including PCNA, RPA, and polδ, but surprisingly, no association with pol was found. XRCC1 has been shown to colocalize and likely to physically interact in vivo with PCNA (Fan et al., 2004). We speculate that the XRCC1-Lig3 complex might function in NER through interactions with PCNA, a key factor to recruit other postincision NER factors. Recruitment of XRCC1-Lig3 to NER sites depends on dual incision activity, as it does not translocate to UV damage in XP-A or XP-F cells (lacking 5′ incision activity). XRCC1 does not interact with the preincision NER components p89 and XPC in any of the ChIP reactions, although weak association with XPA, XPF-ERCC1, and XPG was found. We thus suggest that factors involved in the DNA damage recognition and the postincision steps of NER do not simultaneously reside in the same complex in vivo.

Recruitment of XRCC1 and Lig3 to SSBs in chromatin requires the activation of PARP-1 activity (Okano et al., 2003 and El-Khamisy et al., 2003). Contrary to SSBR, binding of PARP-1 to DNA breaks that arise during NER is not essential for the incision-dependent recruitment of XRCC1-Lig3, because no PAR signal was detected in UV-irradiated quiescent cells and the PARP-1-specific inhibitor KU0058948 had no effect on recruitment of XRCC1-Lig3 to UV damage. Obviously, the NER machinery efficiently targets XRCC1-Lig3 to sites of UV damage without the need for PAR synthesis. Taken together the results indicate that XRCC1-Lig3 plays a central role in NER: the complex accumulates at UV damage throughout the cell cycle and interacts with core NER factors in a UV-dependent manner, without the recruitment of PARP1 or polβ. Interestingly, evidence for a positive role of XRCC1-Lig3 in a reconstituted NER system has been recently found (J.M. Egly, personal communication).

Downregulation of Lig3 Leads to Impaired Repair of UV Lesions and Induction of DNA Strand Breaks

Lig3 has only been identified in vertebrates and is able to take over a number of functions carried out by Lig1 in budding yeast, i.e., mitochondrial DNA replication and repair. One possibility for the involvement of Lig3 in NER in higher eukaryotes is that differentiated nondividing cells of higher eukaryotes have no active Lig1 available, and hence, repair requires specialized ligases, such as Lig3, to carry out these processes. Lig3 is essential for early embryonic development, as targeted inactivation of the *lig3* gene in the mouse leads to embryonic lethality (Puebla-Osorio et al., 2006). However, Chinese hamster ovary (CHO) cell lines (EM9, EM-C11, and EM-C12) in which the XRCC1 protein is mutated and greatly reduced or absent have been generated (Zdzienicka et al., 1992, Caldecott et al., 1995 and Shen et al., 1998). XRCC1 protects Lig3 from proteosomal-mediated degradation

(Caldecott et al., 1995 and Moore et al., 2000), and consequently, EM9 and EM-C11 cells exhibit 4- to 6-fold reduced levels of Lig3 protein and activity. Surprisingly, EM-C11 cells showed no sensitivity to the cytotoxic effects of UV irradiation (Zdzienicka et al., 1992). However, UV-irradiated EM9 and EM-C12 cell strains exhibited a mild sensitivity in a clonogenic survival assay (Shen et al., 1998 and Busch et al., 1980), and UV-irradiated EM9 cells exhibited a reduced rate of rejoining of DNA strand breaks (Nocentini, 1999), implicating a role of XRCC1-Lig 3 in UV repair. In agreement with these results, we found reduced rejoining of UV-induced breaks in proliferating normal human fibroblasts with downregulated Lig3 and further impairment in HeLa-Lig3-KD cells synchronized in early G1 as well as in the Lig3-Lig1 double-knockdown cell line (46BR/SV + Lig3si[1]). Nevertheless, and in line with results obtained with CHO cells, clonal UV survival of Lig3si[1]-transfected normal (VH10/SV) and HeLa-Lig3-KD cells showed only mild sensitivity (data not shown). We were unable to assess UV effects in the double-knockdown cell line (46BR/SV + Lig3si[1]) because depletion of both ligases severely affected cell growth.

Inhibition of polδ/pol in confluent normal human cells by HU/AraC leads to accumulation of DNA strand breaks and abolishment of 6-4PP repair shown in the current work and previously (Mullenders et al., 1985). Obviously, efficient gap filling by DNA synthesis and ligation of the repair patch is required to drive NER to completeness (Erixon and Ahnstrom, 1979). In accordance with this hypothesis, we found that downregulation of Lig3 in proliferating human cells leads to accumulation of DNA strand breaks and significant inhibition of 6-4PP repair, whereas Lig1 deficiency (as manifested in 46BR cells) had no impact on break frequency and repair efficiency. This finding suggests that even in S phase cells Lig3 is the dominant NER ligase. Interestingly, growing cells lacking normal levels of both Lig1 and Lig3 display synergistic impairment of NER-mediated repair. This clearly implicates a compensating role of Lig1 in 6-4PP repair in proliferating human cells with reduced level of Lig3 in spite of the apparent lack of UV sensitivity of Lig1-deficient 46BR cells.

Lig1 Is Involved in the Repair of UV Photolesions in Late G1 and S Phase Cells

Lig1 expression levels strongly correlate with the rate of cell proliferation, consistent with its role in DNA replication (Montecucco et al., 1992 and Prigent et al., 1994). Both mRNA and protein levels increase after serum stimulation of human and mouse fibroblasts, whereas confluency, serum starvation, and cell differentiation lead to a strong decrease of Lig1 gene expression (Montecucco et al., 1992). Interestingly, Lig1 activity is regulated by cell-cycle-dependent phosphorylation allowing Lig1 to be recruited to replication factories in late G1 and S phase, but not in early G1 or G2/M. In mitotic cells, Lig1 is hyperphosphorylated (at serines Ser51, Ser66, Ser76, and Ser91) and functionally inactive (Rossi et al., 1999 and Ferrari et al., 2003). Although Lig1 is present in quiescent human keratinocytes, the protein is inactive and requires phosphorylation for its activation (Bhat et al., 2006). In agreement with these data, we identified hyperphosphorylated Lig1 in G2/M and hypophosphorylated Lig1 in both UV- or mock-treated quiescent cells (G0) cells. We propose that the hypophosphorylated Lig1 is unable to participate in NER, as we failed to detect recruitment of Lig1 to UV spots in quiescent cells.

Our data are consistent with a role of active Lig1/PCNA in the ligation step of NER, but only in late G1 and S phase cells. Lig1 is recruited to UV damage in G1 and S phase cells expressing Ki67, but not in Ki67-negative G0/G1 cells. Moreover, HeLa cells with reduced levels of Lig3 were deficient in 6-4PP repair and accumulated SSBs if synchronized and irradiated in late mitosis/early G1, consistent with the presence of functionally inactive Lig1 in these cells (Ferrari et al., 2003 and Rossi et al., 1999). In contrast, late G1/S HeLa cells expressing functionally active Lig1 (intermediate level of phosphorylation at Ser91 and Ser66 [Ferrari et al., 2003) were capable of sealing UV-mediated DNA breaks, albeit less efficiently than Lig3-proficient human cells. The involvement of Lig1 in NER in S phase cells might provide an explanation for the mild UV sensitivity of Chinese hamster XRCC1 mutant cell lines that display reduced levels of Lig3 (Busch et al., 1980 and Caldecott et al., 1995). It is possible that the presence of a Lig1-mediated process in rapidly proliferating cells can compensate for the absence of Lig3 over extended repair periods.

DNA Polymerases Delta and Epsilon Work in NER via Two Distinct Complexes

Data from immunofluorescence microscopy and ChIP experiments show that polδ and are both involved in the repair of UV damage in vivo, consistent with a role for both polymerases in the gap-filling step of NER in vitro (Aboussekhra et al., 1995). Also, in *Saccharomyces cerevisiae*, polδ and pol are able to carry out DNA repair synthesis in the absence of the other (Budd and Campbell, 1995). Here we show that in vivo the two DNA polymerases participate in different molecular complexes to fulfill their role in NER. ChIP experiments with chromatin from quiescent UV-irradiated NER-proficient cells revealed that the XRCC1-specific antibody pulled down exclusively polδ, suggesting that in nondividing cells XRCC1-Lig3 predominantly associates with polδ at sites of UV lesions. In addition, in both S phase and G0 cells, we could readily show the recruitment of XRCC1-Lig3 and polδ to spots of UV damage, whereas we were unable to monitor accumulation of Lig1 and pol at sites of UV lesions in G0 cells.

In contrast to quiescent cells, Lig1 and pol accumulate at UV lesions in late G1 cells expressing Ki67 and in S phase cells, indicating that functionally active Lig1/pol NER complexes are formed in these cells. Earlier studies using cell-free extracts (Shivji et al., 1995) or purified components (Aboussekhra et al., 1995) revealed that pol was more suitable than polδ for filling in short gaps in an RFC- and PCNA- dependent manner in the presence of Lig1. In addition to pol and polδ, polβ has been implicated in the gap-filling step of NER (Smith and Okumoto, 1984), particularly in the presence of HU/AraC. We were unable to demonstrate recruitment of polβ to UV damage in confluent human fibroblasts in the presence of HU/AraC, and hence, it is possible that the low level of HU/AraC-resistant repair synthesis is carried out by DNA polymerase κ (polκ). Recent evidence emerged that polκ implicated in bypass of replication blocks has a role in the gap-filling step in NER in UV-irradiated cells treated with HU (Ogi and Lehmann, 2006); the choice for polκ in the gap-filling reaction might be dependent and stimulated by the reduced dNTP pools in the presence of HU. Considering that polκ deficiency only delays repair synthesis and that repair synthesis in confluent cells is very sensitive to polδ and pol inhibitors such as AraC/Aphidicolin, we consider polδ and pol as the major DNA polymerases in the gap-filling reaction of NER.

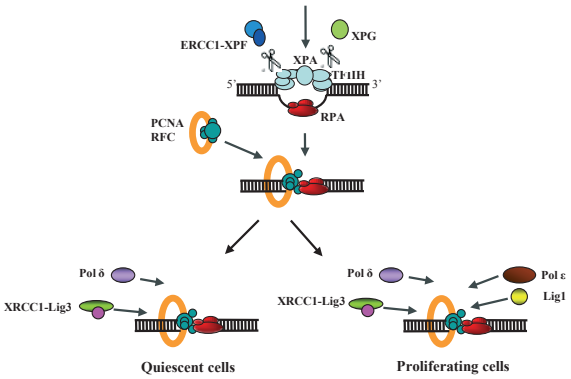

Figure 7. Regulation of NER-Mediated DNA Repair Synthesis In Vivo. NER postincision steps, i.e., DNA repair synthesis and ligation, and the components involved are shown. Two distinct complexes differentially carry out the gap-filling step in NER. XRCC1-Lig3 and polδ colocalize and interact with NER components in a UV- and incision-dependent manner in dividing and nondividing cells. In contrast, Lig1 and pol are recruited to sites of UV damage only in proliferating cells.

In conclusion, XRCC1-Lig3 is the dominant ligase complex involved in NER-mediated gap filling in quiescent and proliferating cells and indispensable in quiescent cells (Figure 7). Two distinct complexes differentially carry out the gap-filling step in NER. XRCC1-Lig3 and polδ colocalize and interact with NER components in a UV- and incision-dependent manner throughout the cell cycle. In contrast, Lig1 and pol are recruited to sites of UV damage only in proliferating cells. Bulky DNA lesions such as cyclopurines are induced by reactive oxygen species generated by X-rays or endogenous cellular processes (Satoh et al., 1993) and require NER for their repair (Brooks et al., 2000). Our data suggest that in quiescent or terminally differentiated cells the sealing of chromosomal DNA nicks that arise during NER would be entirely dependent on functional XRCC1-Lig3 complex. Taken together our data reveal a key role for XRCC1-Lig3 in the maintenance of genomic integrity by the NER pathway in mammalian cells and provide a possible explanation for the impact that XRCC1 polymorphic mutations have on a variety of human cancers, including sunlight-induced squamous cell carcinoma (Han et al., 2004) and lung cancer (Hao et al., 2006).

MATERIALS AND METHODS

Cell Lines. Cells used in this study were cultured in DMEM supplemented with 10% fetal calf serum (FCS) (unless otherwise stated) at 37oC in a 5% CO2 atmosphere and include i) primary normal (VH25), Lig1- (46BR) and Lig4-deficient human fibroblasts (411BR) as well as NER deficient XP-A fibroblasts (XP25RO), ii) telomerase hTert immortalised normal (VH10 hTert), and XP-C, XP-A and XP-F human fibroblasts (XP21RO hTert, XP25RO hTert and XP51RO hTert, respectively), iii) SV-40 transformed normal (VH10/SV) and Lig1-deficient human fibroblasts (46BR/SV) (for siRNA experiments) and also parental HeLa cells and HeLa cells with stable Lig3 knockdown (HeLa-Lig3-KD). Lig4-deficient cells (SC2, kindly provided by Dr. D. van Gent, Erasmus MC, The Netherlands) were grown in Ham's F10 medium. For local and global UV irradiation experiments, the cells were seeded and grown on glass coverslips coated with Alcian blue (Fluka). For repair and ChIP experiments primary and hTert immortalised fibroblasts were grown to confluency for approximately 10 days. Subsequently, cells were synchronised in G0 phase by keeping them for a minimum of 5 days in medium supplemented with 0.2% FCS (serum starved cells). Synchronisation of HeLa and HeLa-Lig3-KD cells in G2/M was achieved by incubation of exponentially growing cells for 16 h in complete medium containing 40 ng/ml nocodazole. Mitotic cells were shaken-off, centrifuged and reseeded in medium that contained (or containing) either 10% FCS (G1) or 0.2% FCS (to delay entrance into the G1) and grown for 2-6 h at 37°C.

Global and Local UV-irradiation. Cells were global or locally UV-irradiated through a filter with pore size 8 μM as described previously (Volker et al., 2001). After irradiation, the cells were returned to culture conditions for the time periods as indicated. When required, cytosine-β-arabinofuranoside (AraC, Fluka) and hydroxyurea (HU, Fluka) were added to the medium 30 minutes (min) prior to irradiation and remained present throughout the time course of the experiment. Final concentrations were 10 μM for AraC and 100 mM for HU. Laser-induced DNA damage was introduced into normal human cells using a pulsed NIR (800nm) laser as described previously (Mari et al., 2006). Images were taken with equal exposure time and over 50 fluorescent images were analyzed for each time point. Signal intensities were determined by measuring the total fluorescence intensity of a spot or nucleus, and dividing by the surface area of the measured spot or nucleus, respectively (Mean +SD).

Antibodies. The following primary antibodies were used: rabbit polyclonal α-XPA, α-p89, α-p21, and mouse monoclonal α-DNA pol δ, α-p21 (Santa-Cruz), mouse monoclonal α-PCNA (PC10), α-XRCC1, α-PARP-1, rabbit polyclonal α-XRCC4, and mouse monoclonal α-pol β, α- XPA, and α-XPF (Abcam), mouse monoclonal α-DNA lig3α (Genetex), mouse monoclonal α-poly(ADP-ribose) (10H; Calbiochem) rabbit polyclonal α-Lig1 (2B1, kindly provided by Dr A. Montecucco, Istituto di Genetica Molecolare, Pavia, Italy), mouse monoclonal α- DNA Lig I (5H5; MBL), rabbit polyclonal α-Lig1 (a gift from A.E Tomkinson, University of Baltimore, Maryland), mouse monoclonal α-pol ε (a gift from Dr. S. Linn, UC, Berkeley, CA), mouse monoclonal α-XPG (8H7, Molecular Probes), mouse monoclonal α-RPA (Ab1, Oncogene), mouse monoclonal α-6-4PP (gift from Dr. O. Nikaido, Kanazawa University, Kanazawa, Japan). Secondary antibodies used for immunofluorescence staining were Cy3-conjugated goat α-rabbit IgG and FITC-conjugated donkey α-mouse (Jackson Laboratories) and Alexa Fluor® 488 goat α-mouse IgG, AlexaFluor 555 goat α-rabbit (Molecular Probes). All secondary antibodies were used according to the manufacturer's instructions.

Immunofluorescence. The fluorescent labelling was performed essentially as described previously (Moné et al., 2001; Volker et al., 2001). Briefly, cells were washed with cold PBS, fixed and subsequently lysed on ice by either addition of 100% Methanol (10 min), or by 2% formaldehyde and 0.2% Triton X-100 (15 min), or alternatively by addition of 2% paraformaldehyde (20 min at room temperature (RT)) followed by 0.2% Triton X-100 and incubation for 5 min at RT. Cells were washed twice with PBS and incubated with 5% bovine serum albumin (BSA) in PBS for 30 min at RT. Subsequently, the cells were incubated with the primary antibodies diluted in wash buffer (WB) (PBS containing 0.5% BSA and 0.05% Tween-20) for 2 h at RT. The cells were washed 3 x with WB and incubated with the secondary antibody (diluted in WB) for 1 h at RT. In experiments requiring double labelling, antibodies were mixed in WB and incubated simultaneously. To visualise only chromatin-bound PCNA, soluble PCNA was extracted by Triton X-100 treatment prior to methanol fixation. Cells were mounted in Aqua/polymount mounting medium containing DAPI (1.5 μg/ml) (Polysciences Inc., Warrington, PA). Microscopy and quantification of fluorescent signal has been described elsewhere (Moser et al., 2005).

RNA Interference. Short interfering RNA (siRNA) duplexes were designed and subsequently synthesized by Invitrogen. The sequences were as follows: 5'-GCAUAAGGACUGUCUGCUA-3' to target the Lig3 transcript and 5'-GCAGACAUGCCUGACUAUU-3' for GFP targeting. A second validated siRNA against the Lig3 transcript, designed and synthesized by QIAGEN (Cat no. S102663549), was also used. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Repair, immunostaining, and Comet assay experiments were carried out 48 hr after transfection. The level of protein knockdown was examined by western blotting.

In Vivo Crosslinking and ChIP. Confluent or serum-starved hTert-immortalized normal human and XP-A fibroblasts were UV irradiated (20 J/m^2) and incubated at 37°C for 30 min to 1 hr prior to in vivo crosslinking (11 min, on ice) and ChIP. In vivo crosslinking, lysis of the cells, chromatin purification and immunoprecipitation, and reversal of the crosslinks prior to protein and DNA analysis were performed as described earlier (Fousteri et al., 2006).

Cell fractionation and western blot analysis. All manipulations with cellular extracts were carried out at 4°C. For western blot analysis siRNA transfected cells and UV- or non-irradiated non-crosslinked cells were lysed in 1 x RIPA buffer for 30 min on ice. After collecting the soluble fraction, the remaining pellet was resuspended again in an equal volume of 1 x RIPA buffer and subsequently snapfrozen. Prior to SDS-PAGE, equal amounts of total protein from the different cell lysates were mixed with one volume of 2 x Laemli-SDS-sample buffer and heated at 95°C for 10 min. The loading was also controlled with an antibody against β-actin. Western blot analysis was performed as described

previously (Fousteri et al., 2006) and protein bands were visualised via chemiluminescence (ECL-Plus, Amersham Biosciences) using Horseradish Peroxidase (HP)-conjugated secondary antibodies and exposure to ECLHyperfilms (Amersham Biosciences).

Comet Assay. Alkaline comet assay was performed as described in (Cramers et al., 2005) with minor modifications. Cells were globally UV-irradiated (15 J/m2), and then incubated in fresh medium for the appropriate repair time. Prior to scoring, DNA was stained with 10 µg/ml ethidium bromide. Mean tail moments were quantified for 130 cells per sample in each experiment using the ColourProc software program as described previously (Cramers et al., 2005). Histograms are the average mean tail moment per sample of at least 3 experiments (reported as means + s.e.m).

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SUPPLEMENTARY FIGURES

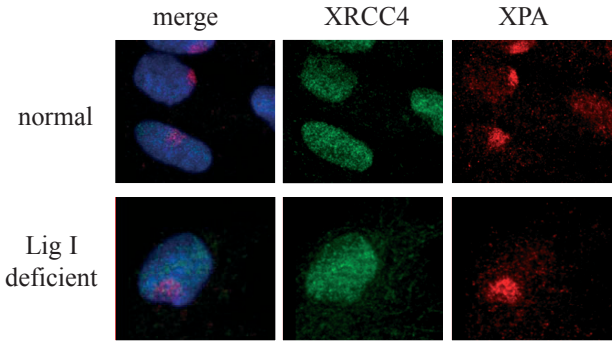
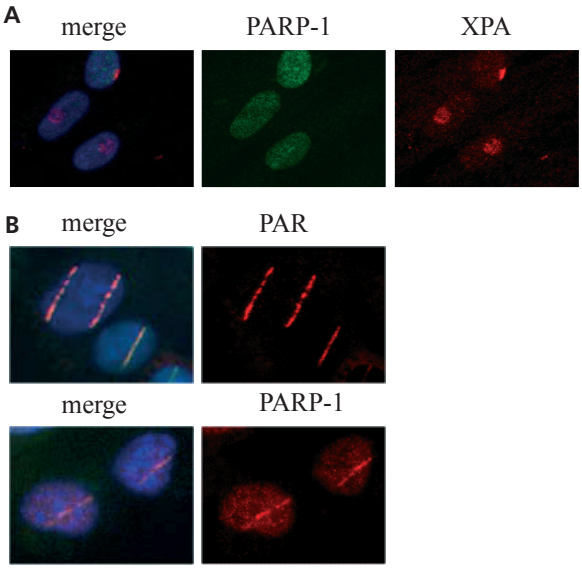


Figure S1. XRCC4 is not recruited to local UV-induced damage. Immunolocalization of XRCC4 in locally UV-irradiated (30 J/m2) confluent primary normal (VH25) or Lig1-deficient (46BR) cells. UV-damage sites are visualized by local recruitment of XPA.



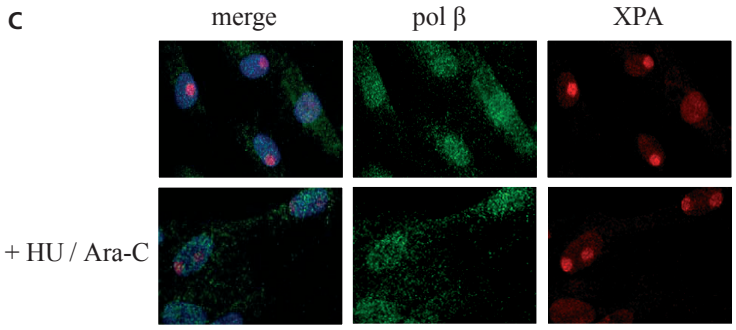


Figure S2. PARP-1 and pol β do not accumulate at local UV-damage spots. (A) Immunolocalization of PARP-1 in locally UV-irradiated (30 J/m2) confluent primary normal cells. UV-damage sites are visualized by local recruitment of XPA. (B) Immunolocalization of PAR and PARP-1 on laser-induced DNA damage in normal human cells. (C) Immunolocalization of polβ in locally UV-irradiated (30 J/m2) confluent primary normal cells. Cells were either mock-treated or treated with HU/AraC. UV-damage sites are visualized by local recruitment of XPA.

REPLICATION PROTEIN A SAFEGUARDS GENOME INTEGRITY BY CONTROLLING NER INCISION EVENTS

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Submitted

SUMMARY

Single stranded DNA gaps that might arise by futile repair processes can lead to mutagenic events and challenge genome integrity. Nucleotide excision repair (NER) is an evolutionary conserved repair mechanism, essential for removal of helix-distorting DNA lesions. In the currently prevailing model, NER operates through coordinated assembly of repair factors into pre- and post-incision complexes; however, it's regulation in vivo is poorly understood. Notably, the transition from dual incision to repair-synthesis should be rigidly synchronized as it might lead to unprocessed repair intermediates. Employing a novel approach of sequential UV-irradiations we monitored NER regulatory events in vivo. Under conditions that allow incision yet prevent completion of repair-synthesis or ligation, pre-incision factors can disassemble and associate with new damage sites. In contrast, RPA remains associated at the incomplete NER sites regulating a feedback loop from complete repair-synthesis to subsequent damage recognition independently of ATR signaling. Our data reveal a pivotal role of RPA in averting further generation of DNA strand breaks that could lead to mutagenic and recombinogenic events.

INTRODUCTION

To counteract genotoxic challenges and maintain genomic integrity, cells have evolved an interrelated network of biological responses including DNA damage detection, signaling and DNA repair systems such as nucleotide excision repair (NER). NER removes DNA helix distorting lesions including DNA photolesions induced by ultraviolet light (UV) i.e. cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP). DNA damage processed by NER is differentially recognized depending on whether the damage is located throughout the genome (global genome repair, GG-NER) or specifically blocks transcription (transcription-coupled repair, TC-NER). The consequences of defective NER are apparent from the clinical symptoms of individuals affected by the rare recessive inherited disorders xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD) that characteristically display severe photosensitivity, as well as high incidence of cancer (XP), multi-system clinical malfunctions, neurological abnormalities and features of premature ageing (CS, XP/CS, TTD) (Tanaka and Wood, 1994).

In vitro reconstituted NER systems (Aboussekhra et al., 1995; Araujo et al., 2000; Bessho et al., 1997; Mu et al., 1995) originally identified approximately 30 polypeptides required for GG-NER and assigned specific roles to the various factors that were later confirmed by in vivo studies (Sugasawa et al., 1998; Volker et al., 2001; Tapias et al., 2004; Moser et al., 2005). The UV-DDB and the XPC-hHR23B heterodimers are responsible for DNA lesion recognition and efficient assembly of the core NER complex (the pre-incision step of NER), which includes the basal transcription factor TFIIH, replication protein A (RPA), XPA, and the structure-specific endonucleases XPG and ERCC1-XPF (Gillet and Scharer, 2006). After excision of the damaged DNA, the gap is filled by DNA repair synthesis (the post-incision step of NER) involving DNA polymerases δ (Polδ), ε (Polε) (Moser et al., 2007) and κ (Polκ) (Ogi and Lehmann, 2006; Ogi et al., 2010). The remaining nicks are sealed by either XRCC1-DNA Ligase IIIα (XRCC1-Lig3) or DNA Ligase I (Lig1) (Moser et al., 2007). Even though the key NER factors involved in the repair of NER substrates have been identified, the coordination between the two stages of NER (pre- and post-incision steps) is still poorly understood. Based on data from reconstituted NER reactions (Wakasugi and Sancar, 1998), it has been suggested that dual incision and/or recruitment of post-incision factors to NER sites lead to the differential release of pre-incision factors with the exception of XPC and RPA. XPC was shown to depart from the complex when positioning of XPG within the pre-incision complex occurs i.e. before incision is complete (Riedl et al., 2003). On the other hand, XPG might reside longer at the repair sites than XPF/ERCC1, since 5'incision generated by ERCC1/XPF does not lead to dissociation of XPG and does not require the catalytic activity of XPG (Staresincic et al., 2009). Among the other factors, RPA was the only pre-incision factor that was found together with post-incision NER factors (Riedl et al., 2003) and it was shown to enhance NER mediated DNA synthesis (Shivji et al., 1995).

More than 30 years ago, it was observed that addition of DNA Polδ and ε inhibitors, cytosine-β-arabinofuranoside (AraC) and hydroxyurea (HU) to UV-exposed cells, led to an accumulation of non-repairable DNA single strand breaks in the genome (Dunn and

Regan, 1979). The number of accumulated breaks was saturated at a dose of 2-5 J/m2 and coincided with complete inhibition of photolesion removal (Snyder et al., 1981). Later it was shown that the saturation of breaks was due to the inhibition of NER-associated DNA synthesis (Moser et al., 2007; Mullenders et al., 1985; Smith and Okumoto, 1984). Together these data suggested that inhibition of the post-incision step of NER by HU & AraC leads to inhibition of further repair incision events. Despite numerous breakthroughs in understanding NER the actual mechanism that controls incision is still unknown. Tight regulation of new repair/incision events when gap-filling/sealing is incomplete is essential to prevent generation of DNA strand breaks that could lead to mutagenic and recombinogenic events (Gillet and Scharer, 2006). Yet it still remains an enigma; how does inhibition of the DNA synthesis step prevent subsequent incisions?

In this study we provide mechanistic insights into the regulation of NER stages in vivo and the central role of RPA therein. We show that dissociation of NER pre-incision factors (including XPC) from the damage sites requires incision. Inhibition of repair synthesis or ligation impairs removal of photolesions and leads to persistent accumulation of all NER factors at sites of UV damage that undergo repair. Under these conditions, we find that pre-incision proteins can dissociate and freely relocate to other damage sites, whereas RPA and post-incision proteins remain engaged at the initial sites of repair. Failure of RPA to relocate to other damages leads to incomplete NER complexes that are unable to perform dual incision. Our data reveal a central role for RPA in coupling NER mediated incision to DNA repair synthesis thereby precluding the initiation of further incision events that could lead to genomic instability.

RESULTS

Impaired DNA repair synthesis/ligation leads to prolonged binding of NER factors and inhibition of repair independent of ATR.

Treatment of UV-irradiated human cells with DNA Polδ and ε inhibitors (HU & AraC) leads to linear increase of NER mediated incisions (measured as accumulated breaks) that comes to saturation at UV-doses of 2-5 J/m^2. Moreover, treatment with these inhibitors appeared to be inhibitory to the bulk repair of photolesions (Moser et al., 2007; Snyder et al., 1981; Mullenders et al., 1985; Smith and Okumoto, 1984). To unravel the mechanism that underlies the saturation of incision events at low UV doses and the inhibition of damage removal in the presence of DNA polymerase inhibitors, we first addressed a possible role of ATR-dependent signalling events initiated by the perturbed gap filling (Matsumoto et al., 2007;O'Driscoll et al., 2003). For this purpose, we compared 6-4PP repair kinetics in the presence and absence of HU & AraC in UV-irradiated non-cycling normal human fibroblasts (NHF) and ATR deficient Seckle syndrome cells (O'Driscoll et al., 2003). Figure 1A shows similar levels of inhibition in NHF and Seckle cells, demonstrating that ATR deficiency does not rescue the HU & AraC mediated inhibition of 6-4PP repair. The severe reduction of H2AX phosphorylation in UV-irradiated Seckle cells compared to NHF cells (Figure 1B) but the normal level recruitment of XPB (a subunit of TFIIH) confirmed that these cells have an impaired ATR-dependent signalling, yet efficient NER complex formation. To

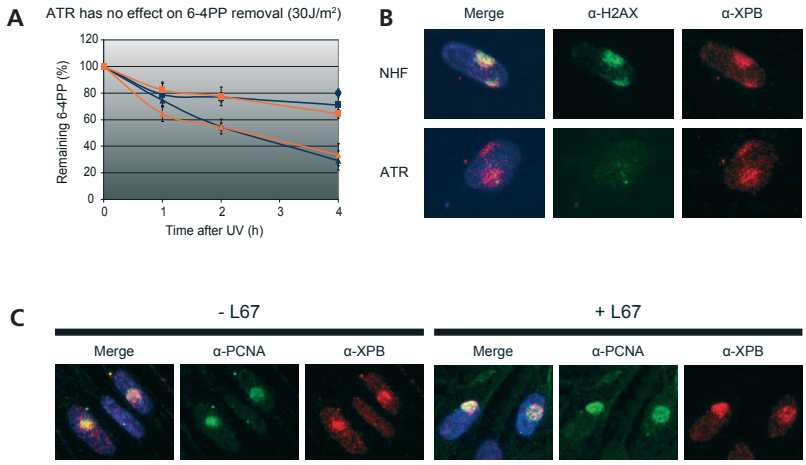

Figure 1. Impairment of DNA repair synthesis and ligation inhibits repair independently of ATR. (A) Removal of 6-4PP in time is measured in the presence (■) and absence (▲) of inhibitors following global UV-irradiation (30 J/m²) in NHF (blue lines) and ATR deficient cells (orange lines); NHF cells are treated with L67 for 4 hours (h) prior to irradiation (●). (B) Seckle syndrome cells (ATR) show impaired γH2AX staining at sites of UV damage compared to NHF. Cells are irradiated with 30J/m² 1 h prior to fixation and processed for immunofluorescent staining with antibodies against XPB and γH2AX. (C) NHF, treated and non-treated with L67 for 4 h prior to local UV irradiation (30J/m²), are stained for XPB and PCNA localisation.

test whether inhibition of 6-4PP repair is a consequence of incomplete gap filling step or merely the result of HU & AraC treatment, we treated confluent NHF with L67, a potent inhibitor of both DNA ligases Lig1 and Lig3 (Chen et al., 2008; Moser et al., 2007) prior to UV irradiation. As shown in Figure 1C, inhibition of DNA ligation by L67 has no effect on the recruitment of either pre or post-incision NER factors to the damage site, however it severely impairs removal of 6-4PP in NHF similar to the HU & AraC treatment (Figure 1A).

To assess the effect of inhibition of UV-induced repair synthesis on assembly and disassembly of NER subcomplexes from sites that undergo repair, we measured the kinetics of (dis)assembly of NER factors in UV-irradiated NHF treated and non-treated with DNA synthesis inhibitors. We then made a comparison with the (dis)assembly of these factors in NER deficient cells that are unable to perform dual incision such as the XP-A and XP-F fibroblasts.

In agreement with earlier reports, pre- and post-incision factors (such as XPB, RPA and PCNA, DNA Polδ, respectively) accumulate at local UV-spots (i.e. regions of locally induced UV damage) shortly after irradiation (30 min, Figure 2A, 2B). In the absence of inhibitors, XPB is barely visible 4 and 8 hrs following UV-irradiation (Figure 2A and S1A) closely mimicking the repair kinetics of 6-4PP in NHF (Figure 1A) (Volker et al., 2001; Wang et al., 2003). On the other hand, Polδ and PCNA can be found at UV-spots up to 8 hrs after irradiation (Figure 2B). Moreover, RPA (a pre-incision factor) is still present at UV-spots 8 hrs following UV irradiation (Figure 2B) similar to post-incision factors and in line with observations from in vitro reconstituted repair systems indicating involvement of RPA in

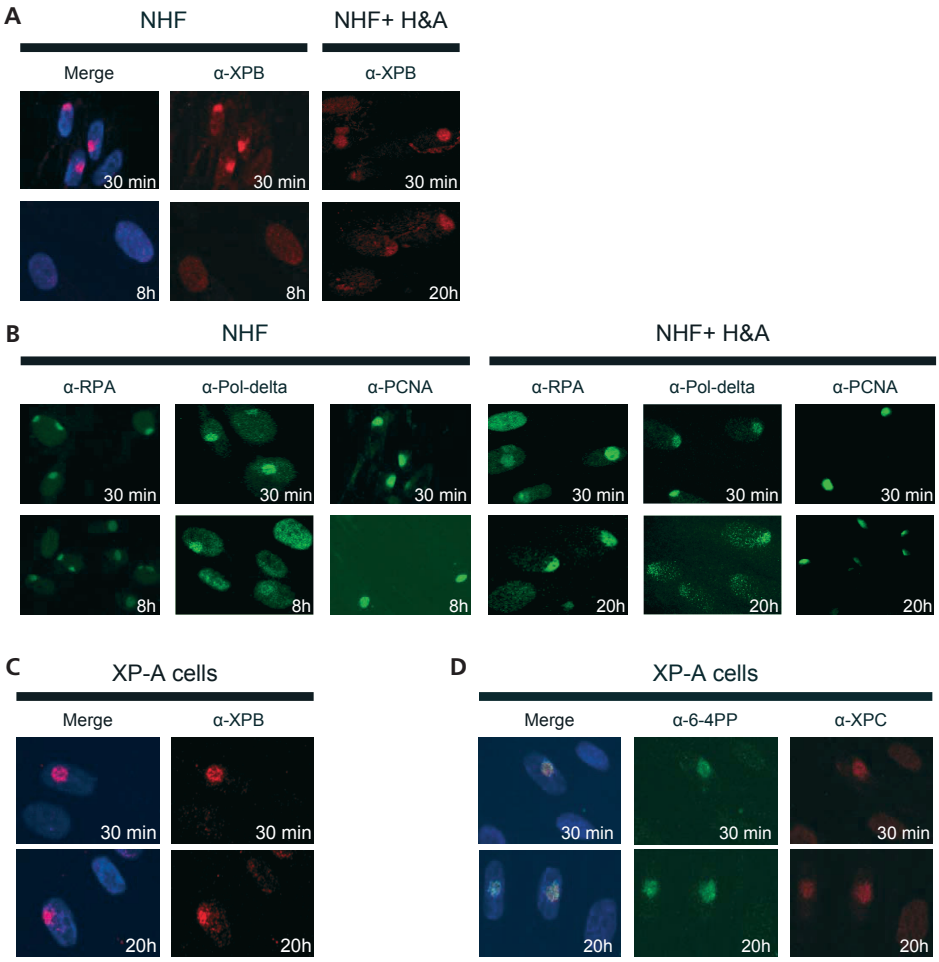

Figure 2. Prolonged accumulation of pre- and post-incision factors at NER sites in the presence of replication inhibitors. (A) Fluorescent immunostaining of XPB localization at damage sites in confluent NHF, and NHF treated with DNA synthesis inhibitors (HU & AraC) after local UV irradiation (30 J/m^2) at time points as indicated. 'Merge' refers to the combined image of DAPI and the antibody used for the panel. (B) Immunolocalization of RPA, Polδ and PCNA in NHF in the presence or absence of HU & AraC at different repair times after 30 J/m^2 local UV-irradiation. (C) Immunolocalization of XPB in confluent XP-A cells at different repair times after 30 J/m^2 local UV-irradiation. (D) XPC and 6-4PP are visualised by immunostaining following local UV-irradiation; cells are irradiated with 30 J/m2 UV and incubated for either 30 min or 20 h.

the repair synthesis step (Shivji et al., 1995). Interestingly, this kinetic analysis revealed that the prolonged binding of post-incision factors (PCNA) at damage sites is independent of TC-NER or CPD repair, as demonstrated by similar kinetics in α-amanatin treated cells and XP-E cells (lacking CPD repair) , respectively (Figure S1A). Despite defective repair of 6-4PP (Figure 1A) and similar to the treatment with L67 inhibitor (Figure 1C), treatment of non-cycling NHF with HU & AraC does not prevent accumulation of NER subcomplexes at sites

of UV-damage when monitored 30 minutes after irradiation (Figure 2A, 2B). Nevertheless, impairment of the DNA repair synthesis step prevents dissociation of NER subcomplexes, since all factors tested (including pre-incision factor XPB) remained visible at the damage spots for up to 20 hrs (Figure 2A, 2B). Closely resembling the effect of DNA repair synthesis inhibition, defective incision leads to persistent binding of pre- incision factors (XPB, XPC) to UV-spots 20 hrs after UV irradiation in XP-A cells (Figure 2C, 2D) whereas accumulation of post-incision factors is absent in these cells (Green and Almouzni, 2003; Moser et al., 2007).

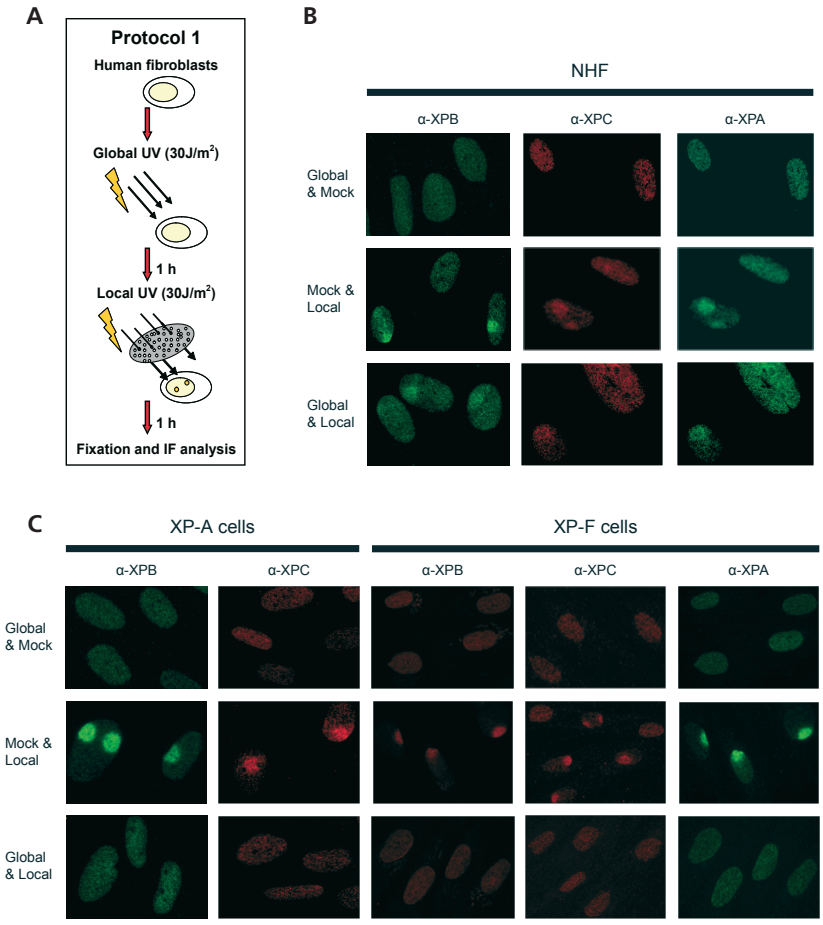

Figure 3. Pre-incision factors remain associated to initial repair sites in the absence of incision. (A) Schematic representation of Protocol 1. Cells are globally UV-irradiated (30 J/m^2) in the absence of HU &AraC, recover for 1 h and subsequently irradiated with the same dose of local UV. (B) Release of XPB, XPC and XPA from repair complexes is dependent upon functional incision. Confluent NHF are treated according to Protocol 1 (A) and immunostained with the indicated antibodies. Exposure times within experiments are equal. (C) Immunolocalisation of XPB, XPC and XPA antibodies in XP-A, XP-F cells treated according to Protocol 1 (A).

NER-mediated incision is required for the release and re-localisation of pre-incision factors to new repair sites.

The persistent localisation of pre-incision factors at local UV spots that we observed in the incision deficient XP-A cells (Figure 2C, 2D) is suggestive of the stable formation of pre-incision NER complex at sites of incomplete repair. However, these data are also consistent with a dynamic equilibrium of processes that allow assembly and disassembly of NER factors at photolesions within the UV-spot (which contains a number of damage sites). To distinguish between the different possibilities we designed an experimental approach i.e. in vivo competition by using two sequential doses of UV irradiation with sufficient time in-between the UV-exposures to allow assembly of repair complexes. The rationale behind this experimental set-up is that NER factors released from DNA damages induced by the first UV irradiation would be targeted to damages induced by the second UV-irradiation, allowing differentiation between stable and dynamic association of endogenous NER factors at sites that undergo repair. In these experiments, cells are exposed to global UV-irradiation of 30 J/m^2, a dose that saturates GG-NER (Smith and Okumoto, 1984; Erixon and Ahnstrom, 1979), cultured for 1 hr and subsequently exposed to local UV (30 J/m^2) or mock-irradiation (Figure 3A i.e. Protocol 1). Following the second UV-irradiation, cells are incubated for 1hr prior to analysis. For Protocol 1, cells were UV-irradiated in the absence with DNA synthesis inhibitors (Figure 3A). In some experiments the order of UV-irradiations was reversed (Figure 4A i.e. Protocol 2).

To validate the experimental setup (Protocol 1) we analysed the distribution of pre-incision NER factors in NHF. Accumulation of XPB, XPC and XPA is observed at damage sites induced by the second (local) UV irradiation (Figure 3B), demonstrating that the experimental protocol allowed detection of the released repair factors from the initially formed (globally irradiated) NER sites after completion of repair. On the other hand, experiments in XP-A and XP-F cells revealed that the same global UV-irradiation of 30 J/m^2 engaged all available XPB, XPC and XPA to UV lesions induced by the initial global irradiation as these cells lack accumulation of the repair factors at damages induced by the subsequent local UV-irradiation (Figure 3C). Similarly, local accumulation of pre-incision factors in XP-F cells (reverse order of UV irradiation, Protocol 2, Figure 4A) could not be competed out by the second globally induced UV damage (Figure S2A, in the absence of inhibitors). Taken together these data demonstrate that release of pre-incision factors from NER sites in vivo is dependent upon incision and/or formation of a functional pre-incision complex. Furthermore, our data suggest that assembly of NER pre-incision complexes at UV damages in the absence of XPA or XPF/ERCC1 leads to incomplete fixed complexes that are unable to dissociate from the specific repair sites.

Inhibition of DNA repair synthesis differentially affects dissociation of NER subcomplexes from damage sites.

We next examined the dynamic nature of the prolonged accumulation of NER factors that was induced by the DNA repair synthesis inhibition (Figure 2A, B). For this purpose, we carried out competition experiments with NHF in the presence of HU & AraC according to Protocol 2 (Figure 4A). Notably, XPA and XPB are able to dissociate from NER sites with

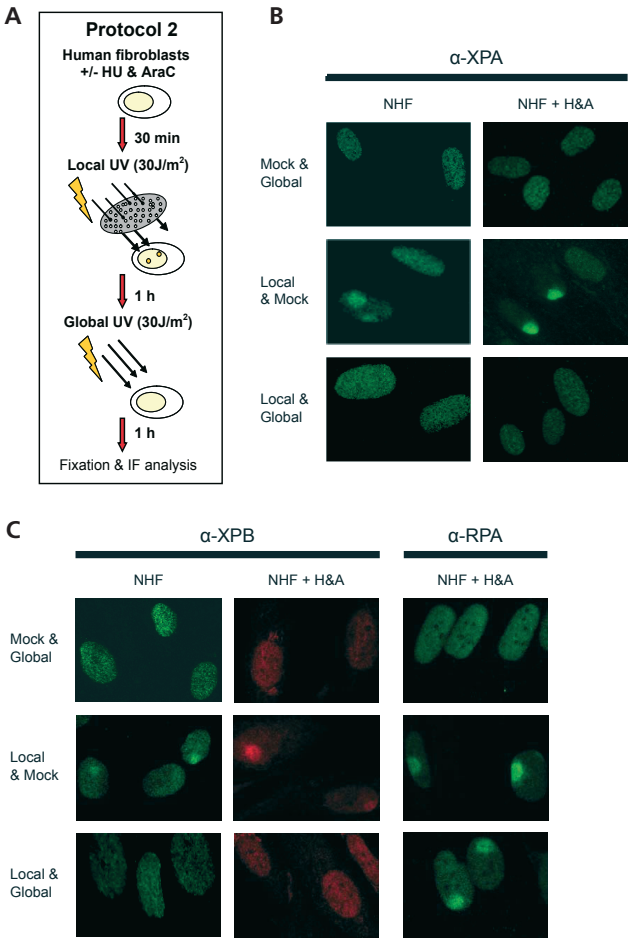

Figure 4. Dissociation of pre-incision factors from repair sites requires incision whereas dissociation of RPA requires DNA synthesis. (A) Schematic representation of the competition experiments according to Protocol 2. Cells are incubated in the presence or absence of inhibitors 30 min prior to the first (local) UVirradiation, which is subsequently followed by the second (global) irradiation. (B) Confluent NHF are irradiated according to Protocol 2 in the absence (NHF) or presence of inhibitors (NHF + H&A) and stained with XPA antibody. (C) NHF treated as in (B) stained with XPB and RPA antibodies.

incomplete NER synthesis engaging in new repair events (Figure 4B, 4C). These data clearly demonstrate that damage incision and not completion of the gap filling/sealing stage of NER is the prerequisite for the release of pre-incision factors. In contrast, RPA remained firmly bound at the initially induced damaged sites and could not be challenged away by the second global UV-irradiation before DNA repair synthesis was complete (Figure 4C) underlining its essential role in the post-incision step of NER.

To exclude possible interference between local (through 8 μm pores) and global UV-irradiation we replaced the global by an additional local irradiation (30 J/m^2) through

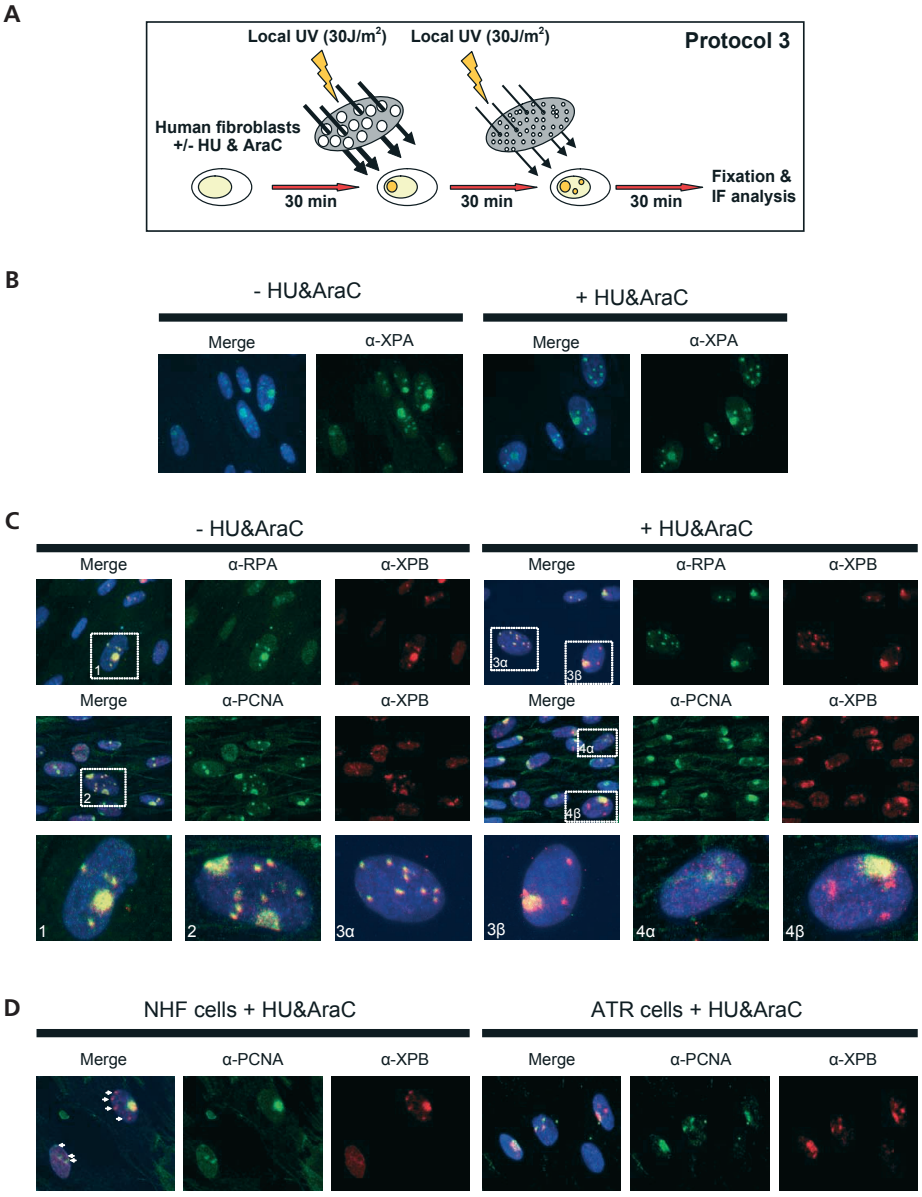

Figure 5. Impairment of DNA synthesis prevents recruitment of RPA and post-incision factors to de novo UV damages. For clarity, representative cells (numbered boxes) are enlarged and depicted below. (A) Schematic representation of competition experiments according to Protocol 3. Cells are treated or mock-treated with DNA synthesis inhibitors 30 min prior to the first local (8 μm) UV-irradiation, which is followed (after 30 min) by the second local (3 μm) irradiation. (B) Competition experiments (Protocol 3) confirm dynamic association of XPA with damage sites in the presence and absence of DNA synthesis inhibitors in confluent NHF. (C) Confluent NHF treated as in (B) and co-stained with RPA, PCNA and XPB antibodies. (D) Confluent NHF and Seckle syndrome cells (ATR) are treated according to Protocol 3 in the presence of HU & AraC, followed by immunofluorescent staining of PCNA and XPB. For clarity small spots are indicated with arrows.

3 μm pores (Figure 5A, Protocol 3). Under these conditions, XPA dissociates from the initial (larger) and is recruited to the subsequently induced (smaller) damage sites, both in the absence and presence of DNA synthesis inhibitors (Figure 5B, cells that have been irradiated twice contain both large and smaller spots), confirming the results of Figure 4B. We then repeated the same experimental approach for XPB, RPA and PCNA as well as XPG and ERCC1 (Figure 5C and S3A). In the absence of inhibitors all factors behave similar to XPA and are recruited to both large and small UV spots within the same cell and with similar intensities. In the presence of the inhibitors however, XPB, XPG and ERCC1 behave similar to XPA (Figure 5C and S3A) whereas PCNA and RPA cannot be competed out by the second irradiation since they are only visible and remain confined at the initial repair sites where synthesis is impaired (Figure 5C). Although we cannot exclude that XPG might reside longer than ERCC1/XPF at NER sites (Staresincic et al., 2009), our data clearly show that XPG (Figure S3A) behaves similar to the other NER pre-incision factors i.e. it is able to dissociate from the incomplete NER sites in the presence of HU & AraC.

Competition experiments (Protocol 3) in the presence of HU & AraC to assess the dynamic nature of the prolonged accumulation of NER factors (pre- and post-incision) in cells with impaired ATR-dependent signalling (Seckle syndrome cells) show similar results to NHF (Figure 5D).

We showed (Figure 3C) that, in vivo, XPC is able to leave the NER sites only after incision has taken place and a functional pre-incision complex has been formed. This result contrasts in vitro observations suggesting that XPC leaves the complex before incision takes place (Wakasugi and Sancar, 1998). To further verify our result, we analysed the localization of XPC in incision deficient XP-A cells employing Protocol 3 (in the absence of inhibitors) and either 30 or 100 J/m^2 of local UV-irradiation (equivalent to a global dose of 4.5 J/m^2 or 15 J/m^2, respectively). The results clearly show that following 100 J/m^2 local UV-irradiation (a dose that saturates NER) (Smith and Okumoto, 1984) XPC is permanently immobilised at the initial non-incised damage sites (Figure S3B) confirming that, similar to the other pre-incision factors, XPC is not able to dissociate from UV-damaged sites in vivo when NER mediated incisions are impaired.

Impediment of NER gap filling and ligation inhibits further incision events.

Single strand DNA gap intermediates generated by NER mediated incisions in non dividing human cells trigger the phosphorylation of histone H2AX (Ser 139) in an ATR-dependent manner (Matsumoto et al., 2007). To examine whether accumulation of the released preincision factors to subsequently induced DNA damages that we observed in the presence of DNA synthesis inhibitors leads to new incisions, we performed competition experiments (Protocol 3) and γH2AX/XPB co-staining in quiescent NHF. Under normal conditions, γH2AX is observed at both the initial and subsequently induced UV-spots, coinciding with XPB (Figure 6A). However, in the presence of HU & AraC, phosphorylation of H2AX occurs only at the initial 8 μm UV spot but is absent from the subsequent 3 μm spots in contrast to XPB.

To examine whether impairment of NER mediated ligation would also generate a response similar to HU & AraC, we performed competition experiments (Protocol 3) in

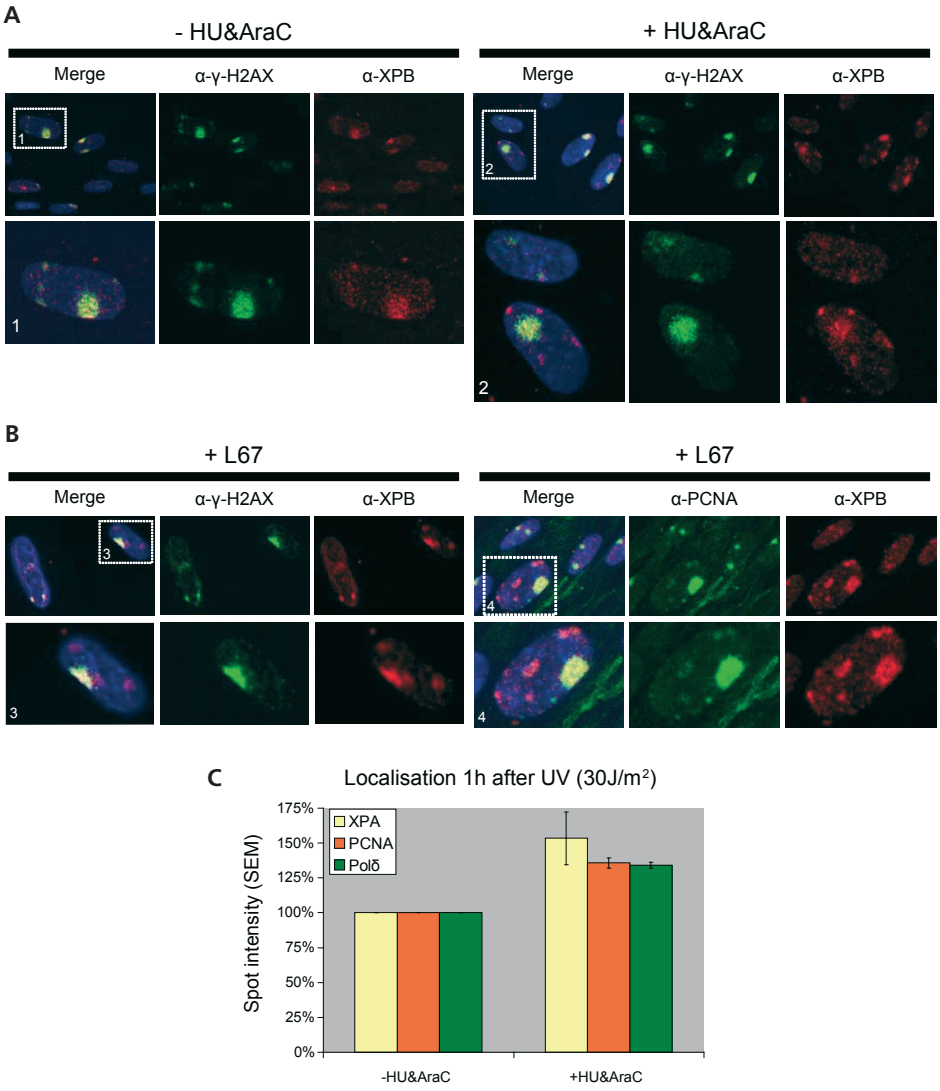

Figure 6. Inhibition of DNA repair synthesis or ligation prevents novel incision events and leads to prolonged accumulation of post-incision factors. (A) Quiescent NHF were irradiated according to Protocol 3. Incision events are visualized by γH2AX staining; counterstaining for XPB revealed areas of damage induction. γH2AX accumulation increases in time, thus the intensity is lower at the second UV spots when compared to the initially induced damage. Also, incubation with inhibitors increases γH2AX accumulation, therefore microscopic exposure time for γH2AX is 3 fold shorter when cells are irradiated in the presence of inhibitors. (B) NHF are irradiated according to Protocol 3 in the presence of L67 and stained for PCNA, XPB and γH2AX. For clarity small spots are indicated with arrows. (C) NHF are locally irradiated in the presence or absence of inhibitors, fixed 1 hr later and stained for XPA in combination with PCNA or Polδ. Average intensity inside local spots is measured and normalized to normal conditions (no inhibitors). Error bars represent the SEM values of 40 nuclei per experiment; out of at least three independent experiments.

the presence of L67 inhibitor (Chen et al., 2008). Treatment of NHF with L67 for four hours before UV irradiation leads to recruitment of pre-incision proteins to newly induced UVspots; in contrast, post-incision factors and RPA are immobilised at the initial UV-spots (Figure 6B). Similarly, phosphorylation of H2AX occurs only at the initial UV-spots, suggesting that under conditions of incomplete DNA repair synthesis, re-localisation of preincision factors (with the exception of RPA) to new NER sites does not lead to further incisions.

In line with published data (Matsumoto et al., 2007), we find that the observed intensity of γH2AX at the initial UV damages is enhanced 3 fold in the presence of inhibitors suggesting that the level of H2AX phosphorylation greatly increases when gap-filling is perturbed. The same increased intensity is observed for all NER factors at UV-damaged spots in the presence of HU& AraC (single UV dose, Figure 6C).

Inhibition of DNA synthesis modulates the ability of RPA to associate with newly formed NER complexes.

We find that RPA is immobilised at incomplete DNA repair synthesis sites whereas the rest of pre-incision factors are able to re-associate to newly induced UV damages. To exclude any limitation of our approach to detect re-localisation of RPA to small spots (Protocol 3), we assessed the effect of RPA knockdown (KD) on the recruitment of NER subcomplexes to

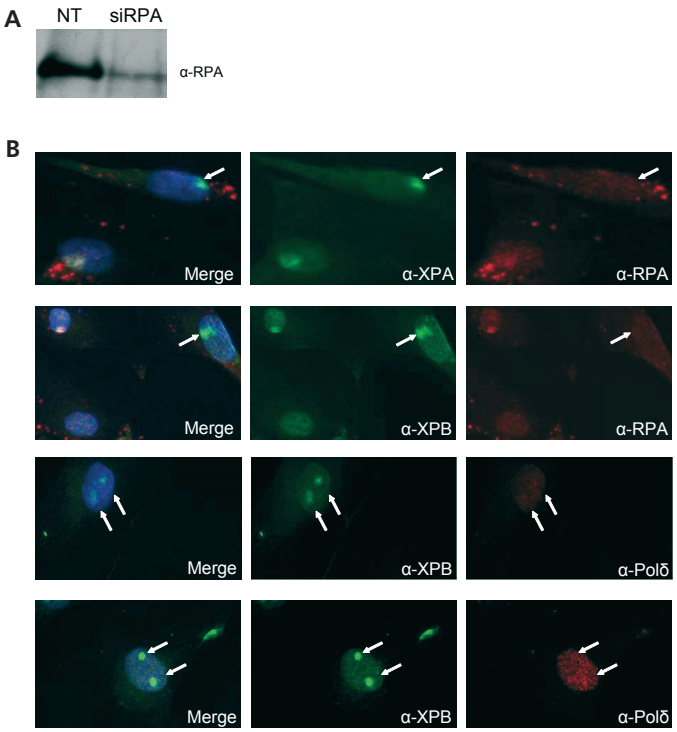

Figure 7. RPA is prerequisite for the functional assembly of NER subcomplexes. (A) Western blot analysis of equal amounts of whole cell lysates prepared from cells treated or non-treated (NT) with siRNA against RPA p70. (B) Cells treated with siRNA against RPA p70 are locally UV-irradiated (30 J/m2) fixed 1 h later and stained with XPA, XPB or Polδ antibodies. Absence of RPA is verified with RPA co-staining (arrows).

locally induced UV damage. KD of RPA p70 (Figure 7A) had no effect on the accumulation of pre-incision NER factors such as XPB and XPA at UV spots, but prevented recruitment of Polδ (Figure 7B). Hence, RPA KD does not impinge on the recruitment of pre-incision factors to UV damage, yet it is absolutely required for the assembly of the post-incision components.

We hypothesised that depletion of RPA by virtue of its binding in post-incision complexes in the presence of inhibitors prevents its subsequent assembly into pre-incision complexes and hence impedes further incisions (γH2AX staining, Figure 6A, B). To support this hypothesis, we assessed the amount of RPA and XPB in the soluble nuclear fraction isolated from non-dividing NHF one hour after exposure to UV in the presence or absence of HU & AraC. In the absence of inhibitors, we monitored a dose dependent decrease of RPA and XPB in the nuclear fraction with the largest depletion in cells exposed to 30 J/m^2 (Figure 8A). Interestingly, in the presence of the DNA synthesis inhibitors, this maximum depletion is observed with a dose as low as 5 J/m^2 and no further depletion is found at increasing doses (Figure 8A). Due to the high amounts of RPA present in chromatin prior to UV exposure we were unable to measure significant changes in this fraction after UV.

To further examine the presence of RPA in the two putative NER subcomplexes at sites of UV damage, we performed a modified protocol of the classic chromatin immunoprecipitation (ChIP) of in vivo crosslinked confluent NHF and analysed the coprecipitated proteins by western blotting (ChIP-western) (Fousteri et al., 2006; Moser et al., 2007; Coin et al., 2008). In line with our previously published data (Moser et al., 2007),

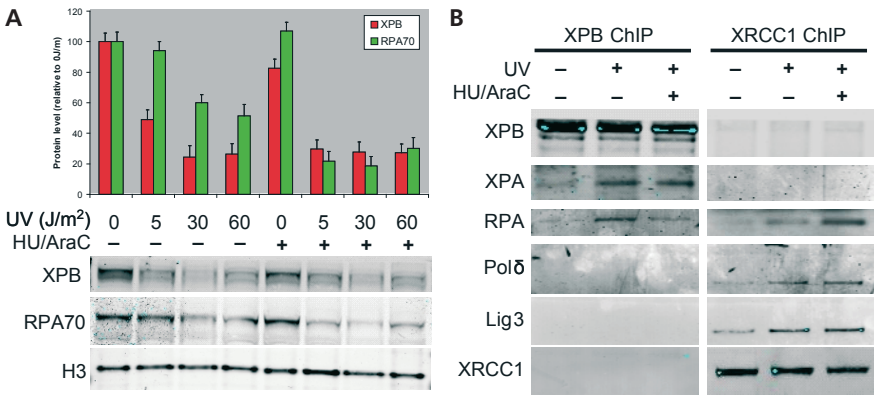

Figure 8. DNA synthesis inhibitors sequester RPA to sites of incomplete NER repair synthesis. (A) NHF irradiated with increasing UV doses (as indicated) in the presence and absence of inhibitors. Nuclear fractions are then isolated 1 h after UV and analysed by western blot analysis using XPB and RPA primary antibodies and fluorescently labelled secondary antibodies, signals are quantified using Odyssey 2.1 and normalised to the loading control. The amount of free nuclear RPA in non-irradiated cells without inhibitors is set at 100%. (B) Confluent NHF are (mock) irradiated with 20 J/m2 in the presence or absence of inhibitors, let to recover and crosslinked 40 min later. ChIP is performed with XPB and XRCC1 specific antibodies and western blot analysis of the co-precipitating proteins is performed with antibodies as indicated.

XPB specific ChIP yields an increased interaction of XPB with pre-incision factors such as XPA and RPA in UV-irradiated cells whereas post-incision factors i.e. Polδ, Lig3 and XRCC1 are virtually absent (Figure 8B), confirming that, also in vivo, NER subcomplexes involved in pre- or post-incision stages reside at different repair sites. When cells are treated with HU & AraC prior to UV irradiation, the XPB interaction with XPA is further enhanced (Figure 8B). This is consistent with the increased accumulation of NER factors and γH2AX staining that we observed at UV spots in the presence of the inhibitors (Figure 6C). However, treatment of cells with HU & AraC has the opposite effect on the interaction between XPB and RPA. These results suggest that the number of chromatin-bound NER subcomplexes that contain both XPB and RPA are reduced when NER synthesis is impaired. The opposite is evident when we performed ChIP-on-western with an XRCC1 antibody; a clear UV increased interaction can be found between XRCC1 and RPA as well as Polδ and Lig3, which is further enhanced in the presence of HU & AraC (Figure 8B). This is in agreement with the increased accumulation and immobilisation of DNA synthesis factors (including RPA) that we observed at sites of incomplete DNA repair synthesis. Similar to XPB specific ChIP, no interaction is observed between XRCC1 and pre-incision factors (Figure 8B) as reported previously (Moser et al., 2007).

DISCUSSION

The results reported here provide novel mechanistic insights in the regulation of NER in human cells. We find that in non-dividing NHF impairment of late NER events (i.e. gap filling and/or ligation) results in a persistent accumulation of NER factors at sites of UV damage and overall repair inhibition in an ATR independent manner. Under these conditions, pre-incision factors may dynamically assemble and disassemble freely associating with other damage sites whereas RPA and post-incision factors remain associated with the perturbed repair intermediates. RPA, an essential component of both NER subcomplexes, plays a unique role in controlling the transition from pre- to post-incision stages by linking the initiation of new repair events to completion of on-going DNA gap filling/sealing events. Incomplete pre-incision complexes, containing RPA, that are unable to perform incision (i.e. XP-A, XP-F cells) are stably bound at damage sites, demonstrating that their disassembly is dependent upon NER-mediated incision. These results reveal an unprecedented role for RPA in regulating NER by coupling novel incisions to completion of already initiated repair events.

Differential requirements for (dis)assembly of NER subcomplexes.

Dissociation of XPC from NER core complexes prior to incision has been observed in in vitro experiments (Riedl et al., 2003; Wakasugi and Sancar, 1998). On the other hand competition experiments in incision deficient XP cells demonstrated that, in vivo the situation might be different given that XPC is stably assembled in NER pre-incision complexes and cannot be challenged away (Figure S3B). Hence, NER-mediated incision is the key determinant for the release of pre-incision NER proteins except RPA. NER incision is also required for efficient recruitment of the post-incision factors (Aboussekhra and

Wood, 1995) and, compared to the pre-incision factors, post-incision factors including RPA remain visible at repair sites for extended periods of time. The average time to repair a UV-lesion i.e. from detection to final DNA ligation is estimated to be four minutes (Erixon and Ahnstrom, 1979), closely mimicking the residence time of most NER pre-incision factors at UV-damage (Mone et al., 2004; Politi et al., 2005). We find that the prolonged association of post-incision factors with repair sites is not due to ongoing NER (i.e. TC-NER or the slower repair of CPD by GG-NER) (Figure S1) suggesting that completion of NER events (i.e. gap-filling and ligation) does not lead to disassembly of proteins involved in the post-incision step in spite of their dynamic nature (Essers et al., 2005; Mone et al., 2004; Zotter et al., 2006). We speculate that this extended association might have a functional role in the restoration of chromatin structure at the repaired sites. In line with this, PCNA has be shown to be the preferred target for chromatin assembly factor CAF1 (Moggs et al., 2000) and NER mediated DNA synthesis occurs in concert with chromatin assembly (Green and Almouzni, 2003) suggesting that these two processes are mechanistically linked.

RPA is indispensable for NER complex stability and incision.

When NER mediated incision is followed by inhibition of repair patch synthesis/ligation, the pre-incision factors but none of the post-incision or RPA can dissociate from sites of ongoing repair and re-associate with unprocessed UV photolesions. Notably, XPF/ERCC1 can also be recruited independently of RPA, raising questions on the exact mechanism of its recruitment. While RPA greatly enhances the binding of XPF to artificial structures in vitro (de Laat et al., 1998; Matsunaga et al., 1996), XPF/ERCC1 was not recruited to sites of damage in XP-A cells despite the presence of RPA (Volker et al., 2001). Taken together, these results suggest that one of the key roles of RPA in NER (in concert with XPA) is its involvement in the correct orientation and activation of the endonucleases XPG and XPF/ERCC1 rather than their recruitment. A number of studies suggest that XPA and TFIIH are sufficient to recruit XPF and XPG, respectively (Li et al., 1994; Park and Sancar, 1994; Volker et al., 2001; Iyer et al., 1996), however in the absence of RPA this will lead to a non-functional pre-incision complex that lacks incision activity (this work).

The number of incisions made by NER in the presence of HU & AraC reaches a maximum one hour after irradiation and at doses as low as 2-5 J/m^2 (Smith and Okumoto, 1984; Mullenders et al., 1985; Berneburg et al., 2000; Snyder et al., 1981) indicating that under these conditions NER is non-catalytic, i.e. NER can only create a limited number of incisions at one time. This limited number of incisions goes along with the impaired removal of 6-4PP (Moser et al., 2007) (Figure 1A), the depletion of the pool of soluble nuclear RPA and XPB (Figure 8A), the enhanced H2AX phosphorylation (Matsumoto et al., 2007) and the increased accumulation of NER factors at UV damage spots (Figure 6C). In addition, no H2AX phosphorylation is observed when pre-incision factors are re-localised to sites of the second UV-irradiation in the presence of NER synthesis or ligation inhibitors (Figure 6A, 6B) suggestive of no incision events at these sites.

We considered two possible mechanisms underlying the limited occurrence of incision events and the impaired repair of photolesions observed when the late steps of NER are inhibited. First, it is conceivable that DNA damage induced signalling (manifested by H2AX

phosphorylation) prevents further incisions when gap filling is not completed. UV-induced signalling has been shown to depend on ATR. Most likely, this signalling is activated by the formation of RPA-bound single stranded DNA patches formed by NER mediated incisions (Matsumoto et al., 2007; Marini et al., 2006; Marti et al., 2006; O'Driscoll et al., 2003) or alternatively by displacement of single strand DNA generated temporally by the initial 5' to the lesion XPF/ERCC1 incision followed by the 3' to the lesion XPG incision (Staresincic et al., 2009). Nevertheless, ATR deficiency has no effect on the persistent accumulation of post-incision proteins and does not lead to further incisions or increased removal of 6-4PP. We therefore conclude that regulation of NER incision does not depend on ATR signalling in confluent human cells.

A second mechanism could be that one or more NER factors becomes 'trapped' inside existing yet inhibited NER complexes and hence unable to associate with other photolesions.

It has been shown that incision by ERCC1-XPF occurs first, allowing the free 3-OH that is generated to initiate repair synthesis before XPG performs the incision 3'to the lesion (Staresincic et al., 2009). This could potentially lead to XPG 'trapping' when repair synthesis step is inhibited. Nevertheless, our data show that in normal cells, under conditions that impair repair synthesis (yet allow the recruitment of post-incision factors), XPG is able to leave the complex suggesting that in the presence of inhibitors the repair site might be a gap generated by dual incision. Based on the current findings, RPA is the only NER protein that is recruited prior to incision and remains associated at the site of repair after incision and recruitment of post-incision factors consistent with in vitro studies (Riedl et al., 2003). The persistent binding of RPA at the initial sites of damage induction and repair in the competition experiments in the presence of DNA synthesis/ligation inhibitors (Figure 4C, 5C, 6B) implies that RPA remains bound to DNA until completion of gap-filling and ligation. Thus, in the presence of inhibitors, all available (free-nuclear) RPA becomes sequestered and trapped in post-incision complexes, thereby preventing it from associating in new repair initiation events. As a consequence, abortive pre-incision repair complexes are formed, which are incapable of incision. In support of this hypothesis, ChIP-on-western experiments (Figure 8B) show an increased interaction of RPA with post-incision factors and a decreased interaction with pre-incision factors under conditions that impair NER mediated DNA synthesis and ligation.

The current observation that NER is independent of ATR in confluent or non-cycling cells (G1, G0) was confirmed by a recent study (Auclair et al., 2008) showing that efficient NER depends on ATR in S but not G1 cells. It should be noted that RPA is released from PML bodies upon UV-irradiation and recruited to UV-induced damages (Park et al., 2005) and this release occurs and requires the kinase activity of ATR in S-and G2-phase but not G1 cells (Barr et al., 2003). Together these observations suggest that during S-phase, due to its large engagement in replication, RPA is unable to associate with repair complexes, and that additional RPA is released from PML bodies in the cell in an ATR dependent manner in order to carry out NER. This is obviously not the case in G1 cells where ATR deficiency has no effect and nuclear RPA is recruited to the damage sites. We speculate that, similar to stalled replication complexes, blocked repair synthesis complexes in G1/

G0 cells also sequesters the majority of free nuclear RPA (indicated by the recruitment of RPA to chromatin after low UV doses in the presence of HU & AraC, Figure 8A), thereby preventing additional NER-mediated incision events to take place.

We propose a model wherein RPA regulates NER by safeguarding the transition from pre- to post-incision stages and coordinating the initiation of new repair events only after completion of on-going repair synthesis (Figure 9). According to this model, pre-incision factors are recruited to sites of DNA damage. In the presence of RPA, NER mediated incision occurs, allowing recruitment of post-incision and release of pre-incision factors. Under normal conditions, the released RPA is able to associate with, and subsequently stabilize and activate newly formed pre-incision complexes enabling further incision events and continuation of repair. In contrast, the persistent association of RPA in post-incision complexes at sites of incomplete repair synthesis prevents release of RPA and thus shields the genome from uncontrolled incisions by coupling repair initiation to completion of DNA repair synthesis and ligation events.

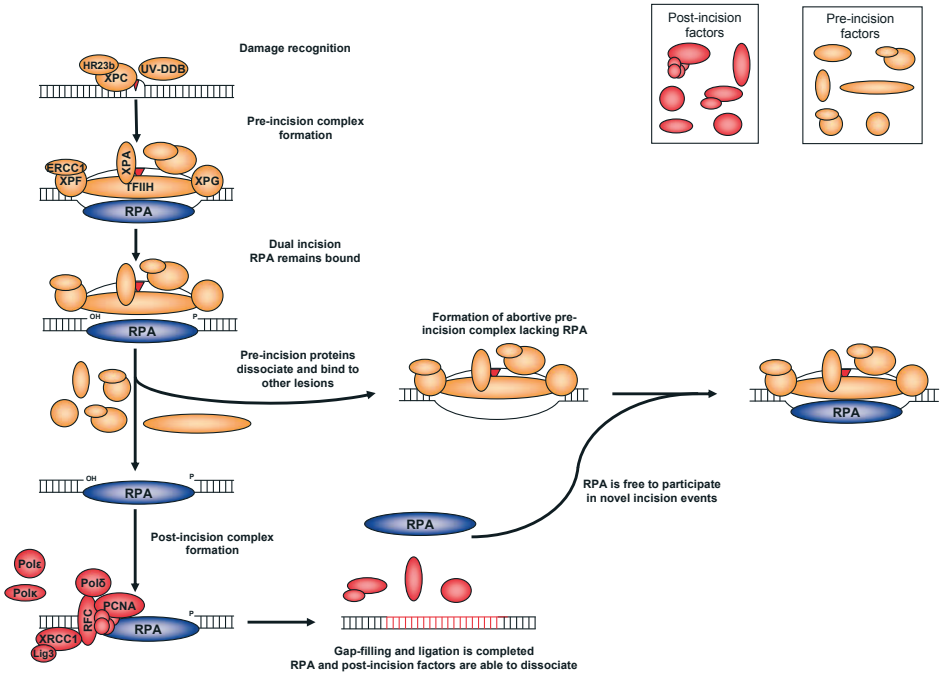

Figure 9. Schematic depiction of regulation of NER mediated incision events in vivo. Upon lesion recognition by UV-DDB and XPC-hHR23B, local opening of DNA by TFIIH provides access to the core GG-NER machinery i.e. XPA, RPA, XPG and ERCC1-XPF. RPA binds to the undamaged single-stranded DNA stabilizing the complex. Subsequently incision is followed by the release of core NER factors, which are then free to associate with other damages with the exception of RPA that, remains bound to the repair site, probably on the undamaged single-stranded DNA. The later stages of repair are carried out by RFC loading PCNA onto the incised DNA, the recruitment of DNA polymerases polδ/polε/polκ and XRCC1-Lig3/Lig1 to fill in and ligate the gap, respectively. After ligation, post-incision factors and RPA are able to dissociate. RPA can then stabilize the otherwise abortive preincision complexes, enabling the initiation of new NER events.

MATERIALS AND METHODS

Cell culture. Cells used in this study have been grown in DMEM supplemented with 10% fetal calf serum and antibiotics at 37°C in a 5% CO2 atmosphere and include i) primary and telomerase hTert immortalized human fibroblasts each of normal NHF (VH10), as well as NER deficient XP-A (XP25RO), XP-F (XP24KY and XP51RO) and XP-E (XP23PV) fibroblasts and ii) ATR deficient Seckle cells (GM18366).

UV-irradiation and immunofluorescence. Global and local UV-irradiation using a 3 or 8μm filter is performed essentially as described (Moné et al., 2001; Volker et al., 2001). After irradiation, the cells are returned to culture conditions for time periods indicated. Cytosine-β-arabinofuranoside (Fluka) and hydroxyurea (Fluka) at final concentrations of 10 μM and 100 mM respectively, are added to the medium 30 minutes prior to irradiation and remained present throughout the time course of the experiment. Ligase inhibitor L67 (Chen et al., 2008) is added 4 hours prior to irradiation at a final concentration of 25 μM. Where required α-amanitin has been added 5 hours prior to irradiation at a concentration of 1 μg/ml. Blockage of transcription by α-amanitin has been verified by measuring 3H incorporation after pulse labelling with 3H-Urd for one hour (van Oosterwijk et al., 1996), which predominantly represents RNA polymerase II transcription.

Immunofluorescence. Immunofluorescent labelling is performed essentially as described (Moser et al., 2007; Volker et al., 2001). Briefly, cells are washed with cold PBS, fixed and lysed on ice by either, 100% methanol for 10 min, or by 2% paraformaldehyde for 20 min at room temperature (RT), followed by 0.2% Triton X-100 incubation for 5 min at RT. Following fixation, cells are washed with cold PBS, and incubated with 5% bovine albumin in PBS for 30' at RT. The cells are subsequently incubated with primary antibodies, diluted in washing buffer (WB) (PBS containing 0.5% bovine serum albumin and 0.05% Tween-20) for two hours at RT. The cells are washed 3x with WB and thereafter incubated with secondary antibody for 1 hour at RT. Cells are mounted in Aqua/polymount (Polysciences Inc., Warrington, PA) containing DAPI (1.5 μg/ml). Microscopy and quantification of fluorescent signal has been described elsewhere (Moser et al., 2007).

Antibodies. The following primary antibodies are used in this study: rabbit polyclonal α-XPA, α-p89 (XPB), α-ERCC1 and mouse monoclonal α-DNA Polδ (Santa-Cruz), mouse monoclonal α- PCNA (PC10), α-XRCC1, α-XPA (Abcam), mouse monoclonal α-DNA ligase 3α (Genetex), mouse monoclonal α-DNA ligase 1 (MBL), mouse monoclonal α-XPG (8H7, Molecular Probes), mouse monoclonal α-RPAp70 and α-RPAp34 (respectively Ab-1 and Ab-3, Oncogene), mouse monoclonal α-6-4PP (Cosmo Bio). Mouse monoclonal α-p89 (XPB), a gift from Dr. J-M. Egly (IGMC, Illkirch, France), affinity-purified rabbit polyclonal α-XPC, a gift from Dr. W. Vermeulen (Erasmus MC, Rotterdam, The Netherlands). Secondary antibodies include Cy3-conjugated goat α-rabbit IgG and FITC-conjugated donkey α-mouse (Jackson Laboratories) and Alexa Fluor 488 goat α-mouse IgG and AlexaFluor 555 goat α–rabbit IgG (Molecular Probes). All secondary antibodies have been used according to the manufacturer's instructions.

In Vivo Cross-linking and ChIP-on -western. Confluent hTert-immortalized NHF treated or mock-treated with HU and AraC are UV-irradiated (20 J/ m^2) and incubated at 37ºC for 40 min prior to in vivo cross-linking. Lysis of the crosslinked cells, chromatin purification and fractionation as well as chromatin immunoprecipitation and reversal of the crosslinks prior to DNA analysis and protein analysis by western blotting are performed as described previously (Fousteri et al., 2006; Moser et al., 2007).

RNA interference. SmartPoolTM siRNA oligos (Dharmacon) are used for all experiments unless otherwise noted. siRNA transfection is performed using HiPerfectTM (QIAGEN) transfection reagent according to the manufacturer's instruction. In typical experiments, 5 nM of siRNA oligos are transfected in suspension, termed 'reverse transfection', and followed by one additional transfection cycle 24 h after the first transfection (double transfection). Experiments are performed 48 h after the first siRNA transfection. Knockdown efficiencies are confirmed by western blotting and immunofluorescence.

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SUPPLEMENTARY FIGURES

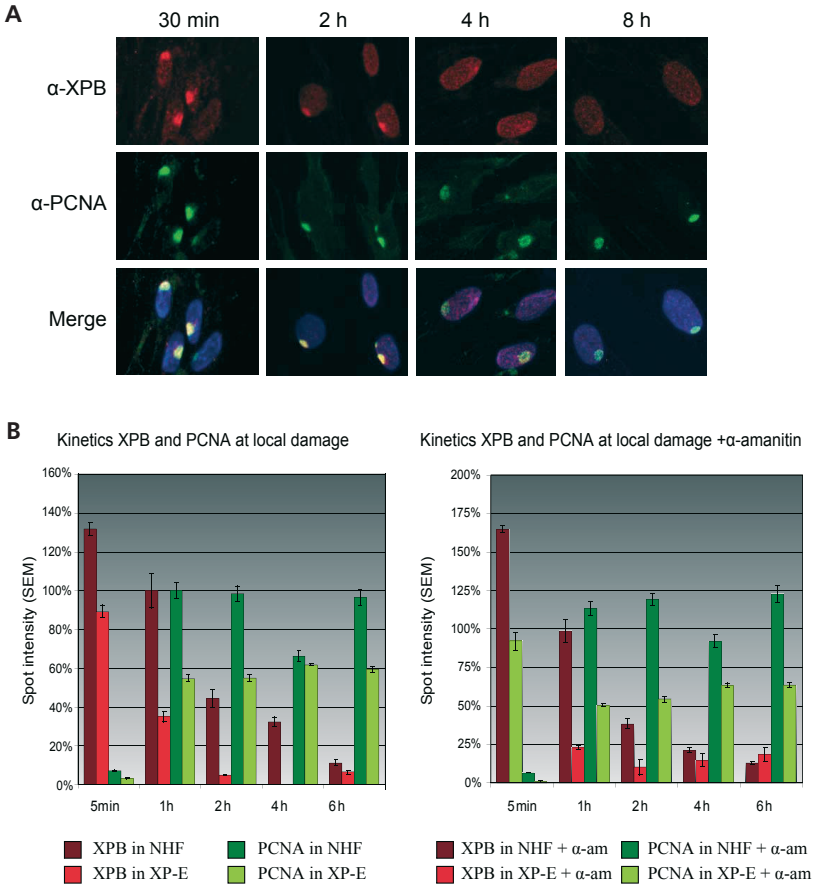

Figure S1. Disassembly kinetics of NER sub-complexes from sites of UV-induced DNA damages and repair. Disassembly is monitored by fluorescent immunostaining of XPB and PCNA in confluent NHF after local UV irradiation (30J/m^2) at different time points prior to fixation. (A) Examples of images used for analysis. (B) Quantitative analysis of the average spot intensity at damage sites as function of time.

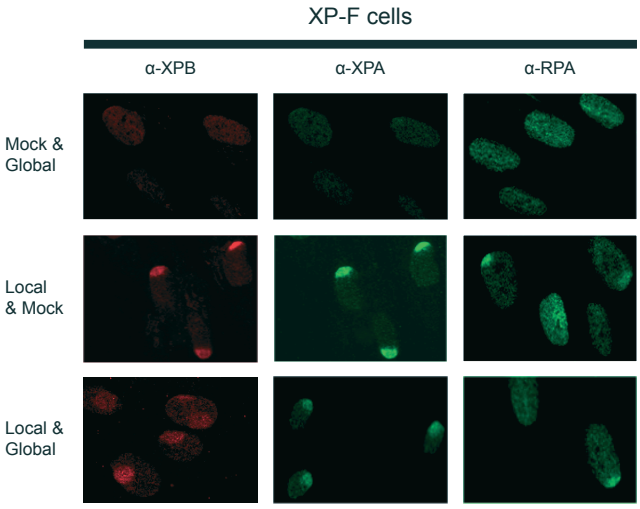

Figure S2. Release of pre-incision factors from NER sites in vivo requires incision. Competition experiments performed in XP-F cells as described in Protocol 2 (first local and then global UV irradiation). The sequential irradiations have been done in the absence of DNA synthesis inhibitors and are followed by immunofluorescent staining of XPB, XPA and RPA antibodies.

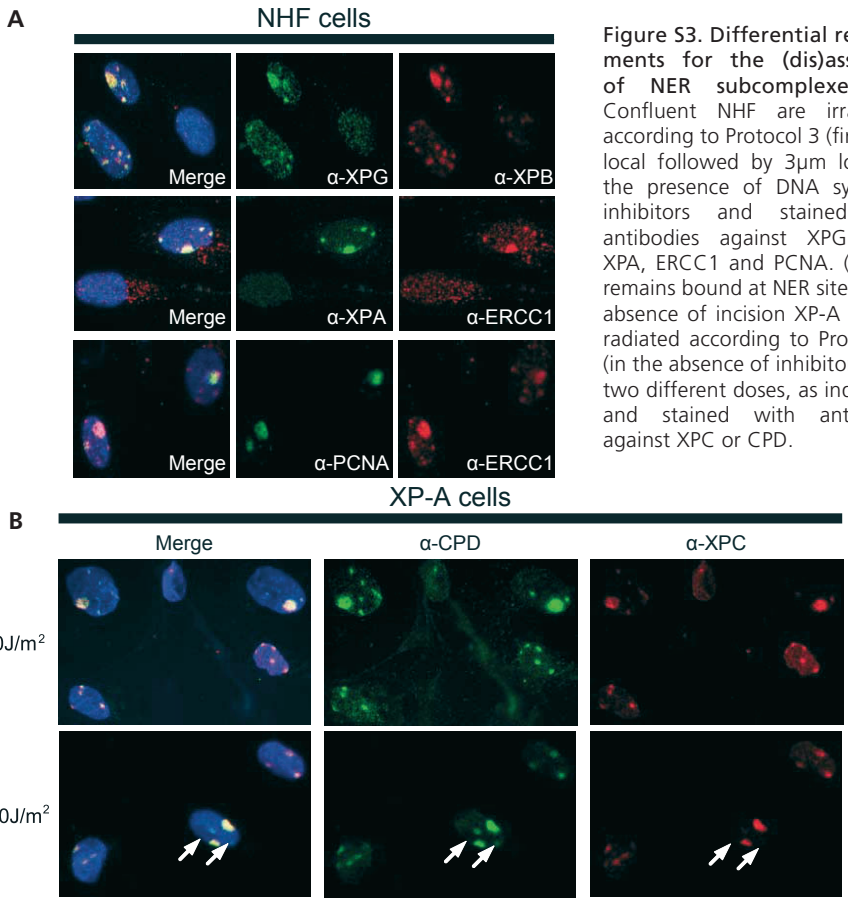

Figure S3. Differential requirements for the (dis)assembly of NER subcomplexes. (A) Confluent NHF are irradiated according to Protocol 3 (first 8μm local followed by 3μm local) in the presence of DNA synthesis inhibitors and stained with antibodies against XPG, XPB, XPA, ERCC1 and PCNA. (B) XPC remains bound at NER sites in the absence of incision XP-A cells irradiated according to Protocol 3 (in the absence of inhibitors) with two different doses, as indicated, and stained with antibodies against XPC or CPD.

8

ARSENIC INHIBITS THE SEALING OF UV-INDUCED CHROMOSOMAL DNA NICKS DURING NUCLEOTIDE EXCISION REPAIR BY INHIBITING DNA LIGASE IIIα

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Submitted

SUMMARY

Arsenic-induced carcinogenesis is a world-wide problem which is threatening to reach epidemic proportions. Epidemiological data and in vivo experiments suggest that arsenic exposure interacts with UV radiation exposure to increase the risk of skin cancer. Inhibition of DNA repair processes is one of the most prominent mechanisms in arsenic-induced carcinogenicity. Impaired gap filling and sealing of chromosomal DNA in nucleotide excision repair (NER) leads to genome instability. Here we show that low concentrations of arsenite (As(III)) disrupt the repair of both CPD and 6-4PP photolesions, and with equal efficiency. We found a slight reduction in accumulation of pre-incision factors accumulating at UV-damage sites in the presence of As(III), yet complex build-up was not significantly altered. Incisions were still made and accumulation of post-incision factors at UV-damage sites was undisturbed by the presence of As(III). The disassembly of pre-incision factors was however delayed in As(III) treated cells. Moreover, we provide data demonstrating that ligation and in particular, DNA ligase IIIα function is especially disrupted leading to impaired NER capacity in As(III) treated cells. Our data reveals new insights into the mechanisms involved in arsenic co-carcinogenesis and provides a possible explanation for the impact that As(III) exposure has on a variety of human cancers, including skin cancer.

INTRODUCTION

Naturally-occurring arsenic in drinking water poses a growing global health risk as millions of people are exposed to elevated levels of arsenic derivatives. According to recent reports chronic low-dose exposure of contaminated drinking water is thought to affect nearly 140 million people in more than 70 countries (Bagchi, 2008). In addition, arsenic contaminated water is widely used for irrigating crops during dry season rice production resulting in elevated arsenic uptake by crops (Meharg et al., 2003) and substantial human intake of arsenic due to consumption of rice (Williams et al., 2005). World Health Organisation guidelines set a safe limit of 10 µg/L of arsenic in water supplies, but millions of people drink water with higher arsenic content (WHO, 2001, ATSDR, 2007). High concentrations of arsenic in drinking water are predominantly found in certain regions of the United States as well as in India, Mexico, Taiwan, Chile and China (Hughes et al., 2007, Huang et al., 2004). However, at present Bangladesh is thought to be the worst-affected country. Epidemiology studies indicate that arsenic is a human carcinogen (IARC, 2004). Long term ingestion is associated with an increased risk of developing tumours of the skin, bladder, liver, kidney, prostate, while inhalation of arsenic compounds increases the risk of lung cancer (Tapio et al., 2006). Arsenic tends to accumulate in the skin, where characteristic non-malignant skin lesions such as hyperpigmentation and hyperkeratosis appear on the palms and soles before skin cancers such as Bowen's disease, squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) appear. (Centeno et al., 2002). Trivalent arsenites appear to be more toxic and are responsible for carcinogenicity; however, in many cases exposure to both trivalent and pentavalent inorganic arsenic occurs but the precise chemical speciation is usually not known (ATSDR, 2007). The underlying mechanisms of arsenic carcinogenicity in humans still remains elusive, mainly due to the fact that arsenic is not a potent mutagen by itself. Many different mechanisms of action have been proposed such as enhanced cell proliferation, altered DNA methylation patterns, induction of oxidative stress, co-carcinogenesis and tumour promotion. However, since arsenic can act as a co-mutagen by compromising the repair of DNA damage induced by a variety of genotoxic agents, several studies have indicated that impairment of DNA repair processes is one of the most prominent mechanisms in arsenic-induced carcinogenicity (Hartwig et al., 1997; Hartwig et al., 2002; Andrew et al., 2006). Low concentrations of arsenite, which are not mutagenic, are able to enhance the carcinogenicity of ultraviolet radiation (UVR) in mice (Rossman et al., 2001; Rossman et al., 2002). Studies suggest that the co-mutagenic and co-carcinogenic effect of arsenic is almost certainly due to an inhibitory effect on NER (Okui et al., 1986; Hartwig et al., 2002).

Nucleotide excision repair (NER) is the major pathway for the removal of UV-induced lesions such as pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) as well as bulky DNA damage induced by a variety of environmental mutagens. NER can operate via two sub-pathways: global genome repair (GGR) that acts on lesions throughout the genome, and transcription-coupled repair (TCR) which repairs lesions on the transcribed strand of active genes. The importance of NER for human health is illustrated when defects in genes encoding for GGR proteins give rise to the UV-sensitive and cancer-prone genetic

disorder xeroderma pigmentosum (XP) (Friedberg, 1996). In human cells, the NER reaction requires at least six core protein complexes for damage recognition and dual incision (XPC-hHR23B, TFIIH, XPA, RPA, XPG and XPF–ERCC1) (Volker et al., 2001; Gillet and Scharder, 2006; Mocquet et al., 2008). Other factors are required for repair DNA synthesis and ligation i.e. PCNA, RFC, DNA polymerase δ or ε and XRCC1-DNA ligase III or DNA ligase I (Kelman et al., 1995; Araujo et al., 2000; Moser et al., 2007; Overmeer et al., 2010). One potential group of molecular targets for arsenic are the zinc-finger containing NER proteins since As(III) has been shown to have a high affinity for vicinyl sulfhydryl groups (Hartwig, 2001). Schwerdtle and collegues (2003) demonstrated the ability of low micromolar concentrations of trivalent arsenic compounds to release zinc from a peptide consisting of 37 amino acids representing the zinc finger domain of the human XPA protein. Other important NER components with zinc finger domains include human replication protein A (RPA), DNA polymerases delta (pol δ) and epsilon (pol ε) and one of the most recently identified NER factors; DNA ligase IIIα (Lig3).

The zinc finger-containing nucleotide excision repair proteins clearly provide a potential target for toxic metal compounds. This mechanism is in agreement with arsenic acting as a co-carcinogen by compromising the repair of DNA damage induced by other DNA damaging agents. Herein, we provide direct evidence that the repair of UV-induced lesions is severely inhibited by the presence of As(III). More specifically, we find that As(III) inhibits repair patch ligation in NER, probably due to a direct inhibition of Lig 3 function.

RESULTS

Arsenite exposure impairs the repair of UV-induced lesions

We investigated the effects of arsenic on gene-specific repair of UV-induced photolesions under non-cytotoxic conditions. The induction and repair of UV-induced CPD (UV dose 10J/m^2) was measured (Moser et al., 2005) in confluent fibroblasts treated with 10μM As(III). A dose of 10μM As(III) is classed as non-cytotoxic in (clonal) cell survival assays carried out previously (Hartwig et al., 1997, Nollen et al., 2009). Repair was measured in a 3' located 18.5kb *Eco*RI fragment of the active ADA gene, in which both strands are transcribed. The intensity of the band representing the ADA gene immediately after irradiation (t=0hr) was diminished after treatment with T4 endonuclease V in both untreated and As(III) treated cells, indicating the presence of CPDs (Fig 1A-B). The intensity of the ADA band after treatment with T4 endonuclease V had completely returned by 24hr, indicating the near complete removal of CPDs from the ADA gene of untreated normal primary fibroblasts. In arsenic treated cells the intensity of the ADA band after treatment with T4 endonuclease V was significantly diminished compared to the intensity of the untreated band after 24hrs, indicating incomplete repair after 24hrs (Fig. 1A-B). Quantification of band intensities and application of the Poisson distribution revealed 90% and 50% repair of CPD after 24hrs in mock - and arsenic treated cells, respectively. These results indicate that there is a clear reduction in the ability of arsenic treated cells to repair CPDs in the active ADA gene compared to untreated normal primary fibroblasts.

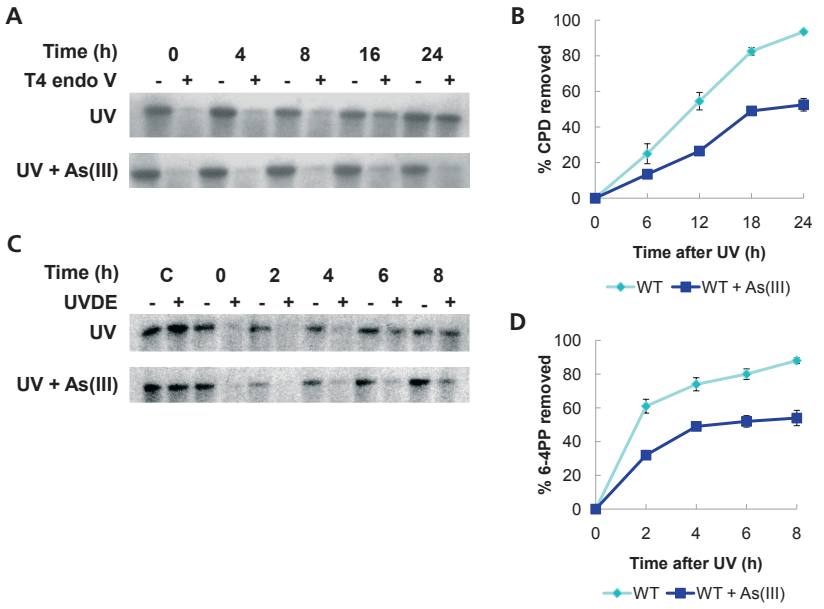

Fig 1. Normal human fibroblasts pre-incubated with As(III) show decreased repair of both CPD and 6-4PPs compared to untreated cells. Removal of CPD and 6-4PP at various times after global UV irradiation was measured in EcoRI restriction fragments of the active ADA gene using a probe recognizing both strands. A. Autoradiogram and B. histogram showing removal of CPD over a period of 24 h in primary normal human (VH25) fibroblasts treated with and without 10μM As(III). C. Autoradiogram and D. Histogram showing removal of 6-4PP over a period of 4 h in primary normal human fibroblasts (VH25) treated with and without 10μM As(III).

Gene specific removal of UV-induced 6-4PP was carried out as described previously (Moser et al., 2005). A high UV dose of 30J/m^2 had to be applied in order to induce sufficient levels of 6-4PP for analysis, i.e. 1-2 6-4PP per restriction fragment of ~20kb. Representative autoradiograms are shown in Figure 1C. The intensity of the ADA band after treatment with UVDE had significantly returned by 8hr and had a similar intensity to the untreated band indicating an almost complete repair (85%) of 6-4PP after 8hr in normal cells. In contrast, the intensity of the ADA band after UVDE treatment in As(III) treated cells was significantly reduced after 8hrs (Fig. 1C-D); the extent of repair was quantified as 50%. Together, these results indicate that there is a considerable reduction of the repair of CPDs and 6-4PPs in normal human cells treated with physiologically relevant concentrations of arsenic. Moreover, the level of repair inhibition of both photolesions was similar.

Core NER components are able to accumulate but persist at UV-lesions in the presence of arsenite

We examined the effect of As(III) on NER complex formation to elucidate which steps are target to inhibition. We applied local UV irradiation and immunofluorescence microscopy

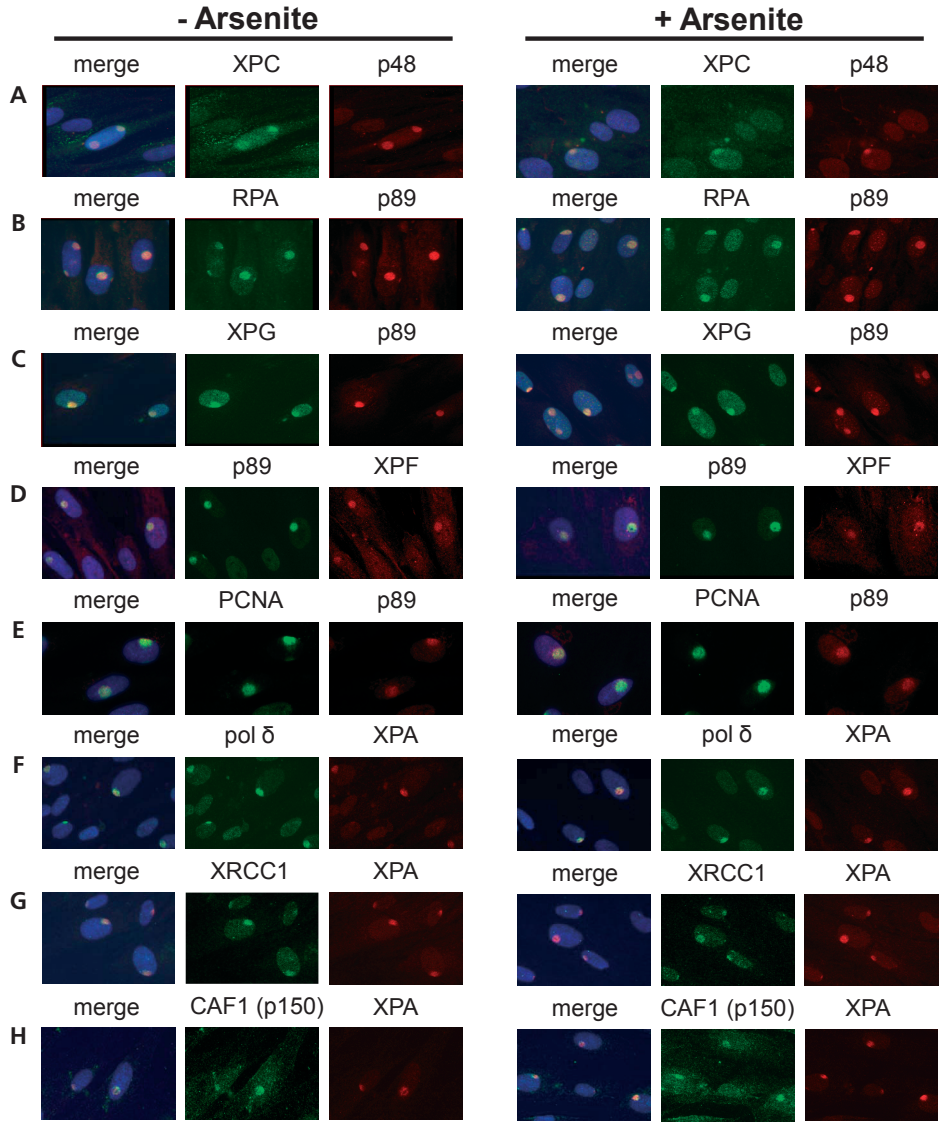

Figure 2. Core NER factors are able to bind to the NER complex despite 24hr treatment with 10μM As(III). Images A-G, were obtained with locally UV-irradiated (30 J/m^2) confluent primary normal fibroblasts. "Merge" refers to the combined image of DAPI and the pair of antibodies used for each panel. Immunolocalization of A, XPC and DDB2(p48) B, RPA(p70) and TFIIH(p89) C, XPG and p89 D, p89 and XPF E, PCNA and p89 F, DNA pol δ and XPA G, XRCC1 and XPA H, CAF1(p150) and XPA.

to investigate the accumulation of core NER components at damage sites in As(III) treated, as well as mock-treated normal human fibroblasts. To assess the impact of arsenic on the pre-incision step we examined the damage recognition factors, UV-DDB and XPC at local UV sites in the presence of As(III) (Fig. 2). As shown in Fig.2, UV-DDB and XPC accumulate at UV spots. In fact, other pre-incision factors such as p89 (TFIIH), XPA, and endonucleases XPG and ERCC1-XPF were also able to accumulate in the presence of As(III) (Fig. 2). Likewise, the core post-incision factors, RFC, PCNA, p21, DNA polymerase δ, the XRCC1-DNA ligase III complex and CAF-1 were also able to accumulate at local UV-induced sites in the presence of As(III) (Fig. 2 and data not shown).

Pre-incision NER components assemble with a slightly reduced efficiency into the NER complex in the presence of As(III). A 24hr pre-incubation with 10μM As(III) led to a slight decrease (approx. 14%) of XPA and XPB/p89 accumulation at local UV damage sites (Fig. 3A). We also checked the disassembly of the NER pre-incision complex in the absence

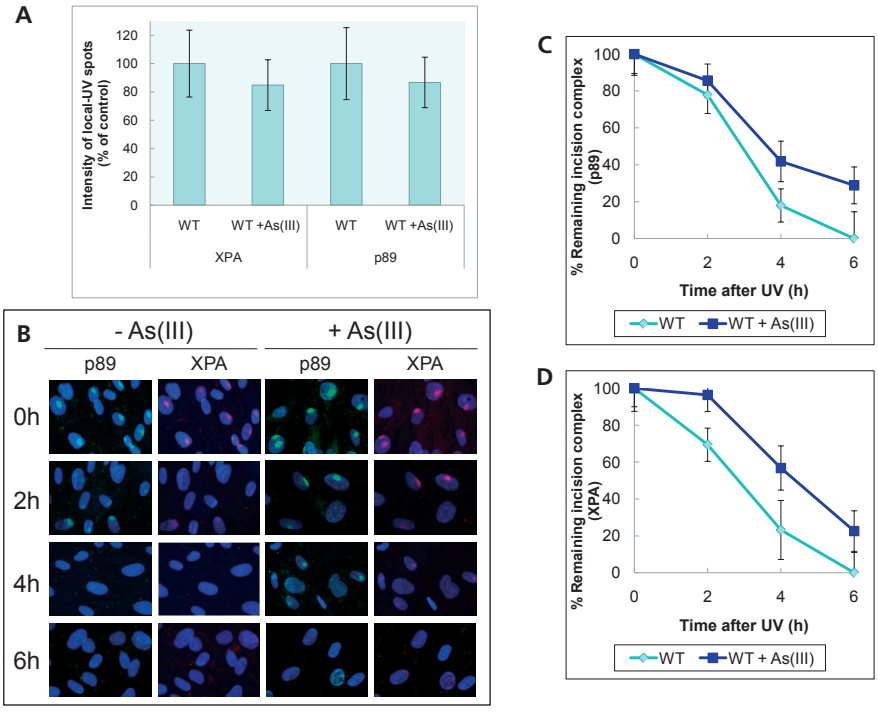

Fig 3. The disassembly kinetics of the NER complex is disrupted in As(III) treated fibroblasts compared to untreated fibroblasts. A, Fluorescent immunostaining of XPA and p89 in the presence or absence of 10μM As(III) 20min after local UV-irradiation. The average intensities of XPA and p89 spots after preincubation with As(III) are normalised to control cells without As(III) incubation (100%). B, Disassembly of the NER complex was monitored by fluorescent immunostaining of both p89 and XPA spots at various time points after local UV irradiation in both untreated primary confluent fibroblasts cells treated for 24hrs with 10μM As(III). C, The removal of p89 from the incision complex over a period of 6h, D, The removal of XPA from the incision complex over a period of 6h. Error bars represent the SEM values of 40 nuclei.

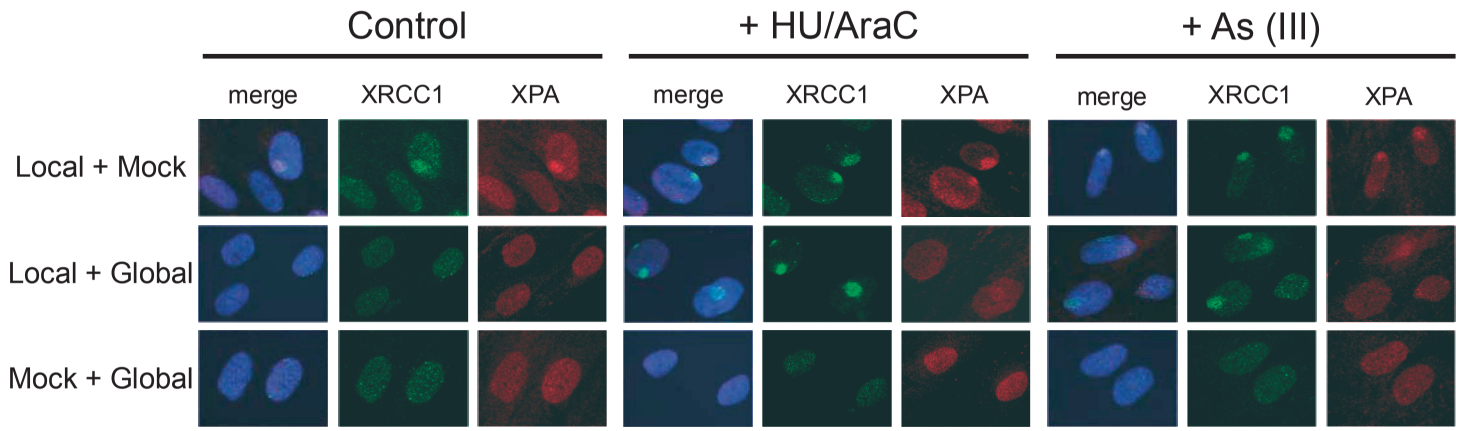

Fig 4. The DNA resynthesis/ligation step is disrupted by As(III) in living cells. A, Normal human fibroblasts, B, Normal human fibroblasts treated with HU and AraC and C, normal human fibroblasts treated for 24hr with10μM As(III) were locally UV-irradiated with 30 J/m^2, incubated for 1 hr, and subsequently globally irradiated with 30 J/m^2. Proteins were immunofluorescently labeled 1hr after the second irradiation showing immunolocalization of XRCC1 and XPA. Controls included cells which had been given only the first local UV-irradiation (25 J/m^2), followed by a mock UV-irradiation, and cells which had been given a mock irradiation followed by global UV-irradiation (25 J/m^2).

and presence of As(III) using local UV-irradiation and immunofluorescence. Previous work has shown that normal cells treated with DNA synthesis inhibitors (HU and AraC) are unable to proceed with the last stages of NER and therefore display retarded disassembly of the NER pre-incision complex and impaired removal of photolesions (Moser et al., 2007). Similarly, the disassembly kinetics of the NER pre-incision complex in As(III) treated cells (monitored by XPA or XPB/p89 TFIIH at local UV spots) was significantly retarded in comparison to that in mock-treated cells (Fig. 3B-3D).

Arsenic inhibits the DNA resynthesis/ligation step of NER

When cells were treated with DNA synthesis inhibitors HU and AraC, the post-incision factor XRCC1 accumulated at sites of local UV-damage (Fig 4B). To establish whether the last steps of NER were disrupted by As(III), we carried out in vivo competition experiments as described in detail elsewhere (Overmeer et al., 2010). Local UV-irradiation to cells in the presence of HU and AraC followed by global UV-irradiation revealed that XRCC1 remained confined to local UV-induced damage spots even after the application of a second competing global UV-irradiation (Fig. 4B). Pre-incision proteins such as XPA are able to leave the NER complex under these conditions (Fig. 4B) (Overmeer et al., 2010). It is therefore likely that the entire DNA re-synthesis/ligation complex will remain assembled at the site of repair and only dissociate after the completion of repair synthesis and ligation. We carried out similar competition experiments in cells which had been exposed to As(III) and found that the post-incision protein XRCC1 was confined to local UV-damage spots despite a second global UV-irradiation (Fig. 4C).

DNA ligase I cells are sensitive to both UV-irradiation and As(III)

To examine which post-incision protein is inhibited by arsenic, we choose a genetic approach. We evaluated the effect of a DNA ligase I deficiency on the UV sensitivity of human fibroblasts pre-treated with As(III). Wild-type and Lig1 (46BR) cells were either mock or pre-treated with low doses (10μM for 24hrs or 40μM for 4hrs) of As(III) prior to UV-irradiation. We find a slight sensitivity of Lig1 cells to UV-irradiation when compared to normal human fibroblasts (Fig. 5A) as reported previously (Teo et al., 1983, Bentley et al., 2002). Treatment of human cells with As(III) in the absence of Lig1, leads to clearly enhanced sensitivity to UV-irradiation (Fig. 5A). We examined the possibility that As(III) may target Lig3 thereby inhibiting the post-incision step of NER. We carried out similar survival assays in HeLa cells and HeLa cells which have a stable Lig3 knock-down (HeLa-L3 KD) but have normal levels of Lig1. We find that the absence of Lig3 did not lead to an increased sensitivity to As(III) pre-incubation prior to UV-irradiation (Fig. 5B). Since we observe residual repair and intermediate UV sensitivity of normal cells treated with As(III) we wondered whether a certain population of cells may be proliferating due to As(III) treatment. Lig1 is highly expressed in proliferating cells and our previously published work indicated that Lig1 is able to take over the role of Lig3 allowing NER-mediated ligation (Moser et al., 2007).

Arsenite impairs ligation of NER-mediated DNA breaks and inhibits DNA ligase IIIα

We assessed the effect of As(III) on repair patch ligation by using the alkaline comet assay. In the absence of UV irradiation, the comet tail size of normal or Lig1-deficient cells did not differ significantly from the tail of As(III) treated cells; in all cells, UV irradiation induced an increase in tail size 30 min after UV irradiation. Normal and Lig1-deficient cells showed efficient sealing of breaks 4 hr after UV irradiation, to a level similar to non-irradiated controls (Fig. 5D). However, normal human cells treated with As(III) showed impaired sealing of UV-induced breaks, coinciding with the observed (partial) inhibitory effect on the repair of UV-induced photolesions. Moreover Lig1 deficient cells displayed severely abolished ligation when treated with As(III) and UV (Fig. 5D). To test for a specific ligase deficiency resulting from As(III) exposure, we specifically analyzed the effect of As(III) on the activities of various purified DNA ligases and DNA ligation activity. For the doses used *in vivo* (around 40 μM), the ligation by DNA Ligase I and III is barely reduced; in addition, no effect on T4 DNA ligase was observed (data not shown).

As shown in figure 6, for higher doses (1-9 mM), we observed a specific effect on DNA ligase III whereas DNA ligase I and T4 DNA ligase activities were unaffected by As(III) pre-incubation. The results of the joining assay were not affected by either pre-incubation of the ligases with arsenite or by omitting DTT from the ligation buffer.

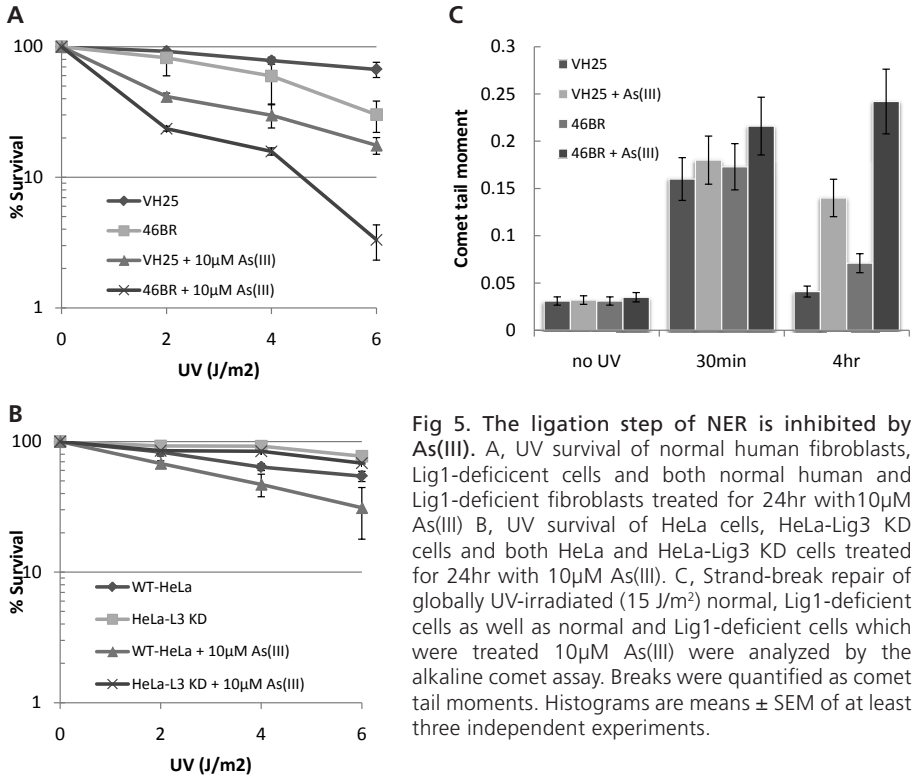

Fig 5. The ligation step of NER is inhibited by As(III). A, UV survival of normal human fibroblasts, Lig1-deficicent cells and both normal human and Lig1-deficient fibroblasts treated for 24hr with10μM As(III) B, UV survival of HeLa cells, HeLa-Lig3 KD cells and both HeLa and HeLa-Lig3 KD cells treated for 24hr with 10μM As(III). C, Strand-break repair of globally UV-irradiated (15 J/m^2) normal, Lig1-deficient cells as well as normal and Lig1-deficient cells which were treated 10μM As(III) were analyzed by the alkaline comet assay. Breaks were quantified as comet tail moments. Histograms are means ± SEM of at least three independent experiments.

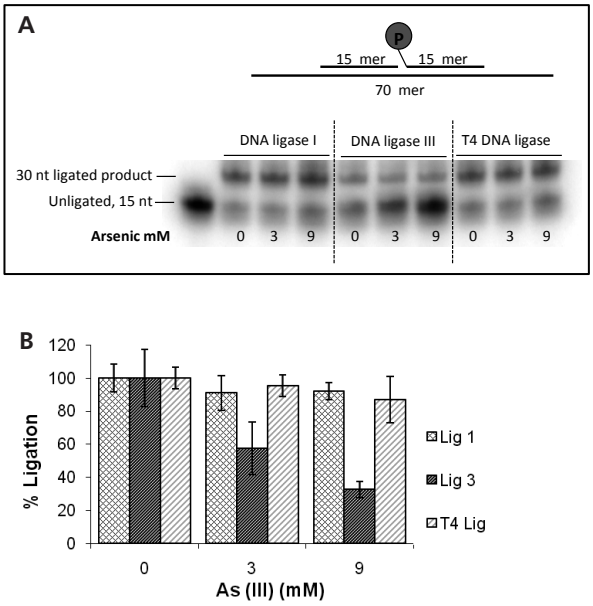

Fig 6. As(III) specifically inhibits Lig3 in vitro. A, The effect of As(III) on DNA ligases (Lig1, Lig3 and T4 DNA Lig) and DNA ligation activity was analyzed as described in the materials and methods. Samples were electrophoresed through a 10% denaturing polyacrylamide gel and autoradiography results quantified. B, quantification of ligation efficiency.

DISCUSSION

Environmental agents may compromise the DNA repair capacity of cells thereby increasing the risk of mutations and cancer from simultaneous exposure to genotoxic agents. Arsenic has been shown to act synergistically with other environmental contaminants, such as tobacco smoke, ionizing radiation and sunlight in causing increased incidence of internal cancers (Rahman et al., 2009, Ferreccio et al., 2000) and skin cancers (Centeno et al., 2002) respectively observed in arsenic-exposed populations. Experimental studies have also shown that low concentrations of arsenite enhanced the tumorigenicity of solar UV irradiation in hairless mice (Rossman et al., 2001, Burns et al., 2004). It is likely that arsenic may act as a human carcinogen, at least in part, through inhibition of DNA repair mechanisms. Previous work using UV-irradiated human repair-proficient and repair-deficient XPC fibroblasts showed that the incision as well as the ligation step of NER were inhibited by As(III) (Hartwig et al., 1997). Herein, we show a clear and similar reduction (around 50%) of the repair of UV-induced CPD and 6-4PP in As(III) treated cells as compared to mock-treated fibroblasts consistent with a 30-40% reduction of UV-induced repair replication in As(III) treated human fibroblasts (Hartwig et al., 1997). In accordance with these observations we find that As(III) treated cells display retarded disassembly of the NER pre-incision complex.

Arsenic may directly impair DNA repair processes by affecting binding to DNA damage and altering the function of proteins within the DNA repair complex. However, in vitro studies have shown that high concentrations of arsenic are needed to disturb purified DNA repair enzymes (Li and Rossman, 1998, Hu et al., 1998). One potential group of molecular targets are the zinc finger proteins. In this family of proteins, zinc is complexed through

four invariant cysteine and/or histidine residues forming a zinc finger domain involved in DNA binding and protein-protein interactions. NER core-factors with zinc finger domains include the pre-incision factor XPA and a recently identified post-incision factor, DNA ligase IIIα (Lig3). In the XPA protein, the zinc finger motif is part of the minimal DNA binding domain and consists of zinc complexed to four cysteine residues; substitution of any of the four cysteines leads to severe reduction in NER activity (Miyamoto et al., 1992). Previous studies have investigated the impact of As(III) on XPA repair protein and its binding to damaged DNA. Trivalent arsenicals have been shown to release zinc from a 37 amino acid-containing XPAzf peptide, representing the zinc finger domain of the human XPA protein (Schwerdtle et al., 2003). More detailed analysis revealed that a ten-fold excess of arsenite partially oxidized the zinc finger structure of XPAzf (Piatek et al., 2008). Thus, zinc binding structures may be sensitive targets for arsenicals. With respect to XPA function, subcellular studies observed no or only a slight decrease on XPA binding to UVC- (Asmuss et al., 2000;) or MMC-damaged (Mustra et al., 2007) oligonucleotides by arsenite.

The current study demonstrates that XPA function is not significantly disrupted by the presence of physiologically relevant concentrations of As(III). XPA is still able to bind to the NER complex in As(III) exposed cells, albeit, with a slightly reduced efficiency. Interestingly, p89 which localises to the NER complex prior to XPA, also binds to the NER complex with slightly reduced efficiency in As(III) treated cells. Similarly, reduced association and dissociation of XPC at sites of local UV-induced DNA damage has recently been reported (Nollen et al., 2009). The latter effect was accompanied by reduced XPC gene expression in the presence of arsenite. Since XPC binds prior to XPA and p89, reduced levels of XPC may account for slightly reduced level of XPA (and p89) at local-UV damage and may also explain reduced incision frequencies after UVC irradiation observed previously (Hartwig et al., 1997). However, although recruitment of XPA to the NER pre-incision complex is only slightly disturbed in the presence of As(III), its function may be impaired. In the absence of XPA function, NER specific incisions would not be introduced and post-incision proteins such as pol δ and XRCC1-Lig3 would be unable to localize at local UV damage sites (Moser et al., 2007). The efficient assembly of post-incision proteins into the NER complex shown in this study and the induction of UV specific breaks in arsenic treated cells also described previously (Hartwig et al., 1997) implies efficient dual incision, even though although reduced as compared to control cells. The observations that all core NER factors are recruited to UV damage and the persistent accumulation of pre-incision proteins in As(III) exposed cells demonstrate that the disassembly process is disrupted. This coincides with the observed NER defect and suggests that a late, post-incision event is likely to be affected by As(III) exposure. These observations parallel previous findings, UV–irradiated human fibroblasts exposed to the DNA polymerase inhibitors HU and AraC, were shown to display persistent accumulation of pre-incision NER proteins at UV damage (Overmeer et al., 2010).

Similarly, inhibition of polδ/pol in confluent normal human cells by HU/AraC leads to accumulation of DNA strand breaks and abolishment of 6-4PP repair (43, 24). Moreover,

components involved in the DNA re-synthesis/ligation step of NER remain assembled at the site of repair and only dissociate after the completion of repair synthesis and ligation (Overmeer et al., 2010). Our in vivo competition experiments as well as reduced repair synthesis (Hartwig et al., 1997) show that As(III) functions with striking similarity to DNA synthesis inhibitors HU and AraC, disrupting the resynthesis/ligation step of NER. Lig3 together with XRCC1 are indispensable for the repair of UV-lesions in quiescent cells, whereas both XRCC1-Lig3 and Lig1 are engaged in the sealing step of NER in dividing cells. The severely impaired repair of UV damage in arsenic treated human cells lacking Lig1 (46BR cells) points to arsenic mediated inhibition of Lig3. In fact, cells lacking functional Lig1 and Lig3 display synergistic impairment of NER-mediated repair. This explains the enhanced sensitivity of UV-irradiated Lig 1 defective cells to As(III) as these cells are solely dependent on Lig3. Proliferating cells involve ligase1 in the post-incision step of NER and hence might be less sensitive to the effects of As(III). The residual repair manifest from the gene-specific repair assay and comet assay experiments as well as the intermediate UV sensitivity of cells lacking Lig3 suggests that there is a percentage of cells which are able to complete NER or that NER is delayed. We observed a higher percentage (50%) of cells expressing high levels of PCNA, a marker for proliferation, in cells which were exposed to As(III) compared to control cells (data not shown). In line with these observations, it is well known that As(III) induces cell proliferation in cultured cells as well as in vivo (Germolec et al., 1996; Hamadeh et al., 2002; Rossman et al., 2004). In these cells, Lig1 is strongly expressed and may partly compensate for the Lig3 deficiency which is induced through As(III) exposure. Taken together, the data suggests that Lig3 function is impaired by As(III) which ultimately results in impaired NER capacity. The zinc finger motif of Lig3, located at the N-termini, has extensive sequence similarity to zinc fingers present in the base excision repair factor, poly(ADP-ribose) polymerase (PARP). Interestingly, PARP activity has been shown to be inhibited by very low, non-cytotoxic concentrations of arsenite (Yager and Wiencke, 1997; Hartwig et al., 2003; Qin et al., 2008). Our in vivo findings are in agreement with previous in vitro results showing that As(III) inhibits the ligation step of base excision repair (Li and Rossman, 1998; Hu et al., 1998) and DNA strand break rejoining in MMS-treated cells (Lynn et al., 1997) attributed primarily to inhibition of Lig3. The specific inhibitory effect of As(III) on Lig3 might support this conclusion although this inhibition is only achieved at millimolar doses of As(III). Previous in vitro studies on DNA repair enzymes showed that Lig1 and Lig3 were both inhibited by 10mM arsenite (37). However, we observe a specific effect on Lig3 only using similar millimolar doses of As(III), Lig 1 was unaffected. For As(III) doses such as those used *in vivo* (10 - 40 μM), the ligation by DNA Ligase I and III is barely reduced and there is no effect on T4 DNA ligase. These data tend to suggest that in vitro experiments only partly mimic the cellular conditions; differences may be due to the formation of more inhibitory arsenic species within cells, by reduced gene expression of lig3 as opposed to direct interactions, by inhibition of poly(ADP-ribosyl)ation as reported previously (Hartwig et al., 2003) or simply by artifical buffer conditions present in the in vitro system, such as the presence of 1 mM DTT which may bind arsenite. Arsenic has been shown to down-regulate the expression of several

DNA repair genes in a time- and dose-dependent manner (Hamadeh et al., 2002; Andrew et al., 2006; Nollen et al., 2009). However, whether XRCC1 or Lig3 are differentially down-regulated as a result of arsenic exposure is currently unknown. Further studies are therefore required to fully understand how As(III) inhibits the ligation step of NER.

In conclusion, we find that As(III) induces its co-carcinogenic effects at least in part by inhibiting the sealing of chromosomal DNA nicks that arise during NER. Our data suggests that the ligation step of NER is significantly inhibited by As(III) and that Lig3 function is particularly impaired. Taken together, our data reveal new insights into the mechanisms involved in arsenic carcinogenesis and provides a possible explanation for the impact that As(III) exposure has on a variety of human cancers, including skin cancer.

MATERIAL AND METHODS

Cell Culture. Cells used in this study were cultured in DMEM (without hypoxanthine and thymidine) supplemented with 10% fetal calf serum and antibiotics at 37°C in a 5% CO_2 atmosphere and include primary normal (VH25) as well as Lig1 (46BR) deficient human fibroblasts, as well as parental HeLa cells and HeLa cells with stable Lig3 knockdown (HeLa-Lig3-KD) (Moser et al., 2007).

Global and Local UV-irradiation. Cells were treated with either medium containing 10μM arsenite ($NaAsO_3$) or medium containing the equivalent amount of water for 24hr or 40μM arsenite for 4hr. Prior to irradiation, the medium was removed and kept at 37°C. The cells were subsequently rinsed briefly with PBS and exposed to global UV irradiation at a dose rate of 0.2 J/m²/s with a Philips TUV lamp (predominantly 254nm). Cells were locally UV-irradiated through a filter with pore size 8μm as described previously (Volker et al., 2001). After irradiation, the cells were returned to culture conditions for the time periods as indicated. When required, cytosine-β-arabinofuranoside (AraC, Fluka) and hydroxyurea (HU, Fluka) were added to the medium 30 minutes (min) prior to irradiation and remained present throughout the time course of the experiment. Final concentrations were 10 μM for AraC and 100 mM for HU.

Clonogenic survival assays. Cells were trypsinized and 500 fibroblasts were plated in P_{90} dishes in duplicate (controls in triplicate), allowed to attach for 4 h, and subsequently treated with 10μM As(III) for 24 h or 40 μM for 4 h. Cells were irradiated with different doses of UV as described above. After 14 days the dishes were rinsed with 0.9% NaCl, dried, stained with methylene blue (0.25%) and colonies were counted under a light microscope (LW Scientific).

Gene specific determination of CPD. Cells grown to confluency were treated with either medium containing 10mM arsenite ($NaAsO_3$) or medium containing the equivalent amount of water for 24hr. The cells were subsequently irradiated with UV (254nm) at 10J/m² incubated in culture medium and lysed. Gene specific repair measurements were performed as described (Venema et al., 1991). Briefly, high-molecular-weight DNA was purified by phenol and chloroform extractions followed by ethanol precipitation. The DNA was restricted with *Eco*RI, further purified and ethanol precipitated before dissolving in TE. Equal amounts of DNA (5mg) were either treated or mock treated with the CPD specific enzyme T4 endonuclease V and electrophoresed in 0.6% alkaline agarose gels. The DNA was transferred to Hybond N+ membranes by vacuum blotting and hybridised with ^{32}P-labeled gene-specific probes. Radioactivity in full size fragments was quantified using a Cyclone Storage Phosphor Scanner (PerkinElmer). The number of CPD was calculated from the relative band densities in the lanes containing DNA either treated or not treated with T4 endonuclease V, using the Poisson expression.

Gene specific determination of 6-4PP. Confluent cells were treated with arsenite as mentioned above and subsequently irradiated with UV (254nm) at 30 J/m². CPD photoproducts were removed from the *Eco*RI digested DNA by in vitro photoreactivation employing the photolyase derived from *Anacystis nidulans*, (kindly provided Dr. A. Eker, Erasmus University, Rotterdam, The Netherlands). Photoreactivation was checked for completeness by treatment of DNA samples with T4 endonuclease V and Southern blotting analysis. Equal amounts of DNA (5mg) were either treated or mock treated with Δ228-UVDE endonuclease (5pmol/μg DNA),(kindly provided by M. de Ruiter, Leiden University,

Leiden, The Netherlands). After incubation, 10mM EDTA and 0.1% SDS were added and the DNA purified and dissolved in TE as described above. The samples were electrophoresed in 0.6% alkaline agarose gels and the DNA transferred to Hybond N+ membranes by vacuum southern blotting. The filters were hybridised with ^{32}P-labeled gene-specific probes. Radioactivity in full size fragments was quantified and the number of 6-4PP was calculated as described above.

Antibodies. The following primary antibodies were used in this study: rabbit polyclonal α-XPA, α-p89, α-p21, α-ERCC1, and mouse monoclonal α-DNA pol δ, α-p21 (Santa-Cruz), mouse monoclonal α-PCNA (PC10), α-XRCC1, α-XPA (Abcam), mouse monoclonal α-DNA lig3α (Genetex), mouse monoclonal α-DNA Lig1 (MBL), mouse monoclonal α-XPG (8H7, Molecular Probes), mouse monoclonal α-RPA (Ab-1, Oncogene) and rabbit polyclonal DDB2 (Acris antibodies). Secondary antibodies used were Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated donkey α-mouse (Jackson Laboratories) and Alexa Fluor 488 goat anti-mouse IgG and AlexaFluor 555 goat α –rabbit IgG (Molecular Probes). All secondary antibodies were used according to the manufacturer's instructions.

Immunofluorescence. The fluorescent labelling was performed essentially as described previously (Volker et al., 2001). Briefly, cells were washed with cold PBS, fixed and subsequently lysed on ice by either addition of 100% methanol (10 min), or by 2% formaldehyde and 0.2% Triton X-100 (15 min), or alternatively by addition of 2% paraformaldehyde (20 min at room temperature (RT) followed by 0.2% Triton X-100 and incubation for 5 min at RT. Cells were washed twice with PBS and incubated with 5% bovine serum albumin (BSA) in PBS for 30 min at RT. Subsequently, the cells were incubated with the primary antibodies diluted in wash buffer (WB) (PBS containing 0.5% BSA and 0.05% Tween-20) for 2 h at RT. The cells were washed 3 x with WB and incubated with the secondary antibody (diluted in WB) for 1 h at RT. In experiments requiring double labelling, antibodies were mixed in WB and incubated simultaneously. To visualise chromatin-bound PCNA, soluble PCNA was extracted by Triton X-100 treatment prior to methanol fixation. Cells were mounted in Aqua/polymount mounting medium containing DAPI (1.5 µg/ml) (Polysciences Inc., Warrington, PA). Microscopy and quantification of fluorescent signal has been described elsewhere (Moser et al., 2005).

Comet Assay. Alkaline comet assay was performed as described (Moser et al., 2007) with minor modifications. Cells grown to confluency were treated with either medium containing 10mM arsenite ($NaAsO_3$) or medium containing the equivalent amount of water for 24hr, globally UV-irradiated (15 J/m^2), and then incubated in fresh medium for the appropriate repair time. Prior to scoring, DNA was stained with 10 ug/ml ethidium bromide. Mean tail moments were quantified for 130 cells per sample in each experiment, with ColourProc software program as described previously (Moser et al., 2007). Histograms are the average mean tail moment per sample of at least 3 experiments (reported as means ± s.e.m).

DNA Nick Ligation Assay. The DNA ligation assay was performed essentially as described (Mackey et al., 1999). In brief, purified ligases I, III and T4 (3pmol of each) were incubated at 37^{0}C in ligation buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.25 mg/ml BSA, 100 µM ATP and 100 mM NaCl without or with arsenite (0-9mM) for 10min. Ligation substrate and product were separated by electrophoresis through a 10% polyacrylamide denaturing gel.

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9

ADDENDUM

Summary
Nederlandse samenvatting
Curriculum Vitae
List of publications
Dankwoord

SUMMARY

DNA is continuously exposed to exogenous and genotoxic insults including ionizing and ultra-violet radiation as well as chemical agents. Endogenous damage is induced by reactive oxygen species and other products generated by normal cellular metabolism. DNA lesions compromise the integrity of the genome and have potentially deleterious effects. It is estimated that endogenous sources induce approximately 50,000 DNA lesions per cell per day in humans (Friedberg, 1995) whereas one hour of sunbathing is estimated to generate around 80,000 DNA lesions per cell (Mullaart et al., 1990). Elaborate genome maintenance machinery counteracts DNA damage and consists of multiple repair pathways and various checkpoint signal transduction and effectors systems (Hoeijmakers, 2001). These cellular responses to DNA damage involve pathways that coordinate DNA lesion recognition and signaling, leading to cell cycle arrest, which facilitates DNA repair. Alternatively, apoptosis or senescence is induced when too much damage has occurred and DNA lesions are irreparable. This network of pathways is known as the DNA damage response. The DNA damage response prevents cells from replicating in the presence of DNA lesions that may result in the development of cancer. **Chapter 2** describes cellular responses to DNA damage and gives an introduction into the field of DNA repair. Several different DNA repair pathways have been identified which are activated by different types of DNA damage. These repair systems differ in the way that the damage is recognized which is dependent on the severity of DNA damage in terms of helix distortion or blockage of DNA replication or transcription (Hoeijmakers, 2001). The main repair pathways in mammals are Nucleotide excision repair (NER), Base excision repair (BER), Homologous recombination (HR), Non-homologous End-joining (NHEJ) and Mismatch repair (MMR) (Friedberg, 2003; Hoeijmakers, 2001). Ultraviolet light (UV) can induce the formation of helix-distorting lesions such as 6-4 photoproducts (6-4PP) and cyclopyrimidine dimers (CPD). NER is a multi-step "cut-and-patch" mechanism that involves more than 30 proteins (Gillet and Schärer, 2006) which is solely responsible for the repair of these lesions. NER is the main topic of this thesis and **Chapter 3** therefore describes NER in detail together with the consequences of defective NER. Two NER subpathways exist; global genome NER (GG-NER) surveys the entire genome for distorting injury, and transcription-coupled repair (TCR) removes DNA damage that blocks elongating RNA polymerases (Tornaletti and Hanawalt, 1999). The NER mechanism can be divided into 5 main steps; recognition of the damaged DNA, unwinding of the DNA helix around the lesion, excision of the damaged oligonucleotide and finally resynthesis of the strand using the undamaged template as well as ligation of the nicks (Moser et al., 2005). Since DNA repair occurs in a chromatin context, repair factors must overcome the restricted access to DNA in chromatin. Chromatin remodeling is not only essential for DNA repair processes but also for key cellular processes such as the regulation of gene expression, apoptosis and DNA replication. **Chapter 4** describes chromatin modifications and ATP-remodeling activities which are necessary for efficient DNA repair.

UV-DDB is a heterodimer of the p48 (DDB2) and p127 (DDB1) proteins which exhibits an affinity for several types of DNA lesions and in particular a high affinity for UV-induced

lesions. In vivo studies show that p48 and p127 relocalise rapidly to sites of UV damage even in the absence of XPC. These observations are consistent with a role for UV-DDB in the repair of lesions within a chromatin context and more specifically DNA damage recognition. In **chapter 5**, we used a number of different methods to examine NER incision complex formation and the repair of 6-4PP at DNA damage sites. When comparing normal and XPE deficient cells we found that accumulation of NER incision complex proteins and the repair of 6-4PP is greatly enhanced in cells proficient for UV-DDB. UV-DDB may therefore have a role in local chromatin remodeling at sites of photolesions, increasing the accessibility for recognition by the XPC-hHR23B complex, especially since it has been shown to interact with p300/CBP. Using different UV doses and therefore introducing different frequencies of DNA lesions we found that when a low number of lesions were introduced, DNA repair was enhanced by the presence of UV-DDB. However, when a high dose was used, this effect was lost. UV-DDB therefore mediates accelerated repair of a limited number of lesions by accelerating and stabilizing the binding of XPC-hHR23B. After binding to 6-4PP, UV-DDB is degraded and therefore cannot assist in subsequent repair of remaining DNA lesions. Therefore, if the amount of 6-4PP exceeds the amount of UV-DDB molecules, most 6-4PP will be repaired through direct recognition by the XPC-hHR23B complex, which is a slower process compared to UV-DDB stimulated repair.

Biochemical evidence suggests that DDB2 is part of the E3 ubiquitin ligase containing CUL4A, ROC1 and DDB1 (Groisman et al., 2003). In order to gain more information on how DDB2 cooperates with the E3 ligase complex in DNA damage recognition, in **chapter 6**, we examined the binding and dissociation kinetics of fluorescently labeled DDB2 to UV-damaged DNA in normal as well as in XPC-deficient cells. We found that DDB2 had similar binding kinetics to that of the other subunits of the ubiqitin ligase complex (CUL4A and DDB1). Thus confirming previous observations (Groisman et al., 2003) and demonstrating that the entire E3 complex binds to UV-induced damage. Although XPC is required for the binding of the other NER factors (Volker et al., 2001), we found that DDB2 accumulated at local sites of damage in cells lacking XPC. Furthermore, we find that there is little physical interaction between the 2 damage sensors at UV-lesions. It is therefore likely that the E3 ligase complex ubiquitylates XPC upon UV irradiation which then enhances its affinity for DNA (Sugasawa et al., 2005) subsequently promoting recruitment of the other pre-incision NER factors resulting in efficient repair.

GGR can be subdivided into pre- and post-incisions stages. The pre-incision stages have been studied extensively. In contrast, the post-incision stages of NER, which involves gap filling by DNA repair synthesis, ligation and chromatin restoration, is less well understood. In **chapter 7** we carried out a number of different experiments which identified 2 unanticipated factors required for the ligation step in NER. We showed for the first time that XRCC1 and DNA ligase IIIα (Lig3) which have an important role in base excision repair are also essential core components of mammalian NER. The involvement of Ligase I in NER in vivo was primarily based on the UV sensitivity of cells derived from a patient deficient in Ligase I (Lig1). However, we found that down-regulation of Lig3 but not Lig1 impaired removal of UV lesions and rejoining of UV-induced nicks in chromosomal DNA (Moser

et al., 2007). In UV-irradiated cells either cycling or arrested, the XRCC1-Lig3 complex is recruited to sites of DNA damage. In contrast, Lig 1 and DNA polymerase ε appear only to have role in proliferating cells. XRCC1-Lig3 associated with other post-incision repair factors at UV-damaged lesions but not with polymerase ε or pre-incision factors suggesting that DNA damage recognition and the post-incision steps of NER do not simultaneously reside in the same complex in vivo. XRCC1 has been shown to interact with PCNA (Fan et al., 2004), a key factor which recruits other post-incision factors. We therefore speculate that the XRCC1-Lig3 complex might function in NER through interactions with PCNA.

NER factors are suggested to sequentially assemble into pre- and post-incision complexes (Hoogstraten et al., 2002; Houtsmuller et al., 1999; Volker et al., 2001). However, much less attention has been devoted to the regulation of NER in vivo, most notably the transition from dual incision to the repair synthesis step. In **chapter 8** we studied the regulation of the pre- and post-incicion stages of NER in vivo and the role of RPA therein. Results suggest that the principal role of RPA in NER (in concert with XPA) is its involvement in the correct orientation and activation of the endonucleases XPG and XPF/ERCC1 by binding to the undamaged strand rather than to recruit the endonucleases. When RPA is absent, XPA and TFIIH are sufficient to recruit XPF and XPG respectively, to form an unstable non-functional pre-incision complex (Li et al., 1994; Park and Sancar, 1994; Volker et al., 2001; Iyer et al., 1996). We show that dissociation of pre-incision factors requires incision. Furthermore, inhibition of repair associated DNA synthesis or ligation leads to accumulation of both pre- and post-incision NER factors at sites of UV damage. Under these conditions we find that pre-incision proteins can dissociate from the initial site of repair and relocate to other sites of UV-damage, whereas RPA and post-incision proteins remain at the initial site of repair. The failure of RPA to relocate to other damage sites leads to an instable NER pre-incision complex that is unable to perform dual incision and as a consequence, fail to attract post-incision factors. RPA therefore plays a key role in safeguarding the transition from pre- to post-incision events during NER. Under normal conditions i.e. repair synthesis and ligation, the released RPA is able to associate with, and subsequently stabilise newly formed pre-incision complexes enabling further incision events and continuation of repair. In contrast, the persistent association of RPA in post-incision complexes at sites of incomplete repair prevents RPA participating in new incisions and therefore shields the genome from uncontrolled incision events by coupling NER mediated incision to completion of DNA repair synthesis and ligation.

Arsenic-induced carcinogenesis is a world-wide problem which is threatening to reach epidemic proportions. According to recent reports chronic low-dose exposure of contaminated drinking water is thought to affect nearly 140 million people in more than 70 countries (Bagchi, 2008). Epidemiological data and in vivo studies suggest that arsenic exposure interacts with UV radiation exposure to increase the risk of skin cancer. Inhibition of DNA repair processes is one of the most prominent mechanisms in arsenic-induced carcinogenicity (Hartwig et al., 1997; Hartwig et al., 2002). In **chapter 9**, we find that arsenite exposure at physiologically relevant concentrations impairs the repair of UV-induced lesions. In line with these observations we find persistent accumulation of

NER pre-incision proteins at UV lesions. We furthermore found that arsenite inhibits the resynthesis/ligation step of NER. As mentioned previously, Lig3 together with XRCC1 are indispensable for the repair of UV-lesions in quiescent cells, whereas both XRCC1-Lig3 and Lig1 are engaged in the sealing step of NER in dividing cells. Severely impaired repair of UV damage in arsenic treated human cells lacking Lig1 (46BR cells) points to arsenic mediated inhibition of Lig3. We therefore conclude that Lig3 function is impaired by arsenic which ultimately results in impaired NER capacity. Our in vivo findings are in agreement with previous in vitro results showing that As(III) inhibits the ligation step of base excision repair (Li and Rossman, 1998; Hu et al., 1998) and DNA strand break rejoining in MMS-treated cells (Lynn et al., 1997) attributed primarily to inhibition of Lig3. In conclusion, we find that As(III) induces its co-carcinogenic effects at least in part by inhibiting the sealing of chromosomal DNA nicks that arise during NER. Our data suggests that the ligation step of NER is significantly inhibited by As(III) and that Lig3 function is particularly impaired.

NEDERLANDSE SAMENVATTING

DNA bevat de code waarin al onze erfelijke eigenschappen zijn vastgelegd. Het is dus van groot belang dat ons DNA niet beschadigd raakt. Ons DNA wordt echter voortdurend blootgesteld aan exogene en endogene factoren die DNA schade tot gevolg hebben. Endogene oorzaken van DNA schade zijn een gevolg van interne biochemische processen, bijvoorbeeld het ontstaan van zuurstofradicalen ten gevolge van normale metabole reacties in de cel. Exogene oorzaken van DNA schade zijn externe invloeden zoals verschillende vormen van straling, waaronder UV-straling van zonlicht en ioniserende straling bijvoorbeeld in de vorm röntgenstraling. Daarnaast kunnen diverse chemische stoffen DNA schade veroorzaken. Het gevolg van DNA schade kan zijn dat de lichaamscellen direct afsterven omdat deze niet meer kunnen functioneren. Wanneer een cel beschadigingen aan zijn DNA niet adequaat oplost, kan dat ernstige gevolgen hebben. Naar schatting treden op moleculair niveau in iedere cel 50,000 DNA beschadigingen per dag op als gevolg van endogene oorzaken. Ter vergelijking met een belangrijke exogene blootstelling: de blootstelling aan zonlicht gedurende één uur op het strand op een zomerse dag veroorzaakt ongeveer 80.000 beschadigingen per cel. Gelukkig treden DNA herstelmechanismen in werking, waardoor schade gerepareerd wordt. Wanneer DNA herstelmechanismen niet goed werken, hoopt de schade op en heeft de cel moeite om zich te delen, door remming van de celcyclus, of de cel gaat dood via een geprogrammeerd proces, apoptosis genoemd. Als de cel zich ongeremd gaat delen, kan dit leiden tot de vorming van tumoren. De DNA schade respons voorkomt dat cellen gaan delen in de aanwezigheid van DNA schade (ook DNA laesies genoemd), een proces dat kan resulteren in de ontwikkeling van kanker. Deze cellulaire processen zijn beschreven in **hoofdstuk 2**. Verschillende DNA herstelmechanismen, die worden geactiveerd door verschillende soorten DNA laesies, zijn geïdentificeerd. Deze reparatiesystemen verschillen in de manier waarop ze de DNA schade herkennen en zijn onder andere voor hun activatie afhankelijk van het soort schade, de mate van de verstoring van de DNA helix, blokkade van DNA replicatie of transcriptie. De belangrijkste herstelmechanismen zijn nucleotide excisie reparatie (NER), base excisie reparatie (BER), homologe recombinatie herstel (HR), en niet-homoloog herstel van DNA dubbele strengbreuken (NHEJ) en mismatch reparatie (MMR). Verschillende eiwitten werken samen en vormen het DNA herstelsysteem. **Hoofdstuk 2** beschrijft kort de verschillende DNA reparatiesystemen.

UV-licht, een deel van zonlicht, kan fotolaesies in DNA introduceren, zoals cyclobutaan pyrimidine dimeren (CPD) en pyrimidine 6-4 pyrimidone fotoproducten (6-4PP). Het herstel van deze laesies is uitsluitend afhankelijk van nucleotide excisie reparatie (NER). Hoewel, niet alleen schade veroorzaakt door blootstelling aan zonlicht wordt hiermee gerepareerd, ook laesies ontstaan door behandeling met het anti-kankermedicijn cisplatin en door onder andere het inhaleren van sigarettenrook. Het onderzoek in dit proefschrift is toegespitst op dit DNA-herstelsysteem en is uitgebreid beschreven in **hoofdstuk 3**. NER kan opgesplitst worden in twee verschillende subsystemen; transcriptie-gekoppeld herstel en globaal genoomherstel. Transcriptie-gekoppeld herstel functioneert om beschadigd DNA in getranscribeerde genen met behulp van transcriptie te herstellen. Globaal

genoomherstel verwijdert laesies uit het hele genoom en bestaat uit een aantal stappen. Het NER mechanisme kan worden onderverdeeld in vijf stappen; herkenning van het beschadigde DNA, opening van de DNA helix rond de laesie, excisie van de beschadigde oligonucleotide en tenslotte resynthese met behulp van de onbeschadigde template alsmede ligatie van de nicks met als eind resultaat hersteld DNA. Meer dan 30 verschillende DNA hersteleiwitten hebben een belangrijke rol in deze georganiseerde processen. De consequenties van een verstoord NER mechanisme zijn hier ook beschreven. Defecten in NER kunnen leiden tot verschillende zeldzame ziektes zoals xeroderma pigmentosum (XP). XP patiënten hebben een extreem hoog risico op het ontstaan van huidkanker in delen van de huid die aan zonlicht blootgesteld zijn. Patiënten die een defect hebben in transcriptie-gekoppeld herstel vertonen meer groei- en neurologische problemen. Deze eigenschappen benadrukken de belangrijke rol die NER heeft voor het bewaken van de integriteit van het menselijk genoom om kanker en andere ziekten te voorkomen. Aangezien reparatie plaatsvindt in een chromatine context, beschrijft **Hoofdstuk 4** chromatine modificaties die nodig zijn voor efficiënt DNA herstel. Hoofdstuk 4 beschrijft ook een model voor het globaal genoomherstel, gebaseerd op het werk gepresenteerd in dit proefschrift alsmede de gepubliceerde literatuur.

UV-DDB is een heterodimeer van p48 (DDB2) en p127 (DDB1) eiwitten die affiniteit vertoont voor verschillende soorten DNA laesies, in het bijzonder een hoge affiniteit voor UV-laesies. In **hoofdstuk 5** gebruiken we verschillende methoden om de vorming van het NER incisie complex en het herstel van fotolaesies te bestuderen. Met behulp van een XPE (p48) deficiënte cellijn vonden we dat het herstel van 6-4PP verbeterd was in cellen met functioneel XPE. UV-DDB speelt mogelijk een rol bij lokale chromatine verandering op de plaats van laesies waardoor de schadedetectie door de XPC-hHR23B complex vergroot wordt.

Biochemische data wijzen erop dat DDB2, evenals CUL4A, ROC1 en DDB1, onderdeel is van een E3 ubiquitine ligase. In **hoofdstuk 6** hebben wij de bindings- en dissociatiekinetiek van fluorescent gelabeld DDB2 aan UV-laesies gemeten in zowel normale als XPC deficiënte cellen om meer informatie te krijgen over hoe DDB2 samen met het E3 ligase complex, DNA schade herkent. DDB2 had dezelfde bindingskinetiek als de andere onderdelen van het ubiquitin ligase complex (CUL4A en DDB1). Het lijkt dus alsof het gehele E3 complex bindt aan beschadigd DNA. XPC is vereist voor de efficiënte binding van de andere NER factoren, hoewel wij weinig interactie vonden tussen de twee schade herkennende eiwitcomplexen op UV laesies. Het is daarom waarschijnlijk dat het E3 ligase complex XPC ubiquitineert na UV bestraling, wat vervolgens leidt tot een verhoogde affiniteit van XPC voor DNA en binding van de andere pre-incisie NER factoren wat resulteert in efficiënt DNA herstel.

Globaal genoom herstel kan worden onderverdeeld in pre- en post-incisie stappen. In tegenstelling tot de pre-incisie stadia, die uitvoerig zijn bestudeerd, zijn de post-incisie stadia van NER minder goed begrepen. De post-incisie stadia zijn DNA herstel synthese, ligatie en chromatine restauratie. Wij hebben een aantal verschillende experimenten uitgevoerd, zoals beschreven in **hoofdstuk 7**, en twee onverwachte factoren geïdentificeerd die

nodig zijn voor de ligatie stap in NER. Wij lieten voor de eerste keer zien dat XRCC1 en DNA ligase 3, die een belangrijke rol spelen in base excisie reparatie, ook van essentieel belang zijn voor NER. De betrokkenheid van DNA ligase 1 in NER was primair gebaseerd op de UV-gevoeligheid van cellen afkomstig van een patiënt met een tekort aan ligase I. Wij hebben geconstateerd dat niet de down-regulatie van ligase 1, maar de down-regulatie van ligase 3 het herstel van DNA schade en de ligatie van de NER geïnduceerde chromosomaal DNA nicks aantast. XRRC1 kan aan PCNA binden, een belangrijk factor die andere post-incisie factoren rekruteert. Wij speculeren dat de XRCC1-Ligase 3 complex in NER functioneert door middel van interacties met PCNA.

Minder aandacht is besteed aan de regulering van NER in vivo, met name de overgang van dubbele incisie naar de DNA synthese stap. Wij hebben, zoals beschreven in **hoofdstuk 8**, de regulering van de pre- en post-incisie stadia van NER onderzocht en de rol van RPA daarin. Onze resultaten suggereren in overeenstemming met vroegere bevindingen dat de belangrijkste rol van RPA in NER, in samenwerking met XPA, de activering van de endonucleasen XPG en XPF is. Remming van de reparatie geassocieerde DNA synthese of ligatie leidt tot ophoping van zowel pre- en post-incisie NER factoren op de plaats van UV-laesies. Onder deze omstandigheden vinden we dat de pre-incisie eiwitten kunnen dissociëren van de oorspronkelijke plaats van reparatie en verhuizen naar andere plaatsen van UV-schade, terwijl RPA en post-incisie eiwitten bij de oorspronkelijke plaats van reparatie blijven. Het falen van RPA om naar andere schadelocaties te verhuizen, leidt tot een instabiel NER pre-incisie complex dat niet in staat is om dubbele incisies te maken. De consequentie hiervan is dat de andere post-incisie factoren niet worden gerekruteerd. RPA speelt dus een belangrijke rol in de bescherming van de overgang van pre- naar post-incisie gebeurtenissen tijdens NER. Onder normale omstandigheden is de vrijgekomen RPA in staat om met de nieuwe gevormde pre-incisie complexen te associëren en te stabiliseren, waardoor verdere incisies en de voortzetting van de reparatie mogelijk is. Daarentegen, persistente associatie van RPA in post-incisie complexen op plaatsen van onvolledige reparatie voorkomt dat RPA deelneemt aan nieuwe incisies en daarmee de integriteit van het genoom beschermt tegen ongecontroleerde incisies van het DNA.

Vroeger werd arseen gebruikt voor het doden van personen, omdat de symptomen op die van acute choleraziekte lijken waardoor een menselijke invloed op de dood minder opviel. Tegenwoordig is vooral het onbewust consumeren van met arseen gecontamineerd drinkwater de oorzaak van arseen geïnduceerde ziektes. Arseengeïnduceerde carcinogenese is een wereldwijd probleem dat epidemische vormen dreigt te bereiken. De wereldgezondheidsorganisatie (WHO) noemt de arseencrisis de grootste massale vergiftiging van de mensheid in de geschiedenis. Momenteel drinken meer dan 140 miljoen mensen in 70 verschillende landen water dat gecontamineerd is met arseenconcentraties ver boven de wettelijke norm. Chronische blootstelling aan arseen leidt tot kanker. Epidemiologische gegevens en in vivo studies suggereren dat blootstelling aan arseen in combinatie met zonlicht het risico op huidkanker verhoogt. Remming van DNA reparatieprocessen is een van de meest prominente mechanismen in arseengeïnduceerde carcinogeniteit. Wij hebben in **hoofdstuk 9** laten zien dat arseen een

duidelijk effect heeft op het herstel van fotolaesies. In lijn met deze bevindingen, vinden we aanhoudende accumulatie van NER pre-incisie eiwitten op UV-laesies. Bovendien hebben wij aangetoond dat de laatste stap van het NER proces, ligatie door DNA ligase III, geremd is. Onze data suggereren dat arseen co-carcinogene effecten induceert gedeeltelijk door het remmen van de ligatie van chromosomale DNA nicks die zich voordoen tijdens NER, met name door remming van Ligase 3.

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