Functions of RING1B and ZRF1 in ubiquitin-mediated regulation of Nucleotide Excision Repair

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This work is dedicated to my late mother

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Index of abbreviations

APS – ammonium persulphate

BER – Base excision repair

BSA – bovine serum albumin

CAK – CDK activating kinase

CPD – cyclopyrimidine dimer

CS – Cockayne syndrome

CSA-CUL4 – CSA-DDB1-CUL4-RBX1 ubiquitin E3 ligase

DAPI - 4',6-diamidino-2-phenylindole

DMEM – Dulbecco minimal essential medium

DSB – double strand breaks

DTT - dithiothreitol

DUB – deubiquitinase

ECL - enhanced chemiluminescence

EDTA - ethylenediaminetetraacetic acid

EdU - 5-ethynyl-2'-deoxyuridine

EGTA - ethylenglycoltetraacetic acid

FBS – fetal bovine serum

GG-NER – global genome NER

H2AK119ubi (H2A-ubiquitin) – Histone H2A ubiquitinated at lysine 119

H2AK13-15ubi – Histone H2A ubiquitinated at lysines 13 and 15

H3K9me3 – Histone H3 trimethylated at lysine 9

H4K16ac – Histone H4 acetylated at lysine 16

H4K20me – Histone H4 methylated at lysine 20

HR – homologous recombination

HU – hydroxyurea

ICL – interstrand crosslinks

IR – ionizing irradiation

K48 – ubiquitin linkage by lysine 48

K63 – ubiquitin linkage by lysine 63

MMR – mismatch repair

NER – Nucleotide excision repair

NHEJ – Non-homologous end joining

PAR – poly-(adenosine-ATP-ribose)

PEI - polyethylenimine

PBS – phosphate buffer saline

PBS-T - phosphate buffer saline, supplemented with 0.1% Tween-20

PIPES - piperazine-N,N′-bis

PMSF - phenylmethylsulfonyl fluoride

PRC1 – Polycomb repressive complex1

PRC2 – Polycomb repressive complex 2

PTM – posttranslational modification

PVDF - polyvinylidene fluoride

ssDNA – single strand DNA

STUbL – SUMO-targeted ubiquitin E3 ligase

SUMO – small ubiquitin-like modifier

TC-NER – transcription-coupled NER

UbA – ubiquitin associated domain

UbL – ubiquitin-like domain

UBD – ubiquitin binding domain

UDS – unscheduled DNA synthesis

UV- ultraviolet

UV-CUL4 – DDB2-DDB1-CUL4-RBX1 ubiquitin E3 ligase

UV-RING1B – DDB2-DDB1-CUL4B-RBX1 ubiquitin E3 ligase

XP – *Xeroderma pigmentosum*

γH2A.X – histone H2A.X phosphorylated at Ser.139

Summary

DNA damage is repaired by a plethora of various DNA repair pathways. Nucleotide Excision Repair (NER) is specifically evolved to deal with helix-distortive DNA damage, arising for example from exposure of cells to UV-light. DNA damage binding proteins DDB2 and XPC are initiating the process of the lesion recognition. Various factors, such as posttranslational modifications of the NER factors as well as the histone at the chromatin region, surrounding the damage site, contribute in the regulation of this process. Ubiquitination of XPC and DDB2 as well as core histones by the ubiquitin E3 ligase DDB2-DDB1-CUL4-RBX1 (UV-CUL4) are accompanying the recognition of the DNA damage in NER. However, little is known about the molecular orchestration of this process.

Ubiquitin E3 ligase RING1B was previously described to be involved the transcriptional repression of genes by monoubiquitination of histone H2A at lysine 119 (H2A-ubiquitin). This activity can be reversed by tethering of the ubiquitin-binding protein ZRF1 which facilitates further removal of ubiquitin from H2A. Previous reports have linked RING1B to the DNA-damage dependent H2A ubiquitination. In this project we have addressed the role of RING1B and ZRF1 in the regulation of NER.

We show that after UV-irradiation H2A is monoubiquitinated by a novel ubiquitin E3 ligase complex, containing RING1B together with DDB proteins and adaptor protein CUL4B (UV-RING1B). This histone modification serves as a recruitment platform ZRF1. ZRF1 is a noverly described factor in NER and it functions by displacing CUL4B and RING1B from UV-RING1B E3 ligase complex and promotes formation of the UV-CUL4 E3 ligase complex which acts downstream. Additionally we demonstrate that ZRF1 may contribute in the proteasomal degradation of the NER proteins, and potentially act in other DNA repair pathways.

Our data expands our understanding of the ubiquitination processes, regulating early steps of the NER pathway as well as the contribution of the chromatin context in this process.

Zusammenfassung

DNA-Schäden werden durch verschiedene DNA-Reparaturmechanismen repariert. Nukleotidexzisionsreparatur (NER) repariert DNA-helix störende Verletzungen, welche durch UV-Strahlung erzeugt werden. Die Proteine DDB2 und XPC erkennen die geschädigte DNA und initiieren NER. NER wird reguliert von verschiedenen Faktoren, zum Beispiel posttranslationale Proteinmodifikationen des NER- und Histonproteinen um die geschädigte DNA-Region. Bei diesem Prozess werden XPC, DDB2 und die Nukleosomenkernhistonproteinen durch die E3 Ubiquitinligase DDB2-DDB1-CUL4-RBX1 (UV-CUL4) ubiquitiniert. Jedoch, sind die Molekularmechanismen und die Konsequenzen dieser Ubiquitinierung unbekannt.

E3 Ubiquitinligase RING1B monoubiquitiniert Histon H2A an Lysine 119 und inhibiert die Geneexpression. Ihre Aktivität wird entgegenwirkt durch die Bindung von Ubiquitin-bindendem Protein ZRF1, welches Deubiquitinierung von H2A fördert. RING1B ubiquitiniert H2A auch bei DNA-Schäden. In dieser Arbeit konnte gezeigt werden, dass RING1B und ZRF1 regulatorische Rollen in NER haben.

Nach UV-Bestrahlung wird H2A durch den neu entdeckten E3 Ubiquitinligasekomplex UV-RING1B monoubiquitiniert. Diese besteht aus RING1B sowie DDB2 und DDB1 Proteine und dem Adapterprotein CUL4B. Die H2A-Monoubiquitinierung bildet die Rekrutierungplatform für ZRF1. ZRF1 ist ein neuartiges NER-Protein, das entfernt CUL4B und RING1B aus UV-RING1B E3 Ubiquitinligase. ZRF1 fördert die Bildung von UV-CUL4 Komplex der wichtig ist für weitere NER-Regulierung. Zusätzlich könnte ZRF1 zu NER-Proteinabbau und auch anderen DNA-Reparaturmechanismen beantragen.

Unsere Ergebnisse erweitern unser Verständnis der Ubiquitinierungsprozesse während der NER-vermitteltes Erkennung der DNA-Schäden. Zudem unterstreichen unsere Resultate die Bedeutung des Chromatinkontexts in diesem Prozess.

Introduction

DNA Repair Pathways

DNA structure is under a constant threat of numerous factors affecting its structure, and hence the encoded genetic information, and causing mutations. Although mutagenesis creates a basis for the evolution, accumulation of mutations can lead to various diseases, related to malignant transformations (cancer) or premature cell death (aging phenotype). Therefore prokaryotic and eukaryotic cells developed various mechanisms to protect DNA from damage.

Types of DNA damage

DNA damage according to its source can be divided in primary and secondary, related to its source (reviewed in De Bont and van Larebeke, 2004; Helleday et al., 2014). Primary DNA damage is caused by the impact of endogenous and exogenous factors on DNA strands and repaired by a variety of DNA repair pathways (Table 1).

Table 1

DNA damage sources and DNA repair pathways

Source	DNA modification	DNA repair pathway
<u>Endogenous</u>		
Reactive oxygen species ($O_2^{-}\cdot$; H_2O_2; $OH\cdot$; $^1O_2$)	Oxidation of bases (8-OH-dG) DNA adducts (ethano-, propano-derived)	Base excision repair (BER) Interstrand crosslink repair (ICL repair)
Endogenous alkylating agents (SAM)	Methylation of bases O^6-methylguanine	BER Direct reversal
Oestrogen metabolites	Oestrogen-DNA adducts Base modification (8-OH-dG) Single strand breaks	BER Mismatch repair
Deamination	Base conversion (C→U; A →hypoxanthine)	Mismatch repair
Replication mismatches and error-prone repair	Base misincorporation	
Misincorporation of nucleotides	rNTP incorporation	RNAse H2

Exogenous		
Chemical adducts	Bulky chemical adducts (benzo[a]pyrene; aflatoxin; cisplatin)	Nucleotide excision repair (NER)
	Alkylation agents (N-methyl-nitrosoguanidine, sulfur mustard, nitrogen mustard)	BER, direct reversal
	DNA intercalators (proflavines)	ICL repair, NER
Irradiation	Ionizing Radiation: double strand breaks	NHEJ, HR
	UV Radiation: photoproducts	NER

NHEJ – non-homologous end joining; HR – homologous recombination.

Although the mechanisms repairing primary DNA lesions are working with a high efficiency, they may interfere with other processes involving DNA, such as replication and transcription. Formation of the single strand DNA (ssDNA) patches by unrepaired DNA lesions due to fail of repair system or nucleotide shortage, unresolved DNA-RNA hybrids (by blocking of transcription), incorporation of ribonucleotides in DNA or formation of secondary DNA structures induce stalling of replication forks. Replication stress is also triggered by treatment with certain genotoxic agents such as hydroxyurea (HU), etoposide or camptothecin (CPT). When not resolved, the replication fork may collapse and form a double strand break (DSB) (reviewed in Zeman and Cimprich, 2014).

Repair of double strand breaks

The presence of a DSB, caused by endogenous of endogenous factors activates a number of proteins, including components of the MRN complex, consisting of MRE11, RAD50 and NBN (reviewed in Petrini and Stracker, 2003) (Figure 1). MRN acts as a sensor of the DSB. It binds and stabilizes broken DNA ends and promotes the activation DSB repair via homologous recombination or nonhomologous end joining (reviewed in Shiloh and Ziv, 2013 and Stracker and Petrini, 2011).

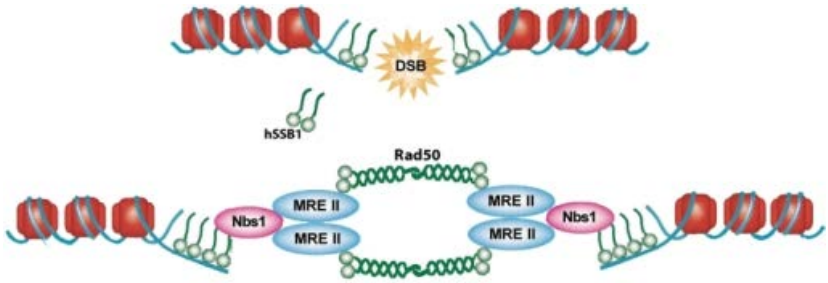


Figure 1

MRN complex is recruited to DSB to stabilize DNA. Adapted from Richard et al., 2011.

Homologous recombination (HR) and non-homologous end joining (NHEJ) operate according to the cell cycle phase and use different approaches to restore the sequence specificity of the damaged DNA. HR uses a DNA template from a sister chromatid or homologous chromosome which leaves this pathway error-free (Figure 2, left). HR is restricted to the S or G2 phase of the cell cycle, when these structures are available. First steps of HR require resection of damaged DNA ends, mediated by the MRN complex, BRCA1-BARD complex together with endonucleases CtIP, Exo1 or others. This leads to formation of a ssDNA stretch, protected by the ssDNA binding protein RPA. RPA is further displaced by the recombinases Rad51 and Rad54. These proteins are required for the search of homologous sequences and completion of DNA repair. HR is the primary mechanism to repair collapsed replication forks.

Unlike HR, repair by NHEJ does not require a sequence template and operates during other phases of the cell cycle, where it directly joins two ends of the DSB (reviewed in Chapman et al., 2012). Classical NHEJ is mediated by the activity of the Ku70/80 dimer which brings together two DSB ends (Figure 2, right). Additionally DNA damage response factor 53BP1 is responsible for the mediation of classical NHEJ by blocking of the resection of DNA ends, characteristic for HR (reviewed in Zimmermann and de Lange, 2014). DSB is repaired further by the activity of XRCC4/DNA ligase IV complex. Alternatively, NHEJ may be independent of Ku70/80 and initiated by a process similar to HR, but generating sites of microhomology, rather than long ssDNA patches as in classical HR pathway.

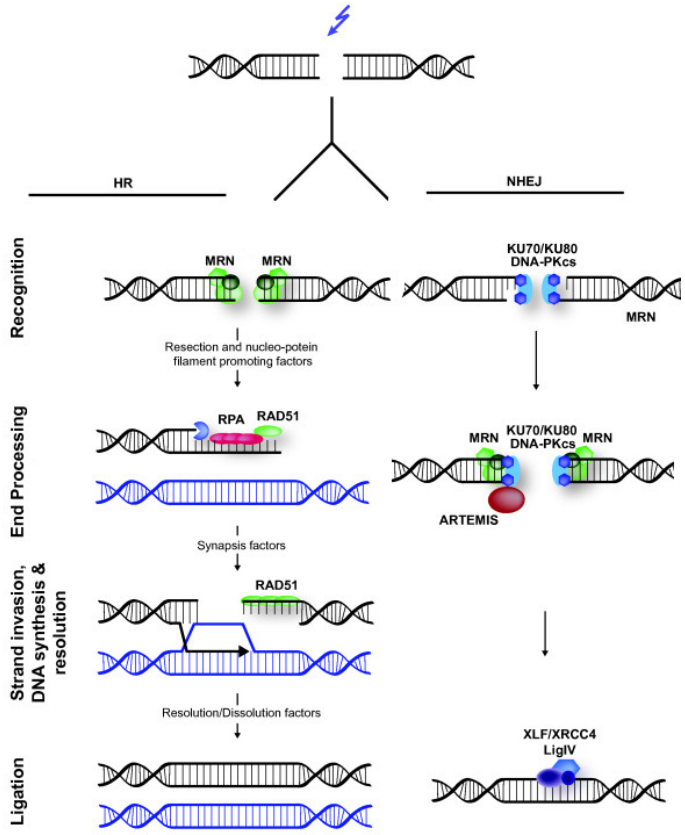


Figure 3

Double strand break repair pathways

Left:
Homologous recombination repairs DSB with the homological sequence provided by a sister chromatid. After end processing homologous pairing is followed by DNA strand exchange, DNA synthesis and ligation.
Right:
Non-homologous end-joining mediated by the activity of the Ku70/80 dimer which brings together two DSB ends. The DNA ends are end-processed and further repaired by the activity of XRCC4/DNA ligase IV complex.

Adapted from Lans et al., 2012.

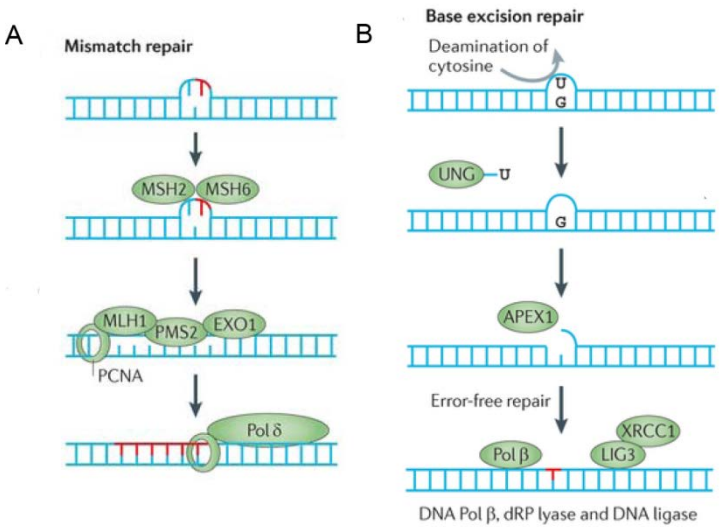


Figure 2

Mismatch and Base excision repair

A) Mismatch repair is initiated by the DNA mismatch recognition proteins MSH2 and MSH6; a segment of DNA is excised between the mismatch and a nearby nick by the MMR endonuclease PMS2 and exonuclease 1 (EXO1). The gap that is left in the DNA is filled by Pol δ.

B) BER is initiated by a number of specific base-glycosylases recognizing a modified nucleotide to create an abasic site (UNG). Further, DNA strand is cleaved by endonuclease APEX1, gap filling and ligation.

Adapted from Helleday, 2003; Lans et al., 2012

Mismatch repair

Aberrantly incorporated nucleotides and products of base conversion are repaired through the mismatch repair (MMR) pathway (reviewed in Jiricny, 2006; Li, 2008). MMR is a highly conserved pathway and consists of proteins, specifically involved in the mismatch repair as well as the proteins involved in further DNA re-synthesis. Recognition step is performed by the MutS heterodimer recruiting further factors to form of a nick and excise the mismatched nucleotide, followed by DNA re-synthesis and ligation (Figure 3A).

Base excision repair

Methylation and hydroxylation of the nucleotides as well as formation of spontaneous abasic sites are one of the main sources of DNA damage. These lesions are repaired by the base excision repair (BER) pathway (reviewed in Krokan and Bjoras, 2013) (Figure 3B). BER is initiated by a number of specific base-glycosylases recognizing a modified nucleotide to create an abasic site. Further, DNA strand is cleaved by the endonuclease APE1, followed by gap filling and ligation. Interestingly, methylated nucleotides can be repaired by a direct conversion of the base. For example, O^6-methylguanine, which is one of the most common alkylated forms of nucleotides, is repaired through activity of O^6-methylguanine-DNA methyltransferase (MGMT) (reviewed in Gerson, 2004; Sharma et al., 2009).

Nucleotide excision repair

BER together with Nucleotide excision repair (NER) share a common approach of dealing with the damaged DNA - an excision step, mediated by endonucleases. Unlike other DNA repair mechanisms, NER is specifically evolved to remove DNA distorting lesions. The most common trigger for NER is exposure of DNA to ultraviolet (UV) irradiation. UV light causes formation of a dimer between two pyrimidines, generating cyclopyrimidine dimers (CPD) as well as 6'-4' photoproducts (reviewed in Franklin and Haseltine, 1986). CPD occur more often when 6'-4' photoproducts and overall their repair kinetics is slower. Additionally CPD are highly distortive to DNA and prone to block replication and transcription (Tung et al., 1996). In contrast, repair of 6'-4' photoproducts is evolved to be repaired much faster. This type of photoproducts does not block replication properly, and if unrepaired 6'-4' photoproducts are highly mutagenic (Kamiya et al., 1998). NER machinery also recognizes various bulky DNA adducts such as benzo[a]pyrene derivatives, cisplatin and others.

Mutations in the NER pathway are related to the disorders of *Xeroderma pigmentosum* (XP) spectrum (Cleaver, 1968). XP is a rare autosomal-recessive decease, characterized by a high

sensitivity to UV light. Individuals with XP have a high risk of developing eye and skin cancer. In some, but not all the cases they develop neurological abnormalities and non-skin tumors. Association of the NER defects and the phenotype of XP patients was demonstrated by Epstein and colleagues (Epstein et al., 1969). Later, XP was divided in 7 genetically heterogeneous groups: from XPA to XPG (Arase et al., 1979; De Weerd-Kastelein et al., 1972). The cells from these lines are sharing low levels of repair DNA synthesis (UDS). Additionally, cells some of XP patients exhibit unaltered level of UDS. These cells were later characterized as XP-variant (XPV) and are known to have mutations in DNA polymerase η which is related not only to NER, but also to replication processes.

Genes and the respective proteins responsible for the XP complementation groups have been described later. This subsequently allowed reconstitution the NER reaction *in vitro* in the pioneer work of Aboussekhra and colleagues (Aboussekhra et al., 1995). Over 30 polypeptides were used to recapitulate this process; however, they do not reflect all the proteins, required for a NER reaction *in vivo*. Overall, the NER pathway could be subdivided in several steps: recognition of the DNA lesion, formation of the preexcision complex, which unwinds DNA and allows excision of the DNA stretch containing the lesion. This process is followed by repair synthesis and ligation of the DNA nicks (Figure 4). Further, we will describe in details the mechanism of the NER.

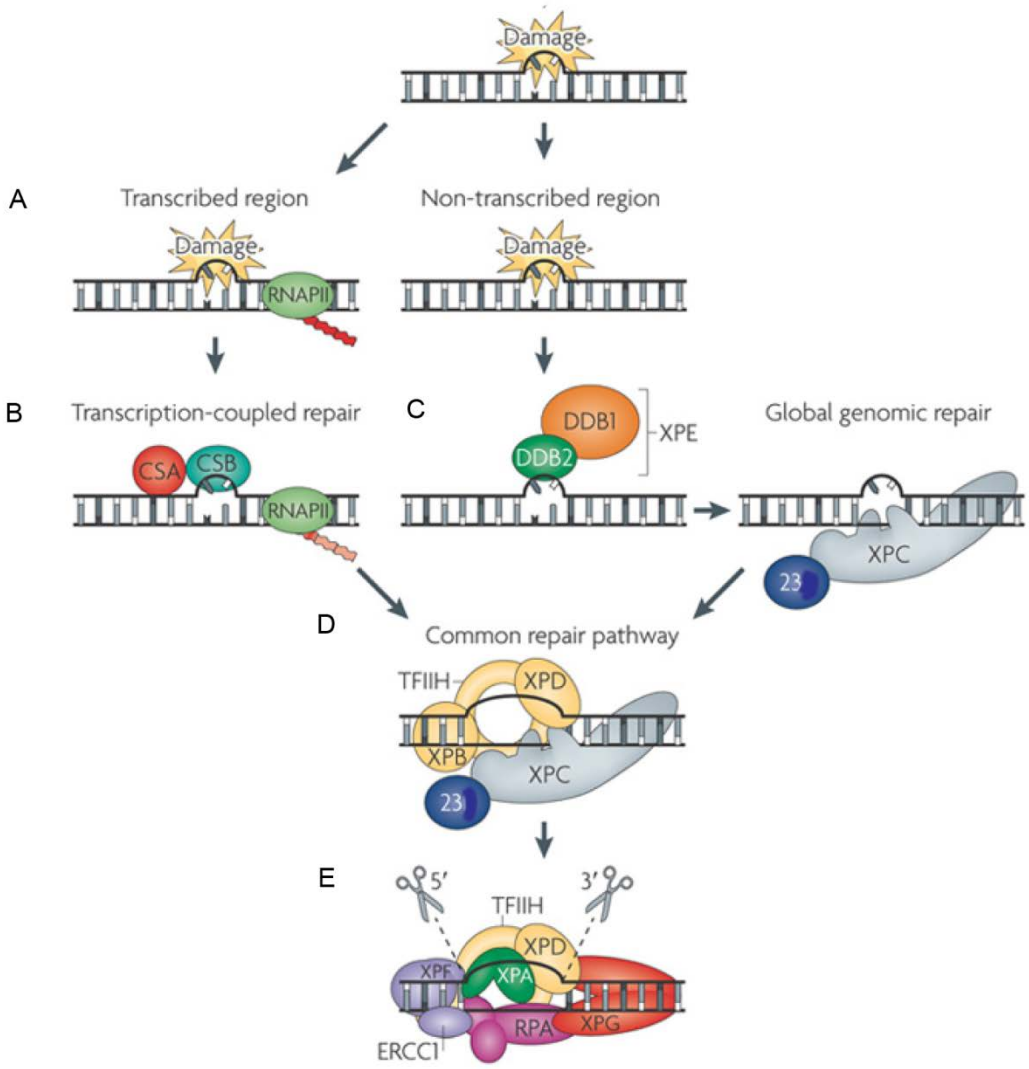


Figure 4

Principal scheme of nucleotide excision repair pathway

A) NER is triggered by the detection of the DNA damage, usually mediated by exposure of DNA to UV-irradiation.
B) Transcription-coupled repair. DNA damage at the transcribed DNA strand is recognized by the stalling of RNApolII and further binding of CSA and CSB proteins
C) Global genome repair. DNA damage is recognized by DDB2 and further by XPC.
D) Formation of the pre-excision complex. TFIIH, including DNA helicases XPB and XPD, and XPA are recruited to the DNA damage site to form open DNA structure.
E) DNA lesions are excised by exonucleases XPF and XPG and the nick is ligated by DNA-polymerases and DNA ligases.

Adapted from Cleaver et al., 2010.

Transcription-coupled NER

DNA damage can occur at different places in the genome. Suggestions, that DNA repair in transcribed and non-transcribed regions of genome goes with different speed, was given by Bohr and colleagues (Bohr et al., 1985). Later, it was identified that only transcribed DNA strand is repaired faster (Mellon et al., 1987). This finding prompted to investigate if there are differences in recognition and repair of the DNA damage in transcribed and non-transcribed genomic regions. Indeed, in NER recognition of the DNA damage on the transcribed strand is performed by a physical stalling of RNApolII (transcription coupled repair sub-pathway – TC-NER), whereas in other cases recognition requires DNA damage binding proteins XPC and DDB2 (global genome repair subpathway – GG-NER) (Figure 4B and 4C). Stalled transcription is causing genotoxic stress as it may interfere with the ongoing DNA replication; potentially causing double strand breaks formation. Blocking of RNApolII leads to activation of DNA damage response pathways and further apoptosis or cell cycle arrest. Therefore, fast recognition is important to resolve these problems. Mutations in the TC-NER proteins CSA and CSB are linked to the Cockayne syndrome (CS). Unlike XP patients, individuals with CS are characterized by the retarded growth and development, progressive neurodegeneration as well as other progeriod features. The symptoms characteristic for CS are demonstrating that defective TC-NER is not only mutagenic, but affects survival of the cells, triggering premature cell death.

As the DNA lesion occurs on the transcribed strand of DNA, elongated form of RNApolII is physically stalled. Importantly, only actively progressing RNApolII can activate the TC-NER pathway. There are several hypotheses regarding the behavior of the stalled RNApolII. First model involves reverse translocation (backtracking) of RNApolII and its subsequent dislocation from chromatin. Alternatively, RNApolI can be dislocated from chromatin to be further subjected to proteasomal degradation. Current research favors the idea that RNApolII stays at the damage site during the early steps of damage recognition (reviewed in Fousteri and Mullenders, 2008, Schwertman et al., 2013). *In vitro* data shows that the incorporation of CPD in the active site of RNApolII leads to its arrest and backtracking. This is followed by the cleaving of the transcript with misincorporated nucleotide and preservation of the remaining RNA oligonucleotide. The transcript cleaving is performed by the transcription elongation complex (Sigurdsson et al., 2010). It remains unclear how this mechanism is orchestrated *in vivo*.

Arrested RNApolII is further sensed by a DNA-dependent ATPase CSB (Troelstra et al., 1992). This protein is essential for the recruitment of the downstream NER machinery, although the exact mechanism behind this remains unknown. CSB specifically recruits another protein, essential for TC-NER: CSA (Fousteri et al., 2006; Henning et al., 1995). This protein has DNA binding

domains and serves as a substrate receptor in the CSA-DDB1-CUL4-RBX1 ubiquitin E3 ligase, regulating TC-NER through series of ubiquitination events (Groisman et al., 2003). Recently, other proteins have been described to be important for progression and regulation of TC-NER such as UVSSA/USP7 complex; XAB2 and HMGN1 (Birger et al., 2003; Fousteri and Mullenders, 2008; Nakatsu et al., 2000; Schwertman et al., 2013). Regulation of TC-NER by these proteins will be described later (see page 24).

Global genome NER

Unlike TC-NER, recognition of the DNA lesion at the untranscribed DNA loci is performed by the proteins XPC and DDB2 (Figure 5). XPC protein exists in a complex with human Rad23 homologue HHR23A/B (RAD23A/B) as well as CENT2 (Araki et al., 2001; Masutani et al., 1994). XPC-HHR23B dimer was shown to bind damaged DNA with a higher affinity compared to non-damaged templates *in vitro* (Sugasawa et al., 1998). XPC has a high affinity to various DNA damages, however, its affinity to UV-mediated CPD is very low (Kusumoto et al., 2001). Unlike other DNA lesions such as 6'-4' photoproducts, CPD are not changing enough the structure of the DNA double helix. Binding of XPC is dependent on small bubble structures (Sugasawa, 2001). Resolving of the crystal structure of yeast XPC homologue Rad4 in complex with Rad23 and CPD-containing DNA oligonucleotide provides an inside of the nature of DNA damage recognition by XPC (Min and Pavletich, 2007). Rad4 binds a stretch of undamaged DNA and flips out the CPD by insertion of the β-hairpin domain. This further stabilizes XPC on DNA. *In vivo* data demonstrates that the initial recruitment of XPC is performed by its probing of DNA for the possible DNA helix altering structures (Hoogstraten et al., 2008).

Little is known about the role of RAD23B and RAD23A in NER. Both these proteins are harboring ubiquitin binding domains which link them to ubiquitin/proteasome degradation pathway (Bertolaet et al., 2001; van der Spek et al., 1996). RAD23A and RAD23B have redundant functions in NER, and their role is proposed to stabilize XPC from proteolysis (Ng et al., 2003; Schauber et al., 1998). However, the role of RAD23 paralogues is not only restricted to this, as XPC overexpression is not rescuing the RAD23 knockout cells from UV-sensitivity. Unlike XPC, RAD23 is not accumulating at the DNA-damage site. In contrary, the XPC-RAD23 complex rapidly dissociates after binding to damaged DNA (Bergink et al., 2012). This proposes that RAD23 enhance the binding of XPC to DNA damage sites. CENT2 is mainly identified a component of a centrosome, important for its duplication during cell division (Salisbury et al., 2002). However, an abundant fraction of this protein is associated with XPC-RAD23 complex (Nishi et al., 2005), where it was shown to enhance the DNA binding activity of XPC. C-terminal domains of CENT2 bind XPC,

while N-terminal interacts with the damage verification protein XPA, stimulating further DNA repair steps (Nishi et al., 2013).

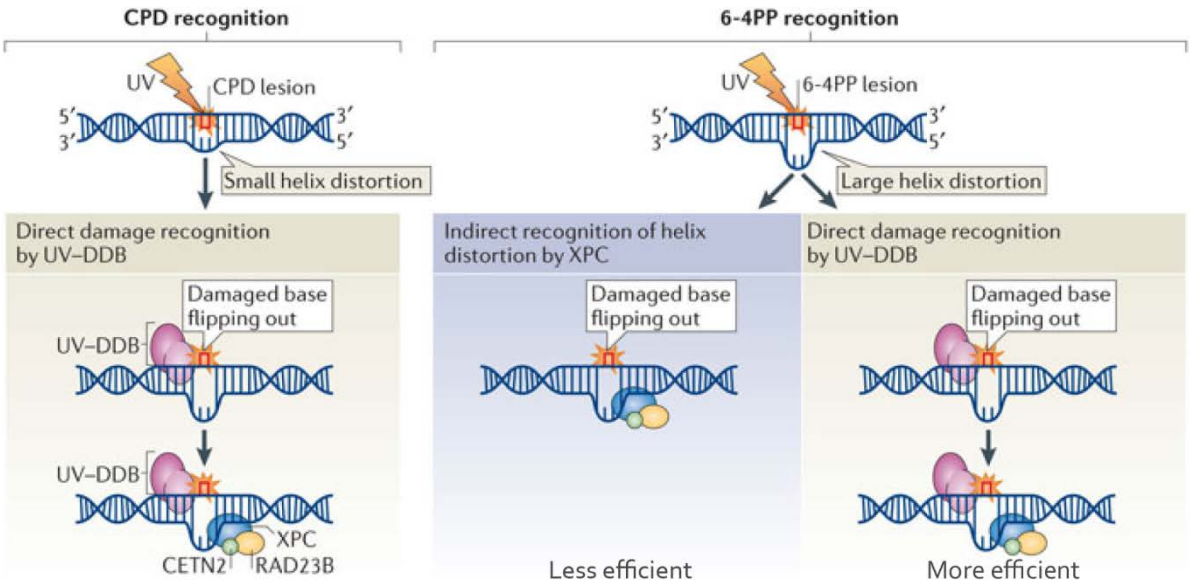


Figure 5

Recognition of DNA damage in GG-NER

CPD and 6'-4' photoproducts (6-4PP) are recognized by UV-DDB complex, which kink DNA and promotes further recruitment of XPC-RAD23 (right and left panel). 6-4PP are also recognized by XPC, although with lesser efficiency (middle panel).

Adapted from Marteijn et al., 2014.

Although XPC seems to be the master regulator of DNA damage recongnition in GG-NER, it is not efficiently recognizing UV-mediated CPDs and 6'-4' photoproducts. The idea of a specific protein, binding UV-induced photoproducts was addressed in the early works of Feldberg and colleagues (Feldberg and Grossman, 1976). They have identified a novel protein specific for binding UV-mediated photoproducts. Later, this protein has been shown to be absent in the fibroblast from XPE patient (Chu and Chang, 1988). Interestingly, further analysis identified that the identified protein exists as a heterodimer consisting of two subunits (Keeney et al., 1993). These proteins are encoded by DDB1 and DDB2 genes respectively, forming the UV-DDB dimer (Dualan et al., 1995). As mentioned before, mutations in DDB2 and DDB1 are characteristic for XPE patients. Compared to other XP affected individuals, these patients are showing milder phenotypes, and the XPE cells are not entirely deficient in UDS. This suggests an auxiliary role of DDB1 and DDB2 in DNA damage recognition. Moreover, reconstitution of NER reaction in vitro is possible

without UV-DDB. It was proposed, that UV-DDB is important in resolving of the damage of chromatinized DNA template, occurring *in vivo* (Aboussekhra et al., 1995).

Purified UV-DDB complex binds with a high affinity to CPD and 6'-4' photoproducts (Fujiwara et al., 1999; Wittschieben et al., 2005). Crystal structures of DDB2 and DDB1 describe their interaction as well as the mechanism of DNA damage detection by DDB2. DDB1 harbors three WD40 B-propeller domains, providing a binding platform for DDB2 (Angers et al., 2006; Scrima et al., 2008). DDB2 is docked to the damaged DNA is through the WD40 domains and similarly to XPC separates the DNA strands at the lesion by insertion of a hairpin structure. This is followed by the unwinding of DNA helix and flipping out of the DNA lesion (Scrima et al., 2008). Although reconstitution of NER reaction *in vitro* is possible without the UV-DDB, it increases the efficiency of the reaction. Furthermore the overexpression of DDB2 enhances recognition of DNA damage by XPC (Fitch et al., 2003; Wakasugi et al., 2002). Additionally, binding of DDB2 prolongs the residence of XPC at damaged chromatin (Luijsterburg et al., 2007). This suggests that DDB2 and XPC are acting successively in the recognition of the UV-induced DNA lesions.

Stabilization of XPC or stalling of RNApolII at the damage site promotes recruitment of a multisubunit basal transcription factor TFIIH (Figure 6) (Coin et al., 2007; Li et al., 2015; Spangler et al., 2001). This complex consists of a main catalytic core, containing XPB, XPD as well as p62, p52, p44, p34 and TTDA proteins and a CDK-activating kinase (CAK) module, composed of CDK7, MAT1 and cyclin H (Egly and Coin, 2011). Both the catalytic core and the CAK module are important for the transcriptional regulation, however, in NER CAK module is dissociated from the TFIIH (Coin et al., 2008). TFIIH is loaded at the lesion site in 5' position and creates an open DNA structure by action of two helicase subunits XPB and XPD (Evans et al., 1997; Sugasawa, 2001). XPB interacts with XPC and loaded on DNA, followed by the binding of XPD to the unwound strand. XPD moves along DNA towards 3' end until it meets the DNA lesion, demarcating the damaged region (Mathieu et al., 2010; Sugasawa et al., 2009). Importantly, TFIIH interacts with XPC and XPA, which facilitates its translocation along the damaged DNA (Li et al., 2015).

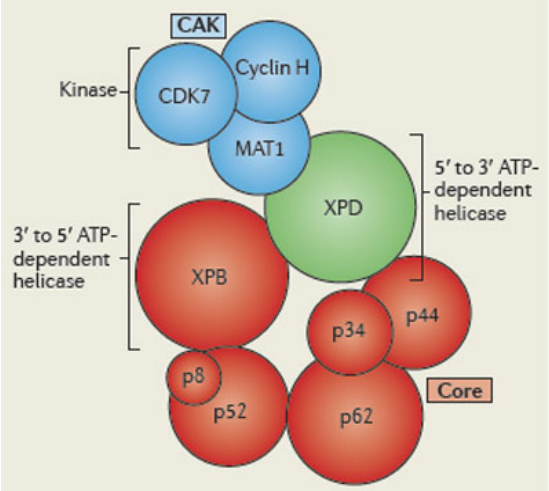


Figure 6

Structure of mammalian TFIIH

Mammalian TFIIH consists of the core complex: XPB, p62, p52, p44, p34 and p8; XPD and the CAK complex: CDK7, cyclin H and MAT1

From Compe & Egly, 2012

XPA is the master regulator of DNA damage verification and proper progression of NER. XPA is able to detect nucleotides with altered chemical structures as well as to interact with DNA, stabilizing 5' end of the lesion site (Camenisch et al., 2006; Krasikova et al., 2010). XPA stimulates the DNA helicase activity of TFIIH and stalls activity of XPB and XPD after the process is complete to facilitate a later excision step (Li et al., 2015; Sugasawa et al., 2009). Presence of XPA promotes further recruitment of the ssDNA binding protein RPA. In NER RPA protects the undamaged DNA strand from cleavage by endonucleases and orientates structure specific endonucleases XPG and ERCC1-XPF to incise the patch of damaged DNA (de Laat et al., 1998). XPG and ERCC1-XPF are recruited sequentially (Volker et al., 2001). Firstly, XPG is recruited either independently or through the binding with TFIIH (Dunand-Sauthier et al., 2005), whereas recruitment of ERCC1-XPF depends on the presence of XPA (Tsodikov et al., 2007). After association with the damage site XPG is required for ERCC1-XPF to perform the 5' nick, followed by the second incision by XPG at the 3' end. Notably, the 5' incision is sufficient to start the gap-filling DNA synthesis, preventing the accumulation of cytotoxic ssDNA stretches. Gap filling DNA synthesis requires the replication-associated proteins such as PCNA and RFC, a DNA polymerase and a DNA ligase. According to the proliferation status of the cell the latter two differ. In replicating cells DNA synthesis is performed by DNA polymerase DNApolε and subsequent ligation is performed by DNA ligase1. In contrast, in quiescent cells this process is performed by DNApolδ and DNApolκ, and ligation step is dependent on XRCC1-DNA ligase III complex (Moser et al., 2007; Ogi et al., 2010).

Regulation of DNA repair by posttranslational modifications

Newly synthetized proteins are often modified by a conjugation of various chemical adducts. Posttranslational modifications (PTM) vary from simple chemical groups such as phosho- or methyl-group to small proteins such as ubiquitin and SUMO. Modification of a target protein is usually a multistep mechanism, which includes an interplay of a substrate, conjugation enzymes as well as enzymes which edit and remove the modification. PTMs play an exceptional role in the regulation of protein functions. First of all, they expand a variety of protein functions and impact their activity. As an example, activation of protein kinases is often dependent on their phosphorylation status. Moreover, proteins can be modified with several PTMs in the same time, further expanding their functional variety. Addition of PTMs may play a role in the modulation of the protein structure, altering its binding to substrates, such as other proteins or nucleic acids. In aforementioned kinases, phosphorylation of the catalytic center reorganizes it to the active mode. However, most commonly PTMs serve as the intermediates of protein-protein interactions, as many proteins have PTM-interacting domains. Combination of subsequent PTMs followed by sequential binding of proteins is the key mechanism to regulate various signaling pathways such as DNA damage repair processes (reviewed in Dantuma and van Attikum, 2016).

Phosphorylation

In the process of phosphorylation a phosphate moiety is conjugated to the tyrosine, serine or a threonine of the target protein by protein kinases. This process uses an ATP molecule as the source of a phosphate and is reversible by dephosphorylation. Three kinases ATM, ATR and DNA-PK are responsible for the main phosphorylation events in DNA repair. ATM acts mainly in the repair of DSB, whereas ATR is active in a presence of single strand breaks and after UV irradiation. DNA-PKs are generally related to the later stages of DNA repair. ATM and ATR are responsible for phosphorylation of the histone variant H2A.X at serine 139 (γH2A.X) (Rogakou et al., 1998). This modification is extremely important in the orchestration of DSB repair pathways. It is required for the initial steps of the DNA damage recognition such as recruitment of MDC1. Activated ATM plays an important role in the formation of DNA repair foci as it phosphorylates such proteins as MDC1, 53BP1 and BRCA1 (Figure 7) (Matsuoka et al., 2007). These events lead to sequestration of the proteins at the damaged site and further amplification of the signal, ensuring proper DNA repair.

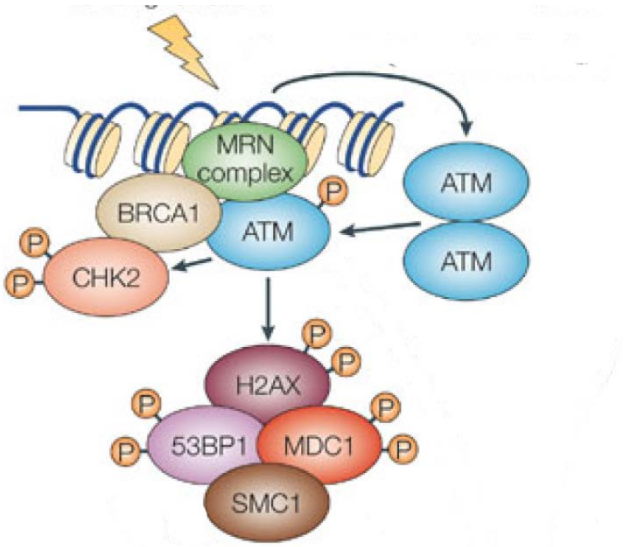


Figure 7

Phosphorylation is integral for the recognition of the DSB

DSB promotes activation of ATM which in turn phosphorylates DNA damage proteins MDC, 53BP1 and histone variant H2A.X. These phosphorylation events lead to sequestration of the proteins at the damaged site and further amplification of the signal, ensuring proper DNA repair

Adapted from Sengupta and Harris, 2005

PARylation

PARylation of proteins is manifested by addition of PAR (poly-(adenosine-ATP-ribose)) moieties at aspartate, glutamate and lysine of the target proteins. PAR-conjugation protein PARP1 is recruited to the sites of DNA damage as it binds DSB, CPD and 6'-4' photoproducts. At the damage site it modifies a variety of proteins with PAR chains, including itself (reviewed in Luo and Kraus, 2012). These events are important for the structural organization of the DNA damage site, as the PAR-chains are able to create a scaffold platform for the docking of the repair proteins. Importantly, PAR-chains have a strong negative charge which leads to attraction of the DNA and RNA binding proteins. Presence of the PAR-chains is shown to dissociate histone-DNA interactions. Therefore it promotes local changes at chromatin, relaxing its structure and preparing it for the DNA repair machinery (Poirier et al., 1982).

Ubiquitination

Ubiquitin-conjugation system

Ubiquitination of proteins was identified as the first PTM and promoted further extensive research on the role of this modification (Goldknopf and Busch, 1977). Conjugation of ubiquitin as well as ubiquitin-like proteins, such as SUMO or NEDD8 is a multistep process (Figure 8). It requires an activation enzyme (E1), a conjugating enzyme (E2) and a ligase (E3) (reviewed in Pickart, 2001). In a multistep ubiquitination mechanism E1 activates ubiquitin and transfers it to the active site of E2. E3 ligases are usually binding the substrate and the E2~ubiquitin conjugate,

assisting the transfer of ubiquitin to the substrate. Ubiquitin E3 ligases are characterized by the presence of HECT (Homologous to the E6-AP Carboxyl Terminus) or RING (Really Interesting New Gene) domains, responsible for their enzymatic activity.

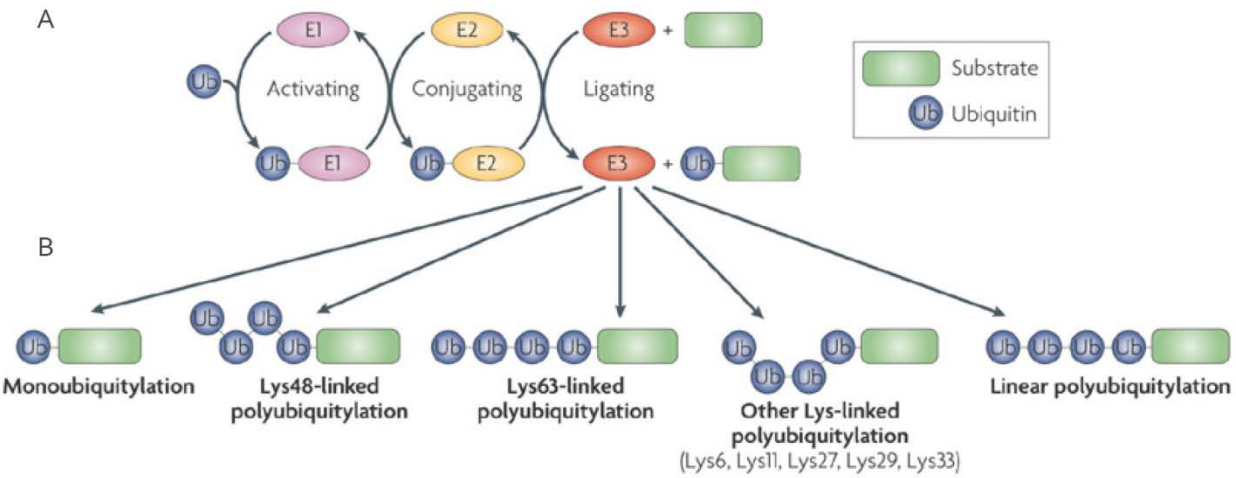


Figure 8

Ubiquitination cascade and formation of ubiquitin chains

A) Ubiquitination is a multistep reaction, which requires activity of E1, E2 and E3 ubiquitin ligases.
B) Ubiquitin can be conjugated to the target protein as a monomer, or form a polymer chain through different lysines of the ubiquitin molecule.

Adapted from Dikic et al., 2009.

Ubiquitin is a small 8.4kD protein attached to the lysines of a target protein in a form of monomer or polymer chains. The polymer chains are formed through one of the seven lysines of a ubiquitin molecule and their structure defines the further fate of the protein. Individual E3 ligases can interact with various E2 to modify the substrate with ubiquitin chains with different linkages (Christensen et al., 2007; Ye and Rape, 2009). The most common ubiquitin linkage in the polymer chains is through the lysine-48 (K48) of ubiquitin. These compact chains serve as a signal for shuttling the protein to a proteasome where it is degraded to small oligopeptides by proteolysis. Other linkages with the exception of K63 chains, are also implicated in degradation of the proteins by proteasomes, however the detailed mechanism remains elusive (Xu et al., 2009). Monoubiquitination as well as the formation of K63 chains have primarily a non-proteolytic function. K63 chains are not directly involved in proteasomal degradation; instead they are implicated in the protein-protein interactions as well as stabilization of proteins and modulation of their activity. Interestingly, K63 ubiquitin chains also exist in unanchored forms. At the site of the DNA damage they may serve as a scaffold to attract the proteins to the DNA damage sites (Xia et al., 2009).

Protein monoubiquitination also acts as a protein regulation mechanism. Initially the monoubiquitination of histones H2A and H2B has been identified, and their function is primarily characterized in regulation of transcription and DNA repair. However, other proteins can be subjected to monoubiquitination. For example, monoubiquitination of components of Falconi pathway FANCD2 /FANCI promotes recruitment of the downstream nuclease FAN1 (Joo et al., 2011).

Proteins, modified by ubiquitin are recognized by the ubiquitin binding domains (UBD) of the interaction partners. These proteins can be responsible for the ubiquitination or deubiquitination of a substrate or serve as ubiquitin receptors, recognizing and interpreting signals of the conjugated ubiquitin chains (reviewed in Hicke et al., 2005). UBDs can be specific for binding specific ubiquitin linkages, enhancing specificity of the protein interactions. Importantly, these interactions play an important role in crosstalk of the PTMs, as the proteins may contain various domains binding other PTMs. A well-studied example of such interplay is generation of “phospho-degrone”, where a protein targeted to degradation is phosphorylated, prior to formation of K48 ubiquitin chain (reviewed in Ravid and Hochstrasser, 2008). The complex interaction between phosphorylation, PARylation and various ubiquitination events is a key mechanism to regulate DNA damage response. In this process crosstalk between PTMs plays an important role in recruitment and removal of the DNA repair factors from chromatin, ensuring progression of DNA repair.

Role of ubiquitination events in DNA repair

Phosphorylation of H2A.X is an early DNA damage response event and an integral signal to orchestrate DSB repair. The phosphate on H2A.X is recognized by the fork-head domain of the ubiquitin E3 ligase RNF8 (Figure 9). Together with E2 UBC13 RNF8 initiates series of local ubiquitination events at the DNA damage site. One of the main substrates of RNF8 is histone H1, which is decorated by K63 ubiquitin chains (Thorslund et al., 2015). These chains are further recognized by the ubiquitin dependent module 1 of the ubiquitin E3 ligase RNF168. Recruitment of RNF168 to the DNA damage site promotes monoubiquitination of histone H2A at lysines 13 and 15 (H2AK13-15ubi), a histone modification, characteristic exclusively for DNA damage response (Mattiroli et al., 2012). The role of H2AK13-15ubi is probably to recruit the downstream factor 53BP1 (Fradet-Turcotte et al., 2013). The ubiquitin moieties can be further prolonged by RNF8 to K63-linked chains, contributing to overall increase in ubiquitinated proteins in the DNA damage locus. However, these chains can be prolonged with K27 linkage by RNF168, which are important for recruitment of the downstream factors 53BP1, RAD50 and RNF168 itself (Gatti et al., 2015). Such a system of wave ubiquitination and recruitment of the factors ensures amplification of DNA repair signaling and accumulation of the excessive amount of DNA repair proteins. The

ubiquitination by RNF168 is counteracted by its paralog RNF169. Although RNF169 has a RING domain, its main function is binding the products of RNF168 activity through its UBD domains (Panier et al., 2012; Poulsen et al., 2012). Overexpression of RNF169 prevents recruitment of 53BP1 as well as RAD18, restricting accumulation of the repair factors at the damage sites.

BRCA1/BARD ubiquitin E3 ligase was shown to be the next ubiquitin E3 ligase in this cascade. Its recruitment to the damage site is facilitated by the interaction of BRCA1/BARD with a UBD-containing protein RAP80 (Kim et al., 2007). RAP80 recognizes K63 chains at the damage site, generated by RNF168 (Sims and Cohen, 2009; Sobhian et al., 2007). Interestingly, this protein also recognizes K6 chains on BRCA1, probably facilitating the docking of RAP80 to the damaged chromatin (Nishikawa et al., 2004; Wu-Baer et al., 2003). BRCA1/BARD substrates are not well studied. It has been proposed that it ubiquitinates histone H2A at the lysines 127 and 129, however, the role of this histone modification remains uncharacterized (Kalb et al., 2014).

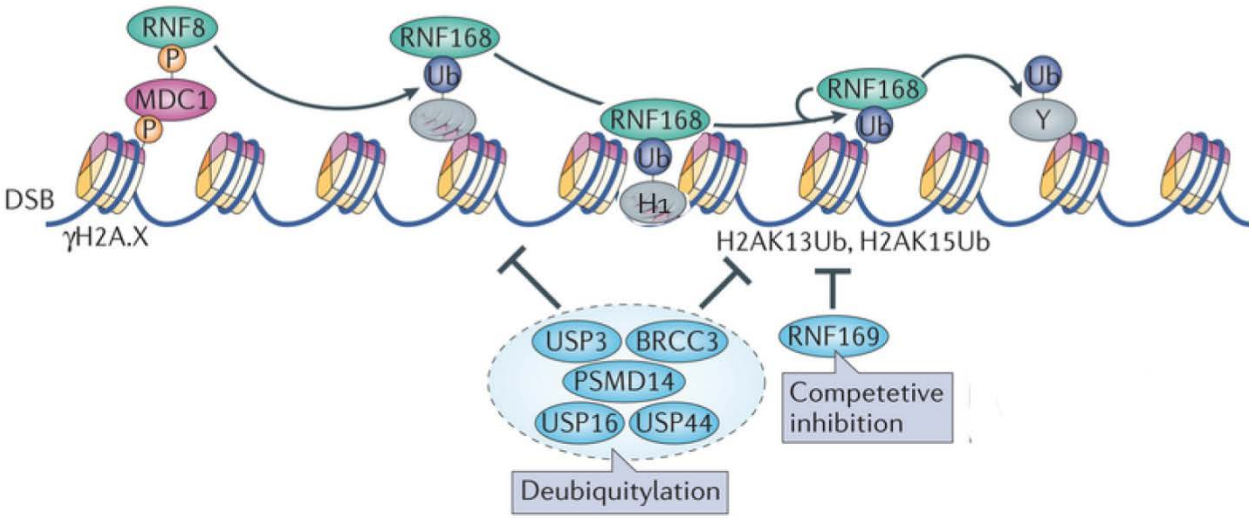


Figure 9

Ubiquitination events in the recognition of the DSB

Recruitment and phosphorylation of MDC1 promotes ubiquitination activity of activity of RNF8 towards a number of substrates, including H1 (Thorslund et al., 2015), which further mediates recruitment of ubiquitin E3 ligase RNF168 and ubiquitination of histone H2A and other substrates. Ubiquitination is counteracted by deubiquitination enzymes or competitive inhibition of E3 ligase activity and is important for the downstream steps of the DSB repair.

Adapted from Panier and Durocher, 2013.

SUMOylation

Small ubiquitin-like modifier (SUMO) is conjugated to a substrate protein via the same E1-E2-E3 ligase cascade as ubiquitin. *In vitro* SUMO is able to form polymer chains, however, *in vivo* it attaches to the substrate proteins in a monomeric form. SUMOylation of proteins is less abundant, compared to ubiquitination. There is only one known E2 ligase, participating in SUMOylation (UBC9), and only few characterized SUMO E3 ligases. However, in DNA repair pathways different proteins are shown to be SUMOylated (MDC1 (Galanty et al., 2012; Luo et al., 2012; Yin et al., 2012); BRCA1 (Morris et al., 2009). SUMOylated proteins are further processed by the downstream factors. For example, SUMO sequesters a family of SUMO targeted ubiquitin E3 ligases (STUbL) (Prudden et al., 2007; Uzunova et al., 2007). Apart from a RING domain which interacts with an E2 ligase, these proteins harbor a SUMO interaction motif mediating its interaction with SUMOylated substrate. RNF4 is a STUbL which is recruited to the sites of DSB during early damage recognition (Galanty et al., 2012; Yin et al., 2012). RNF4 can mediate different types of ubiquitin chains. However, its depletion coincides with the reduction of K63 chains at the damage site and depletion of RPA and Rad51 loading. Additionally, RNF4 targets SUMOylated MDC1 to promote its ubiquitination and subsequent degradation. This is important for the further clearance of the DNA damage site and the mediation of HR pathway (Luo et al., 2012).

Modification of the replication proteins PCNA by SUMO and ubiquitin is a prominent example of a SUMO/ubiquitin interplay which regulates resolving of the replication stress. During S-phase PCNA is constitutively modified by SUMO at K164 site to inhibit potential recombination process (Hoege et al., 2002). However, after DNA damage, this site is recognized by a STUbL RAD18, which together with E2 RAD6 deposits an ubiquitin moiety at the same site (Parker and Ulrich, 2012). This moiety is further recognized by a translesion DNA polymerase polη which can operate irrespectively of the presence of the DNA lesion, allowing error-prone DNA damage bypass (Kannouche et al., 2004). Alternatively, the same site can be polyubiquitinated by RAD5/UBC13 and lead to an error-free DNA repair by HR. Incites on the interplay of SUMO, ubiquitin and other posttranslational modifications of DNA repair proteins are important for understanding how DNA damage repair is orchestrated in the temporal and spatial manner.

Regulation of NER by posttranslational modifications

Phosphorylation in NER

As other multistep DNA repair pathways, NER is also regulated by different posttranslational modifications, however these are remain poorly understood. After loading of RPA to the ssDNA

stretches ATR is recruited to the damage site through their common binding partner ARTIP (Namiki and Zou, 2006). In NER-proficient cells this leads to phosphorylation of H2A.X (Vrouwe et al., 2011). Interestingly, UV-irradiation triggers recruitment of γH2A.X reader MDC1 and subsequent recruitment of ubiquitin E3 ligase RNF8 which ubiquitinates H2A at the site of DNA lesion (Marteijn et al., 2009). A major fraction of RPA, MDC1 as well as 53BP1 and BRCA1 are recruited as part of the post-incision process. This recruitment ensures a proper repair of the potential DSB in case NER fails. Interestingly, it remains unclear whether this signaling after UV-irradiation is dependent on ATR and ATM as inhibition of these kinases doesn't affect the recruitment of RNF8. Activation of ATR promotes phosphorylation of NER factors XPA and XPC and stimulates their activity (Wu et al., 2006). Dephosphorylase WIP1, expressed in an ATR-dependent manner, further quenches the activity of ATR and suppresses NER (Nguyen et al., 2010). This feedback loop is important for the regulation of NER reaction.

PARylation in NER

Similar to the response to DSB, PARP1 is recruited to the sites of UV-irradiation (Pines et al., 2012). PARP1 modifies DDB2 and promotes attraction of chromatin remodeling factor ALC1. Moreover, PAR chains at the UV-damage site may serve a recruitment platform for NER machinery. It was reported that treatment of cells with inhibitors of PAR reduces recruitment of XPC, as the XPC-RAD23B dimer is modified by PAR chains (Luijsterburg et al., 2012; Maltseva et al., 2015). PARylation of these proteins is proposed to contribute to their recruitment to chromatin. At the same time, covalent and non-covalent binding of PAR to the damage site weakens the interaction of proteins with DNA. This suggests a role of PAR in eviction of NER proteins from the DNA damage site (Fischer et al., 2014; Maltseva et al., 2015). Another role of PAR chains in NER is proposed after observation that its function is related to activity to TC-NER factor CSB. PAR chains potentially can serve as a source of ATP, which is essential for the chromatin remodeling activity of CSB (Flohr et al., 2003; Oei and Ziegler, 2000). These data can give alternative explanation why PARylation events lead to local relaxation of chromatin at the damage site.

Ubiquitination in NER

Recent studies have identified various ubiquitination events after UV-irradiation, tightly regulating the NER (reviewed in Figure 10). Cullin-RING ubiquitin E3 ligases are the key regulators of the DNA damage recognition in NER. This class of E3 ligases is responsible for a large part of all ubiquitin-related events in the cell (Soucy et al., 2009). The feature of Cullin-RING ligases is their modular structure. They consist of a ubiquitin E3 ligase module RBX1, a scaffold protein, which belongs to the Cullin family and a substrate-recognition module. The principal E3 ligases in NER

use CUL4 as a scaffold subunit. In mammals this protein has two paralogues, CUL4A and CUL4B. These proteins share 80% similarity but exhibit distinct functions when abrogated in mice. *Cul4b* knockout mice display embryonic lethality, whereas mice with non-functional *Cul4a* show no visible developmental defects (Jiang et al., 2012; Liu et al., 2009). Under unchallenged conditions Cullin-RING E3 ligases are interacting with a COP9 signalosome complex, which regulates their activity by counteracting association of a ubiquitin-like protein NEDD8 to the Cullin subunit (Furukawa et al., 2000) Upon activation stimulus Cullins are NEDDylated, which increases E3 ligase activity by promoting recruitment of an E2 (Kawakami et al., 2001).

Both CUL4B and CUL4A interact with DDB1 - a component of UV-DDB complex, which serves as critical factor for the assembly of the CUL4-RING ligases. DDB2 and CSA interact with DDB1 to form the complete E3 ligase and bind to the substrate proteins, mediating their ubiquitination (Fischer et al., 2011). The DDB2-DDB1-CUL4A-RBX1 E3 ligase (UV-CUL4A) plays a specific role in GG-NER. The main ubiquitination targets are DNA damage recognition proteins DDB2 and XPC as well as the core histones. Ubiquitination of DDB2 is observed as the first ubiquitination-related modification of NER proteins (Nag et al., 2001). DDB2 is modified by UV-CUL4 complex with K48 chains priming DDB2 for degradation. UV-mediated removal of ubiquitinated DDB2 is mediated by VCP/p97 protein (Puumalainen et al., 2014). VCP/p97 is an ATP-driven molecular chaperone which remodels proteins to facilitate their degradation by shuttling it to the proteasome or recycling. It is previously described to play a role in restoration of stalled replication forks in cooperation with an adaptor protein DVC1, linking it to DNA repair (Davis et al., 2012; Mosbech et al., 2012). In NER VCP/p97 recognizes ubiquitinated DDB2 using an adaptor protein NPL4. Absence of this protein or VCP/p97 itself stabilizes DDB2 on chromatin which coincides with failure in progression of NER (Puumalainen et al., 2014)

XPC is another target of the UV-CUL4A mediated ubiquitination. This E3 ligase was shown before to ubiquitinate XPC; however this ubiquitination is not promoting proteasomal degradation, but rather a stabilization of XPC on chromatin (Sugasawa et al., 2005). It was proposed that HHR23B may shield ubiquitinated XPC, preventing its proteasomal degradation. Nevertheless, dissociation of RAD23B coincides with its ubiquitination, suggesting that this protein may have other functions in the NER (Bergink et al., 2012). Apart from the ubiquitination by UV-DDB-CUL4, XPC is a target of another E3 ligase – RNF111/Arcadia (Poulsen et al., 2013). This protein is a typical STUbL: its activity is triggered by SUMOylation of XPC. RNF111 ubiquitinates XPC with K63 chains promoting the release of XPC from chromatin and further loading of NER factors ERCC1/XPF and XPG (van Cuijk et al., 2015). It remains unclear how ubiquitination of XPC regulates its behavior at chromatin. The type of ubiquitin chain on XPC produced by UV-CUL4A is

not yet characterized, and it remains unclear whether it causes proteasomal degradation of the protein. XPC is evicted from chromatin by VCP/p97 and the adaptor protein UFD1 (Puumalainen et al., 2014). Interestingly, VCP/p97 weakly interacts with the K63 chains, compared to the K48 chains. Therefore the association of activity of RNF111 and VCP/p97 is not yet understood. Possibly, XPC is not degraded by a proteasome at the site of DNA damage, but rather just displaced from the chromatin by VCP/p97, to promote further degradation. Alternatively the ubiquitin chains on XPC can be modified or removed by the deubiquitination enzymes. Indeed XPC is the primary sensor of UV-mediated DNA damage and exists in a pull to quickly perform its function in presence of the damage. Therefore modification of the ubiquitin chains may participate in the maintenance of the pull to ensure proper damage recognition.

In TC-NER CSB is the main substrate for CSA-DDB1-CUL4A-RBX1 (CSA-CUL4A) E3 complex (Groisman et al., 2006). Interestingly, interaction of this E3 ligase with CSN complex is different from the similar DDB2-containing E3 ligase. CSA-CUL4A becomes associated with the CSN complex after UV-irradiation, and the CUL4A subunit remains unNEDDylated (Groisman et al., 2003). Recent data suggests that CSB important to reinitiate transcription after completion of TC-NER. Unlike XPC and DDB2, it is ubiquitinated and degraded at the late stages of NER, corresponding with its role of transcriptional initiation (Proietti-De-Santis et al., 2006) CSB is protected from degradation by the activity of UVSSA protein in complex with the USP7 deubiquitinase (Nakazawa et al., 2012; Schwertman et al., 2013; Zhang et al., 2012). Depletion of both proteins is abolishing recovery of UV-mediated transcriptional inhibition. Recruitment of UVSSA/USP7 is mediated by CSA, probably repressing the activity of CSA-CUL4A E3 ligase. UVSSA is also shown to bind ubiquitinated and stalled forms of RNApolII, which may imply its role in the regulation of NER through the regulation of the ubiquitination status of RNAPolII (Zhang et al., 2012).

Ubiquitination and subsequent degradation of the largest subunit of RNAPolII – RBP1 is associated with UV-mediated DNA damage (Bregman et al., 1996). Earlier it was regarded to be the primary mechanism to remove stalled RNApolII from chromatin. However, degradation of RNApolII is probably the last option in NER, which occurs when a NER reaction fails. RPB1 is mono- or polyubiquitinated with K63 chains by the E3 ligase NEDD4. These chains can be trimmed and further elongated with K48 chains by another E3 ligase complex, consisting of Elongin A/B/C, Cullin5 and an E3 subunit RBX2 (Yasukawa et al., 2008). Ubiquitinated RBP1 is further extracted from chromatin by the VCP/p97 complex and transferred to the proteasome (den Besten et al., 2012). It is still unclear whether the ubiquitination status of RBP1 can affect the backtracking mechanism to remove RNApolII from the DNA lesion site.

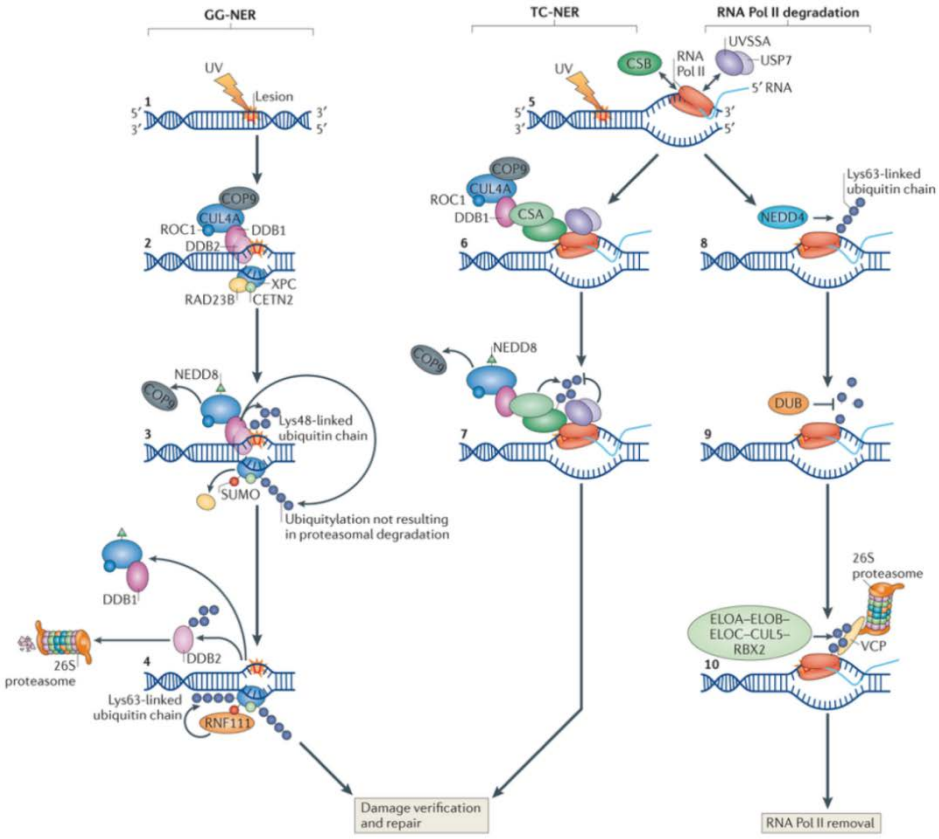


Figure 10

Ubiquitination events in the recognition of UV-mediated DNA damage

Left: in GG-NER DDB2 is a component of a CUL4-RING ubiquitin E3 ligase complex. It is activated after UV-irradiation by dissociation of the COP9 signalosome from CUL4 and further ubiquitinates itself and XPC. Ubiquitination of DDB2 promotes its proteasomal degradation, whereas XPC is supposedly stabilized at chromatin by ubiquitination. XPC is futher modified by a STUbL RNF111, which is important for further damage verification and repair.

Right: in TC-NER CSA is a component of a CUL4-RING ubiquitin E3 ligase complex, which binds CSB after UV-irradiation. COP9 signalosome is dissociated from the complex at the later stages of NER to ubiquitinate and degrade CSB. This is counteracted by the USP7/UVSSA dimer.

Stalled RNApolII can be ubiquitinated by the ubiquitin E3 ligase NEDD4 by K63 chains. This ubiquitin chains are further modified by deubiquitinating to create a substrate for ELOA/B/C-CUL5-RBX2 E3 ligase to polyubiquitinate RNApolII and promote its proteasomal degradation.

Currently our understanding of NER regulation is expanded by the recent progress in mass-spectrometry analysis of PTMs. It allows not only identification of the novel proteins, but also mapping the sites of the potential modifications. However, regulation of NER-related PTMs remains unclear, as there are more potential players in this process, including the proteins, previously implicated for other processes like replication, cell cycle regulation and transcription.

Chromatin reorganization in DNA repair

DNA in a eukaryotic nucleus exists in a complex with histone proteins. 147bp of DNA is wrapped around a nucleosome core, which consists of two H3-H4 and two H2A-H2B histone dimers. Nucleosomes are separated by 80-90bp linker DNA, associated with histone H1. DNA wrapped around nucleosomes in form of 10nm “beads on the string” fibers. They are further associated in more compact structures, forming interphase and even denser mitotic chromatin (reviewed in Felsenfeld and Groudine, 2003). Association of DNA with histones and formation of chromatin organizes DNA in the nucleus. Nevertheless, as a consequence, chromatin structures also restrict access of to the DNA (Lee and Workman, 2007). Therefore, various factors evolved to regulate chromatin structure in response to the DNA-related processes such as transcription, DNA replication and DNA repair. In DNA repair chromatin reorganization involves regulation of access of DNA repair factors to damaged DNA, transcriptional repression of genes in the loci of DNA damage and restoration of chromatin structures after DNA repair is complete.

Incorporation of histone variants

Although the core histones H2A, H2B, H3 and H4 are extremely conservative between species, histone variants can be incorporated in nucleosomes to regulate chromatin-related processes. Incorporation of histone variants is actively mediated by the specific histone chaperones in mostly replication-independent manner (De Koning et al., 2007). Histone H2A.X was the first histone variant, identified to be important for DNA repair. H2A.X is rapidly phosphorylated in response to DNA damage (γH2A.X) (Rogakou et al., 1998). This histone variant is associated with 10% of nucleosomes and similarly to H2A incorporated to nucleosomes by the histone chaperone FACT (Heo et al., 2008)). Phosphorylation of H2A.X is not only critical for recruitment of the factors of DSB repair (see Chapter 2), but also for amplification of the checkpoint signal and the demarcation of the chromatin region, containing the DNA damage site (reviewed in Yuan et al., 2010). These functions are related to the ability of γH2A.X to spread bidirectionaly from the DNA breaks (Iacovoni et al., 2010). Additionally γH2A.X is prone to dissociate from the nucleosomes, therefore it contributes in the relaxation of the chromatin after DNA damage. Other variants of H2A such as H2A.Z and MacroH2A are also involved in the DNA repair (Kalocsay et al., 2009; Papamichos-Chronakis et al., 2011; Timinszky et al., 2009). Dynamics of H3 variants in the DDR is less studied; however, their role is possibly related mainly to restoration of chromatin after DNA repair. Histone H3.1 is deposited to the sites of UV-induced DNA damage and single strand breaks by CAF1 factor, consistent with the previous observations in replication process (Polo et al., 2006; Schopf et al., 2012). Moreover, newly synthetized histone variants H3.3 are incorporated to

chromatin at the DNA damage sites by the histone chaperone HIRA1 to promote restoration of transcription after DNA repair (Adam et al., 2013).

Posttranslational modifications of histones

Posttranslational modifications of the core histones and their variants play an important role in dynamics of the chromatin structures. Deposition of histone modifications on damaged chromatin is performed either by specific DNA damage factors ATM/ATR/DNA-PK (γH2A.X) and RNF8/RNF168 (ubiquitinated H1 and H2AK13-15-ubi) or related to the factors playing role on global chromatin processes.

Acetylation

Acetylation of histones was described to be the first histone modification and typically is associated with chromatin relaxation (Kouzarides, 2007). After induction of DNA damage core histones are rapidly acetylated, promoting enhancement of the DNA repair (Ramanathan and Smerdon, 1989). Histone acetyltransferases such as MOF, GCN5 and TIP60 promote acetylation of core histones at the DNA damage site (Ikura et al., 2000; Ikura et al., 2007; Lee et al., 2010; Li et al., 2010). Acetylation of lysine 16 of histone H4 (H4K16ac) plays an important role in chromatin structure as it inhibits chromatin folding, locally relaxing chromatin at the damage site (Shogren-Knaak et al., 2006). H4K16ac deposited by TIP60 plays a role in regulation the pathway choice in DSB repair, where it inhibits recruitment of 53BP1, promoting HR (Tang et al., 2013). Recruitment and activation of TIP60 is dependent on the presence of another histone modification – H3K9me3, deposited by the histone methyltransferase SUV39H1 (Ayrapetov et al., 2014; Sun et al., 2009). Interestingly, H3K9me is generally associated with the formation of heterochromatin (Grewal and Jia, 2007), suggesting that the chromatin at the site of DNA damage is subjected to local dynamical structural changes.

Methylation

Methylation of H3 at the lysine 27 (H3K27me3) is associated with close chromatin structures. It is deposited by the enzymatic activity of the histone methyltransferase EZH2 (Kuzmichev et al., 2002). EZH2, together with other subunits EED and SUZ12 are components of the Polycomb repressive complex 2 (PRC2). PRC2 is important for the transcriptional repression in differentiation, regulation of cell cycle and senescence (reviewed in (Morey and Helin, 2010)). Although, EZH2 is deposited at the sites of laser and enzymatically mediated DSB, deposition of H3K27me3 at the sites of DNA damage remains controversial (Campbell et al., 2013). However, it is possible that

PRC2 can act at the site of DNA damage transiently, mediating recruitment of other proteins, related to closed chromatin structure.

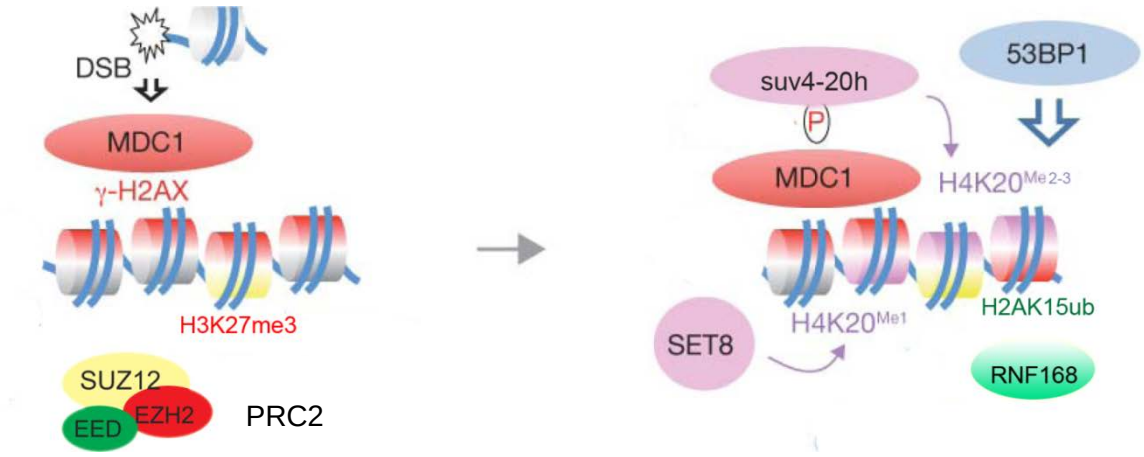


Figure 11

Methylation of histones in the recognition of DSB

PRC2 complex is recruited to the vicinity of the DSB to possibly mediate H3K27me3. SET8 mediates H4K20me1 to promote further formation of H4K20me2 by sub20h. H4K20me2 together with H2AK15ub, deposited by RNF186, are essential for recruitment of 53BP1.

Adapted from Lukas et al., 2011.

Interestingly, the chromatin environment, for example preexistence of histone variants such as H2A.X or histone modifications, can influence DNA repair. Methylation of lysine 20 of H4 (H4K20me) is one of the most abundant histone modifications and plays role in genome stability as well as chromatin organization. H4K20me3 is regarded as the marker for centromeres, telomeres and imprinted regions. However, H4K20me1, deposited by the histone methyltransferase SET8, and H4K20me2, deposited by SUV4-20H1, are distributed broadly across genome (Schotta et al., 2004). Ablation of SET8 and SUV4-20H1 promotes genome instability (Oda et al., 2009; Schotta et al., 2004). While H4K20me2 is very abundant, after induction of DNA damage it serves as a docking platform for 53BP1 which interacts both with H4K20me2 and H2AK15-ub (Fradet-Turcotte et al., 2013). Importantly, the ability of DNA repair machinery to interplay with the chromatin organization factors ensures fast and reliable DNA repair process (Figure 11).

Ubiquitination

Monoubiquitination of H2A is also regarded one of the most abundant histone modifications. As was mentioned previously, H2A is modified after induction of DSB by the ubiquitin E3 ligase

RNF168 at lysines 13 and 15 (Mattiroli et al., 2012). However, these sites are not regarded canonical, and in 10% of nucleosomes H2A is modified at lysine 119 (H2AK119ubi, further H2A-ubiquitin). This histone modification is related to the transcriptional repression of the genomic loci as it inhibits transcription at the elongation steps (Stock et al., 2007). H2A-ubiquitin prevents formation of histone modifications, associated with the active genomic loci, and hence is related to the transcriptional repression of the genomic loci inhibiting transcription at the elongation steps (Nakagawa et al., 2008; Stock et al., 2007). FACT complex is responsible for removing of H2A/H2B dimers from nucleosomes, allowing RNApolII move along transcribed DNA strand (Reinberg and Sims, 2006). H2A-ubiquitin abolishes recruitment of the FACT complex, therefore inhibits transcription. (Zhou et al., 2008).

PRC1 complex

H2A-ubiquitin is mainly deposited by the ubiquitin E3 ligase RING1B with a help of a RING-containing protein BMI-1, which are components of Polycomb repressive complex 1 (PRC1) (Wang et al., 2004). Other subunits of PRC1 include PH and CBX paralogues (Cao et al., 2005; Wang et al., 2004) (Figure 12). Apart from its association with PRC1, RING1B is a component of other PRC1-like complexes, including RYBR and E2F6/L3MBTL (reviewed in Gil and O'Loghlen, 2014). PRC1-like complexes seems to be highly dynamic and differ at various genetic loci not only in subunit composition, but also in their biochemical functions. Currently, little is known regarding the regulation of the PRC1-like complexes as well as the level of their redundancy. Recruitment of the PRC1 was long thought to be dependent on the activity of PRC2 and presence of H3K27me3; however, recent data opposes this idea.

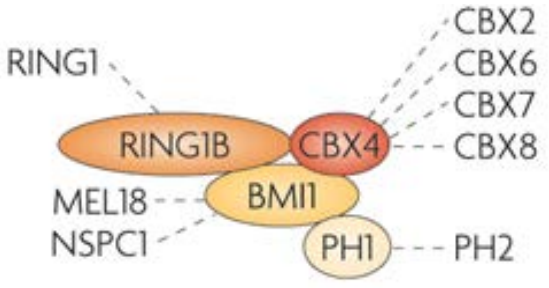


Figure 12

Mammalian PRC1

Core subunits of mammalian PRC1 complex: RING1B, BMI-1, CBX4 and PH1. Dotted lines show possible substitution of the core subunits with alternative proteins.

Adapted from Simon & Kingston, 2009

Various subunits of PRC1 complex were shown to be recruited to the DNA damage sites induced by ionizing radiation, laser irradiation or treatment with HU (Ginjala et al., 2011; Ismail et al., 2010). Their recruitment is dependent on the activity of ATM, suggesting that it marks early stages of DNA repair. Additionally PRC1 components are recruited independently of the PRC2. Interestingly, it remains unclear, what is the molecular composition of the PRC1 complexes associated with DSB. There are controversial reports, regarding the recruitment of the PRC1

subunits to the DNA damage sites. RING1B was shown to be recruited both in absence and presence of BMI-1. Additionally absence of RING1B is not abolishing BMI-1 recruitment. BMI-1 seems to be acting in the pathway parallel to RNF8/RNF168 possibly to promote HR (Ginjala et al., 2011). However, little is known about the molecular functions of PRC1 components at the DNA damage site. Recent reports were focusing on the potential transcriptional repression role of PRC1 at the DSB (Kakarougkas et al., 2014; Shanbhag et al., 2010; Ui et al., 2015). First reports have shown that that H2A-ubiquitin is responsible for local chromatin condensation at the damage site (Shanbhag et al., 2010). Later, BMI-1 was revealed to have a major role in this process as it interacts with transcriptional elongation complex ENL1 in presence of DNA damage. This interaction allows local transcriptional repression and, as proposed by Ui and colleagues, promotes accessibility of the damaged site to the repair factors (Ui et al., 2015). However, it remains unclear how local transcriptional repression and formation of the compact structures, normally promoted by PRC1, can influence DNA repair. Most probably, there are some additional proteins which interact with PRC1 components, facilitating progression of DNA damage repair.

Deubiquitination of H2A

Histone modifications can be enzymatically reversed. This is particularly important as a part of the regulation of the rapid chromatin response in DNA repair. As discussed previously, deposition of H2A-ubiquitin at the DSB loci stimulates local transcriptional repression. Post-repair removal of this mark is crucial for the restoration of a local chromatin structure. The ubiquitin moiety from H2A can be removed by the activity of the deubiquitinase enzymes USP3, USP16 and USP21 (Nakagawa et al., 2008; Nicassio et al., 2007). Although depletion of these proteins results in increase in the basal levels of H2A-ubiquitin, little is known about the mechanisms of their action, particularly their recruitment to the ubiquitinated substrate. Recently, it has been proposed that the ubiquitin on the H2A molecule serves as a binding platform for a protein called ZRF1. Its recruitment to H2A-ubiquitin displaces PRC1 from chromatin during cellular differentiation of NT2 progenitor cells. Residual H2A is further proposed to be removed with the help of USP21, however possibly USP16 also has a role in this process (Richly et al., 2010) (Figure 13). It remains unclear whether recruitment of ZRF1 has functions other than facilitation of H2A-deubiquitination. Apart from its function in the nucleus, ZRF1 acts as a chaperone, providing folding of a newly synthetized polypeptides (Hundley et al., 2005). This function could be related to the possible role of ZRF1 in processing of the proteins in vicinity of its binding locus.

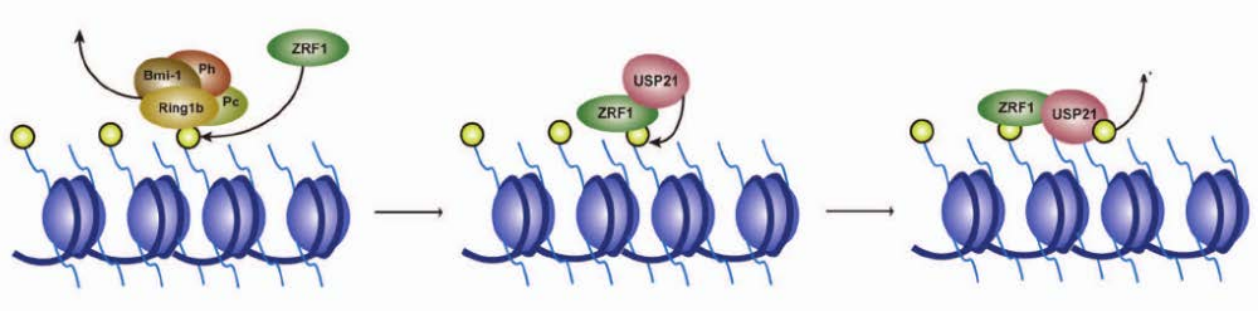


Figure 13

ZRF1 facilitates deubiquitination of H2A

ZRF1 binds monoubiquitinated H2A and displaced PRC1 from chromatin. Binding of ZRF1 promotes deubiquitination of H2A and transcriptional activation of the genes (adapted from Richly and Di Croce, 2011).

USP16 was recently shown to reverse local transcriptional stalling at the DSB loci and prevent spreading of newly synthetized H2A-ubiquitin along the chromatin (Shanbhag et al., 2010). This function can potentially be important for restoration of proper chromatin functions after DNA repair is complete. Indeed, the peak of USP16 recruitment to chromatin is shifted to the later stages of DNA repair (Zhang et al., 2014). H2A deubiquitinases USP3 and USP44 were also implicated in regulation of DSB repair by removing ubiquitin deposited on H2A by RNF168 and RNF8 (Sharma et al., 2014). However, their function in regulation of PRC1-mediated transcriptional repression is not yet described.

ATP-dependent chromatin remodeling

Deposition and removal of histone modifications require a combination of the relevant enzymes, substrates and axillary factors. ATP-dependent chromatin remodeling acts as an active and rapid mechanism to restructure chromatin after DNA damage. Indeed, many histone modifications are involved in further deposition of various chromatin remodelers after induction of the DNA damage. Chromatin remodelers are multisubunit complexes, which contain a large ATPase motor and can be divided four families: SWI/SNF, CHD, INO80 and ISWI (reviewed in Clapier and Cairns, 2009). Their activity is related to removing nucleosomes from chromatin as well as shifting them along DNA strands. Additionally, they facilitate an exchange of histone variants, further impacting chromatin reorganization in DNA damage repair. Chromatin remodelers are implicated in different types of DNA repair, including BER, NER and repair of DSB (Czaja et al., 2012; Price and D'Andrea, 2013). The hNuA4 is a histone acetyltransferase complex, containing HAT TIP60 as well as the member of INO80 family ATPase p400. Subunits of the hNuA4 are recruited to the site of DSB in dependence of MDC1 (Xu et al., 2010). Presence of p400 facilitates

exchange of H2A to H2AZ. This is further important for the acetylation of the histones in the DSB and formation of the open chromatin structures (Xu et al., 2012). NuRD complex counteracts the activity of TIP60. It is also rapidly recruited to the DSB, where it flanks the sites of the DSB, promoting downstream recruitment of RNF8 and RNF168 (Smeenk et al., 2010). Members of the SWI/SNF family are also recruited to the DNA damage sites in dependence of both NuRD complex and histone acetyltransferase Gsn5 (Bennett and Peterson, 2015). Collectively, this data shows, that chromatin dynamics in the process of DNA damage recognition is dependent on the interplay of histone modifications, chromatin remodelers as well as incorporation of histone variants.

Chromatin rearrangements are currently investigated largely in the context of the DSB repair. As mutations in the DSB repair pathways have high clinical relevancy and associated mainly with malignancy, various methods were developed to investigate its regulation through chromatin. Apart from traditional approaches, inducing DSB by genotoxic drugs or irradiation, it is possible to artificially create a DSB site in the genome. In these loci it is performed by the activity of the endonuclease ISceI (Bellaiche et al., 1999; Rouet et al., 1994). This method can combine traditional approaches such as microscopy with sequence related methods, such as chromatin immunoprecipitation or RNA-sequencing. This approach allowed identification of histone modifications related directly to the DSB site as well as identification of RNA species associated with the DNA damage sites (Beishline et al., 2012; Francia et al., 2012). However, investigation of other DNA repair pathways, such as ICL repair, BER or NER, is restricted by the absence of the sequence specificity. Nevertheless, other methods allow having an insight in the chromatin-based regulation of these pathways.

Chromatin reorganization in NER

Efficiency of NER is affected by the presence of nucleosomes, based on the *in vitro* studies, using a DNA template with a photoproduct coupled to recombinant nucleosomes (Hara, 2000). Therefore, rearrangements of chromatin, mediated by histone modifications and chromatin remodelers, are extremely important for fulfilling NER. Phosphorylation of H2A.X, which is the hallmark of DSB-induced DNA damage signaling, is also triggered by irradiation of cells with UV (Matsumoto et al., 2007). However, in contrast to DSB repair, its function is mostly related to the later stages of NER, when stretches of ssDNA are formed. Consistently with formation of γH2A.X, H2A is ubiquitinated in response to UV-irradiation (Bergink et al., 2006; Marteijn et al., 2009). These ubiquitination events are related to the activity of the PRC1 component RING1B, as well as RNF8, previously implicated in DSB repair. The activity of these E3 ligases is dependent on ATR and mainly contributes to the later stages of NER. Interestingly, DSB repair proteins MDC1 and 53BP1 are recruited to the sites, marked with ubiquitinated H2A (Marteijn et al., 2009). This

preassembly of DSB recognition machinery allows rapid repair of a potential DSB. However, the role of NER components in this process remains unclear as well as the possible active contribution of RING1B and RNF8 in the recognition of photoproducts.

DNA damage recognition process in NER is dependent on the transcriptional status of the damaged DNA strand. DDB2, the primary recognition factor of UV-mediated photoproducts in GG-NER, is a component of a ubiquitin E3 ligase complex UV-CUL4. This protein complex ubiquitinates GG-NER proteins DDB2 and XPC as well as the core histones H2A, H3 and H4 (Guerrero-Santoro et al., 2008; Kapetanaki et al., 2006; Wang et al., 2006). These ubiquitination events are mainly related to the early steps of NER, consistent with the early recruitment of DDB2. Nevertheless, little is known regarding the functional role of the histone ubiquitination in NER and the contribution of different variants of UV-CUL4 complex. As mentioned previously, a scaffold protein CUL4 has two paralogues – CUL4A and CUL4B. Complexes including both paralogues were implicated in the ubiquitination of core histones (Guerrero-Santoro et al., 2008; Kapetanaki et al., 2006). It remains unclear whether both UV-CUL4A and UV-CUL4B complexes ubiquitinate H3 and H4. However, it was proposed that UV-CUL4B complex is more efficient towards ubiquitination of H2A (Guerrero-Santoro et al., 2008). Given the similarity of the CUL4A and CUL4B proteins the complexes might be redundant, and the preferential activity of a complex containing CUL4A or CUL4B might depend on some unknown factors. Ubiquitination of histones H3 and H4 is suggested to facilitate histone release from the damaged chromatin (Wang et al., 2006). Similar idea was put forward regarding the function of H2A ubiquitinated by UV-CUL4B E3 ligase (Lan et al., 2012). This publication also suggests that H2A is ubiquitinated by UV-CUL4B at lysine 119 – same site as the substrate for the activity of RING1B. In contrast to the general compactization of chromatin in response of RING1B-mediated H2A ubiquitination, the activity of UV-CUL4B is proposed to facilitate the eviction of histone H3 from nucleosomes. However, the *in vitro* data, presented in this study so far is not supported with *in vivo* observations. Another important question, whether PRC1 subunits are related to the activity of RING1B in NER. For example BMI-1 is not described to be recruited to chromatin at the NER-repaired sites. It was proposed that the ubiquitination of H2A is facilitating restoration of chromatin structure after completion of NER, however, it is related to the late ubiquitination events, and was not linked to the activity of PRC1 subunits (Adam et al., 2013).

Chromatin reorganization after UV-irradiation has features similar to the response to the DSB. Histone acetylation occurs rapidly after UV-irradiation and performed by GCN5, which is a component of histone acetyltransferase complexes STAGA and TFTC (Brand et al., 2001; Martinez et al., 2001). These complexes are recruited in the early stages of NER, in dependence of DDB2 or in parallel with XPA recruitment, respectively. GCN5 is recruited to the damage site with help of the

transcription factor E2F1, facilitating further recruitment of XPC and XPA (Guo et al., 2010). Interestingly, TIP60 described being important for the DSB repair, was shown to be dispensable for NER regulation.

One of the important features of NER is regulation of the transcriptional response to UV-irradiation and further restoration of transcription after completion of NER. CSB – a component of TC-NER - possess a chromatin-remodeling ability (Citterio et al., 2000). CSB repositions nucleosomes in an ATP–dependent manner *in vitro* and this activity is enhanced by the NAP1-like histone chaperones (Cho et al., 2013). Recently SMARCA5 was shown to promote early stages of TC-NER, facilitating deposition of CSB at the UV-damaged chromatin (Aydin et al., 2014). Restoration of transcription is dependent on FACT complex, depositing novel H2A histones, as well as deposition of Histone H3.3 done by the histone chaperone HIRA (Adam et al., 2013; Dinant et al., 2013). Initiation of GG-NER also includes activity of chromatin remodeler BRG1, associating with XPC and DDB2 (Zhang et al., 2009). Similarly, INO80 is implicated in the early stages of NER (Jiang et al., 2010).

Aims of the study

The main aim of this thesis is to characterize the role of RING1B and ZRF1 in NER. Specific aims were to discover the

- mechanistic role of RING1B in the NER pathway, including the possible interactors of RING1B in this process;
- possible recruitment of ZRF1 to chromatin after induction of UV-mediated DNA damage;
- possible functions of ZRF1 in the ubiquitin-mediated regulation of NER.

Materials and methods

Cell culture

HEK293T (human embryonic kidney cells), U2OS (human osteosarcoma cells) and HeLa (human cervical adenocarcinoma cells) cell lines were cultured in DMEM (Gibco) supplemented with 10% FBS (PAA), 2mM L-glutamine (Gibco) and 100U/mL Penicillin/Streptomycin (Gibco) at 37°C and 5% CO_2. HeLa Kyoto cells stably expressing cherry-PCNA were cultured in DMEM supplemented with 10% FCS and 1 µM/ml Gentamycin, 2,5 µg/ml Blasticidin. MRC5 (human embryonic lung fibroblasts) (AG05965), human skin fibroblasts (GM15876), XPE (GM01389), XPE (GM02415), XPC-complemented (GM16248), XPC (GM15983), XPA-complemented (GM15876), XPA (GM04312 and GM00710) fibroblasts were purchased from Coriell Cell Repositories and cultured in DMEM, supplemented with 15% FBS.

For subculturing cells were washed once or twice with PBS (137 mM NaCl, 2,7 mM KCl, 4,3 mM Na_2HPO_4, 1,47 mM KH_2PO_4, IMB Mainz) followed by addition of Trypsin solution (0,005% trypsin with EDTA in PBS) (Gibco). After dissociation from the cell culture plates, cells were collected in complete DMEM, spun down (4minutes, 1200rpm), resuspended in fresh medium and plated at desired density.

Cell line production

U2OS-shRING1B

U2OS cells were transfected with pSUPER-shRING1B (Richly et al., 2010) using Lipofectamine 2000 (Life technologies) according to the manufacture's protocol and selected with 2 ug/ul Puromycine for 3 days. Single cells were further sorted in 96-well plates and protein expression of RING1B was futher tested by Western Blot.

shRNA and siRNA mediated gene knockdown

shRNA transduction

To mediate gene knockdown in MRC5 fibroblasts, cells were transduced with viral particles containing the respective shRNA, packaged using 3rd generation lentivirus system (Dull et al., 1998).

Viral particles were produced in HEK293T cells. Briefly 5mln cells were transfected with 4ug of pLKO.1 (Sigma Aldrich) (Table 2), 2ug pMDLg/pRRE (Addgene), 2ug pRSV-Rev (Addgene) and 2ug pMD2.G (Addgene) vector using polyethylenimine (PEI) (Sigma Aldrich). Medium was collected 48h and 72h post transfection and filtered through 0.45mm PDVF filter. After collection the medium was used for viral transduction of MRC5 cells in complete growth medium in presence

of 4ug/mL of Polybrene (Sigma). For transduction 3mL medium with viral particles were added to MRC5 fibroblasts plated 24h before first viral transduction. Transduction was facilitated by a spinofection protocol (1000rpm, 60min). After two rounds of transduction, medium was replaced to complete growth medium and used for further experiments.

Table 2

shRNA sequences used in this study

Gene	TRC code	Sequence
NMC	TRC1/1.5	CCGGCAACAAGATGAAGAGCACCAACTCG AGTTGGTGCTCTTCATCTTGTTGTTTTT
DDB2	TRCN0000083995	CCGGGCTGAAGTTTAACCCTCTCAACTCG AGTTGAGAGGGTTAAACTTCAGCTTTTTG
ZRF1	TRCN0000254058	CCGGCTGGAAGAACCAAGATCATTACTCG AGTAATGATCTTGGTTCTTCCAGTTTTTG
RING1B	TRCN0000033697	CCGGGCCAGGATCAACAAGCACAATCTCG AGATTGTGCTTGTTGATCCTGGCTTTTTG
XPC	TRCN0000307193	CCGGGCAACAGCAAAGGGAAAGAAACTCG AGTTTCTTTCCCTTTGCTGTTGCTTTTTG

siRNA transfectrion

siRNA transfection was performed using Lipofectamine 2000 according to the manufacture's protocol. The following siRNAs were used (Table 3)

Table 3

siRNA sequences used in this study

Gene	siRNA source	Oligo name, Sequence
Negative control	Sigma Aldrich	SIC001. Sequence not disclosed by the company
DDB2	Sigma Aldrich	SASI_Hs01_00101645. Sequence not disclosed by the company SASI_Hs01_00101647. Sequence not disclosed by the company
DDB2	Dharmacon	D-011-22-01. CAACUAGGCUGCAAGACUU D-011-22-02. GAUAUCAUGCUCUGGAAUU D-011-22-03. GACCUCCGAGAUUGUAUUA D-011-22-04. AGAGCGAGAUCCGAGUUUA
RNF168	Dharmacon	D-0071520-18. GAGUAUCACUUACGCGCUA

		D-0071520-03. AGAAGGAGGUGGAUAAAGA D-0071520-02. GAAAUUCUCUCGUCAACGU D-0071520-01. GGAAGUGGCUGAUGACUAU
CUL4A	Sigma Aldrich	EHU020361. Sequence not disclosed by the company
BMI1	Sigma Aldrich	EHU004421. Sequence not disclosed by the company
CUL4B	Sigma Aldrich	EHU064911. Sequence not disclosed by the company
XPC	Sigma Aldrich	SASI_Hs01_00086530. Sequence not disclosed by the company SASI_Hs01_00086531. Sequence not disclosed by the company

Transfections

Plasmid transfection was performed using Calcium Phosphate precipitation protocol (^{FLAG}RING1B; ^{FLAG}ZRF1; ^{FLAG}DDB2; ^{FLAG}H2A.X) or PEI transfection (UV-RING1B complex transfection; BMI-1-HA). The construct name and the source of the vectors are shown in the table 4.

Table 4

Plasmid vectors used in this study

Contruct name	Source
RING1B-YFP	Gift from the di Croce lab (CRG Barcelona, Spain)
pCMV2-FLAG-RING1B	Richly et al, 2010
pCMV-TAG2b	Sigma Aldrich
pT3-EF1a-BMI1	Addgene (31783)
pDNA3-FLAG-DDB2	Gift from the Tanaka lab (Osaka University, Japan)
pGEX-UbcH5	Gift from the Rape lab (UC Berkeley, USA)

pET-UBA1	Gift from the Rape lab (UC Berkeley, USA)
pGEX-RING1B	Richly lab
pEGFP-C1-DDB2	Gift from the Dantuma lab (Karolinska university, Sweden)
pcDNA3-FLAG-DDB1	Addgene (19918)
pcDNA-FLAG-STREP-CUL4B	Gift from the Beli lab (IMB Mainz, Germany)
pcDNA3-HA-RBX1	Addgene (19897)
pcDNA3-HA-CUL4A	Addgene (19907)
mCherry-ZRF1	Richly lab
pCMV2-FLAG-H2A.X	Richly lab
pCMV2-FLAG-ZRF1	Richly et al, 2010
pCMV2-FLAG-ZRF1-ΔUBD	Richly et al, 2010
HIS-HA-GFP-XPC	Gift from the Cardoso lab (TU Darmstadt, Germany)
pCS2-HIS-Ubiquitin-WT	Gift from the Rape lab (UC Berkeley, USA)

Genotoxic treatment and enzymatic inhibition

UV-irradiation was performed using Stratalinker® UV Crosslinker. Chemical treatment was performed as indicated in table 5. Inhibitors of RING1B activity (Ismail et al., 2013) and protein synthesis were added to the cells as indicated in the table 6.

Table 5

Genotoxic treatment performed in this study

Chemical	Stock concentration/ solvent	Working dilution	Incubation time	Type of DNA damage
Etoposide	10mM in DMSO	20ug/mL	1h	Stalled replication

				forks, ssDNA breaks
Bleomycine	100ug/mL in DMSO	300ng/uL	3h	Double strand breaks
Camptothecin	10mM in DMSO	10uM	1h	Stalled replication forks, ssDNA breaks

Table 6

Chemical inhibition used in this study

Chemical	Stock concentration/ solvent	Working dilution	Incubation time
PRT4165	100 mM in DMSO	50µM	4h
Cycloheximide	50mg/ml in DMSO	50ug/mL	0-8h

Biochemical methods

SDS-PAGE and Western Blot

Protein samples were analysed by SDS-PAGE and subsequent Western Blot using Biorad Mini Trans-Blot® Cell system.

SDS-polyacrylamide gels were prepared as follows: Separating gel (4x separating buffer, 8-12% Acrylamide, 0.1% APS, water); Stacking gel (4x stacking buffer, 6% Acrylamide, 0.1% APS, water). Gels were run in Running buffer (25mM Tris-base, 200mM glycine, 0.1% w/v SDS) at 25mA per gel and transferred on nitrocellulose membrane (Biorad) at 300mA in ice-cold Transfer buffer (25mM Tris-HCl pH 8.3, 200mM glycine, 20% v/v methanol). To detect ubiquitinated proteins and free ubiquitin, gels were transferred on PDVF membrane activated for 5min with methanol. To determine transfer efficiency and protein loading membranes were incubated in 1% Ponceau S solution in water and imaged by Biorad Chemidoc system. Membranes were blocked with 5% skim milk or 5% BSA in PBS-T (0,1% Triton X-100 in PBS) for 1h and incubated overnight with the corresponding primary antibody at 4C. After incubation membranes were washed 4 times for 5 minutes in PBS-T and incubated with HPR-conjugated secondary antibody in PBS-T for 1h at room temperature, followed by 4 washes for 5 min in PBS-T. Protein detection was performed with Pierce ECL Western Blotting Substrate (Thermo Scientific) using Biorad Chemidoc.

The list of antibodies used in this study is presented in Appendix 1

Coomassie blue staining

Polyacrilamide gels containing the samples were incubated with Coomassie Blue staining solution (0.1% Coomassie Blue R250 in 10% acetic acid v/v, 50% methanol v/v in water) on a shaker at room temperature. After incubation, gels were distained by incubation in destaining solution (10% acetic acid v/v, 50% methanol v/v in water) until the protein bands are visible. Protein bands were visualized by Biorad Chemidoc.

Cell extracts

Cells were harvested by trypsinization or scraping. Cell pellet was washed with PBS and resuspended in 2x Lämmli buffer (2% SDS, 120mM Tris-HCl (pH 7.2), 20% glycerol, 10% β-mercaptoethanol, 0,02% Bromphenol Blue).

Chromatin association assay

Cell pellets were cross-linked with a solution of 1% formaldehyde (Sigma) in PBS for 10 minutes. The cross-linking reaction was quenched by addition of 0.125M glycine and incubated at room temperature for 10 minutes on a wheel. Cell pellets were resuspended in Buffer A (100mM Tris pH 7.5; 5mM $MgCl_2$; 60mM KCl; 125mM NaCl; 300mM sucrose; 1% NP-40; 0.5mM DTT), kept on ice for 10 minutes and centrifuged (3000 rpm, 3 min, 4°C).Supernatant was removed and the nuclei pellet was lysed in a hypotonic solution (3mM EDTA; 0.2mM EGTA; 1 mM DTT) and incubated on ice for 30min. Nuclei were centrifuged for 5 min, 14800 rpm, 4°C, and the nuclear pellet was lysed again for 15min.The chromatin containing pellet was solubilized in 2X Lämmli buffer and sonicated in Diageonde Bioruptor for 10-12 min at High setting (30s ON/30s OFF). Samples were boiled in one for 30min to reverse the cross-linking before loading on polyacrylamide gels.

Immunoprecipitations and affinity purifications

Cells were treated with UV and harvested 1 hour after exposure unless stated overwise. Cells were resuspended in buffer A (10 mM HEPES pH 7.9; 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT, 1mM PMSF, Protease inhibitors (Roche)) and homogenized by 10 strokes in a Dounce homogenizer with a B-type pestle. After centrifugation, nuclei were resuspended in lysis buffer (20mM Hepes, 150mM NaCl, 2.5mM EGTA, 2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 1 mM PMSF, Protease inhibitors (Roche)) and sonified using a Diagenode Bioruptor for 20min (30s ON/30s OFF) at High setting. To verify sonification efficiency DNA from the extracts analyzed by agarose gel electrophoresis. Only samples containing DNA of 300bp or smaller were used in the experiments. Protein extracts were then subjected to centrifugation (21.000g, 4 °C, 15min). Protein concentration was measured by Pierce Coomassie (Thermo Scientific) and samples were diluted

with Lysis buffer to obtain the same protein concentration. Supernatant was incubated with antibodies overnight at 4°C on rotating wheel. After incubation with ProteinA agarose beads for 2 hours at 4°C, the immune complexes were washed extensively in lysis buffer and material retained on the beads was analyzed with Western blot. Affinity purifications using FLAG-M2 agarose beads (Sigma Aldrich) and Anti-HA−Agarose beads (Sigma Aldrich) were performed using the protocol stated for immunoprecipitations. Purifications involving the STREP tag were performed with STREP-Tactin® beads (Iba lifesciences) and Desthiobiotin (Sigma Aldrich) according to the manufacturer's instructions.

In vitro ubiquitylation assays

In vitro ubiquitylation reactions were performed with 3µg purified histone H2A (New England Biolabs) or 5µg recombinant nucleosomes (Active Motif), 200 ng purified HIS-UBA1 (E1), 20 ng purified GST-UBC5H (E2), 150 ng purified UV-RING1B (E3) or 150 ng GST (Control) in UBAB buffer (25 mM Tris/HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) supplemented with 20 mM ATP, 1.5 mg/ml Ubiquitin, 10mM DTT, and 1 U creatine phosphokinase. Reactions were kept at 37°C for the indicated times and subsequently subjected to Western blotting.

Purification of recombinant proteins

BL21 *E.coli* transformed with HIS-UBA1 or GST-UBCH5 vectors were grown in LB growth medium. Protein expression was induced by adding 0.1mM IPTG at OD600= 0.5 to the growth medium for 4h. Bacterial pellets were lysed after incubation in lysis buffer (PBS, 0,1% Triton X-100, Protease inhibitors (Roche), lysozyme (Sigma Aldrich) using Bramsen sonicator. Lysate was spun down (12000 rpm, 15min, 4ºC) and incubated with NiNTA Agarose beads (Quagen) or GST Sepharose beads (Amersham). His-UBA1 was eluted in HIS elution buffer (PBS, 300mM NaCl, 0,1% Triton X-100 and 200mM imidazole). GST-UBCH5 was eluted in GST elution buffer (100mM Tris-HCl (pH 7.5) and 10mM glutathione). Eluates were dialyzed in PBS and concentrated simultaneously using Pierce™ Protein concentrators. Protein purity was analyzed by band visualization using Coomassie staining. For storage 10% glycerol was added to the eluate.

Mass spectrometry analysis

Mass spectrometry sample preparation, measurement and database search were performed as described previously (Bluhm et al., 2016). Gradient lengths of 45 or 105 min were chosen depending on the immunoprecipitated material obtained. Raw files were processed with MaxQuant (version 1.5.2.8) and searched against the Homo sapiens UniProt database (February

25, 2012) using the Andromeda search engine integrated into MaxQuant and default settings were applied. Proteins with at least two peptides, one of them unique, count as identified.

Microscopy methods

Fluorescent microscopy on fixed cells

Localisation microscopy experiments were performed with MRC5 fibroblasts and patient derived fibroblasts. Cells were transfected with mCherry-ZRF1 and GFP-DDB2 expressing plasmids using Lipofectamine 2000, according to manufacturer's instructions.–Cells were exposed to localized UV damage (100J/m^2) using a polycarbonate micropore membrane with 5µm pore size (Millipore) as described in (Katsumi et al., 2001). Preextraction was performed with ice-cold CSK buffer (100 mM NaCl; 300 mM sucrose; 3mM $MgCl_2$; 10 mM Pipes (pH 6.8)) supplemented with 0.2% Triton at 30 min post UV and then fixed in 4% PFA. After permeablization in ice-cold PBS supplemented with 0.5% Triton X-100 and blocking in 5% FBS in PBS-T, cells were stained with XPA (Novus Biologicals) or XPC (Cell Signaling) antibodies overnight at 4°C. After washing coverslips were incubated with Alexa-488 fluorophore-conjugated secondary antibodies (Life technologies) and mounted in Vectashield with DAPI. Images were acquired with the LAS AF software (Leica) using a TCS SP5 confocal microscope (Leica) with a 63x/1.4 oil immersion objective. For co-localization studies, about 100 lesions were counted per condition.

To analyze distribution of γH2A.X in shControl and shRING1B U2OS cells were irradiated with 20J/m^2 UV and fixed with 4% PFA. Cells were stained with RING1B (Cell signalling) and γH2A.X (Millipore) antibodies overnight at 4°C. After washing coverslips were incubated with Alexa-488 and Alexa-542 fluorophore-conjugated secondary antibodies (Life technologies) and mounted in Vectashield with DAPI.

Live microscopy and imaging

For microirradiation HeLa-Kyoto Cherry-PCNA or wildtype HeLa cells were grown on cover slide dishes and transfected with the indicated constructs using PEI. Imaging and microirradiation experiments were performed using an UltraVIEW VoX spinning disc confocal system (PerkinElmer) in a closed live-cell microscopy chamber (ACU, Perkin Elmer) at 37°C with 5% CO_2 and 60% humidity, mounted on a Nikon TI microscope (Nikon). Images were taken with a CFI Apochromat 60x/1.45 NA oil immersion objective. GFP and cherry or were imaged with 488 and 561 nm laser excitation and 527 ± 55 and 612 ± 70 nm emission filters, respectively. For microirradiation, a preselected spot (1 µm diameter) within the nucleus was microirradiated for 1200 ms with the 405nm laser resulting in 1 mJ. Before and after microirradiation, confocal image series of one mid

nucleus z section were recorded in 2 seconds intervals. For evaluation of the accumulation kinetics between four and 12 cells were analyzed. Images were first corrected for cell movement (ImageJ plugin StackReg, transformation mode Rigid body) and mean intensity of the irradiated region was divided by mean intensity of the whole nucleus (both corrected for background) using ImageJ software. Maximal accumulation represents the highest ratio from each experiment.

DNA repair assays

Colony formation assay

Cells were transfected with the respective siRNAs with Lipofectamine according to the manufacturer's protocol. Cells were plated on tissue culture plates at a density of 1000 cell per plate 24 hours after transfection. 48 hours post transfection growth medium was aspirated and cells were irradiated with the indicated UV dose. Colonies were counted 7 days after irradiation. Numbers of colonies formed after irradiation were normalized against non-UV treated control.

Unscheduled DNA synthesis

UDS experiments were performed as described previously (Jia et al., 2015). Briefly, MRC5 fibroblasts were transduced with lentiviral particles expressing the respective shRNAs. After viral transduction, the cells were serum starved for 24 hours in DMEM, containing 0.5% FBS, irradiated with UV light (20J/m^2) and incubated with 10μM EdU in DMEM (Thermo Fisher) for 2 hours. Alexa-555-azide (Thermo Fisher) was conjugated to EdU using the Click-reaction. The coverslips were mounted in Vectashield with DAPI. Images were acquired with the LAS AF software (Leica) using a AF-7000 widefield microscope (Leica) with a 63x/1.4 oil immersion objective and an ORCA CCD camera (Hamamatsu). Images were analyzed using ImageJ. DAPI was used to define nuclei, and EdU intensity within nuclei was measured after background subtraction. 150-300 nuclei were analyzed per sample. Mean intensities of +UV and –UV conditions for all cells were calculated, and used to estimate the DNA repair occurring in the particular sample.

Removal of CPDs

MRC5 fibroblasts were transduced with lentiviral particles expressing the respective shRNAs. 24h after viral transduction, the cells were replated on coverslips, exposed to UV light (10J/m^2) and fixed at the indicated timepoints. Cells were stained with CPD antibody (Cosmo Bio) using manufacture's protocol, followed by incubation with Alexa-488 fluorophore-conjugated secondary antibodies (Life technologies). The cells were mounted in Vectashield with DAPI and images were acquired with the LAS AF software (Leica) using an AF-7000 widefield microscope (Leica) with a 63x/1.4 oil immersion objective and an ORCA CCD camera (Hamamatsu). Images

were analyzed using ImageJ. DAPI was used to define nuclei, and CPD intensity within nuclei was measured after background subtraction. 100-200 nuclei were analyzed per sample. Mean intensities of +UV and –UV conditions for all cells was calculated, and used to estimate the DNA repair occurring in the particular sample.

Results[1]

Identification of RING1B and ZRF1 as components of NER

The first aim of this study is to characterize RING1B and ZRF1 as the possible components of DNA repair pathways. RING1B was previously described to be recruited to chromatin after induction of DNA damage with IR, laser irradiation or HU treatment (Ginjala et al., 2011; Ismail et al., 2010). We decided to expand these observations after induction of DNA damage with etoposide, bleomycin and CPT. After treatment with the respective agents, cells were harvested and crosslinked with formaldehyde at the selected time points and used for the further isolation of chromatin. Using this technique we observed a minor recruitment of RING1B to chromatin after induction of different types of the DNA damage, with the most prominent effect after treatment with CPT. Additionally, we detected the enrichment of chromatin with H2A-ubiquitin, consistent with the previous observations (Figure 14A).

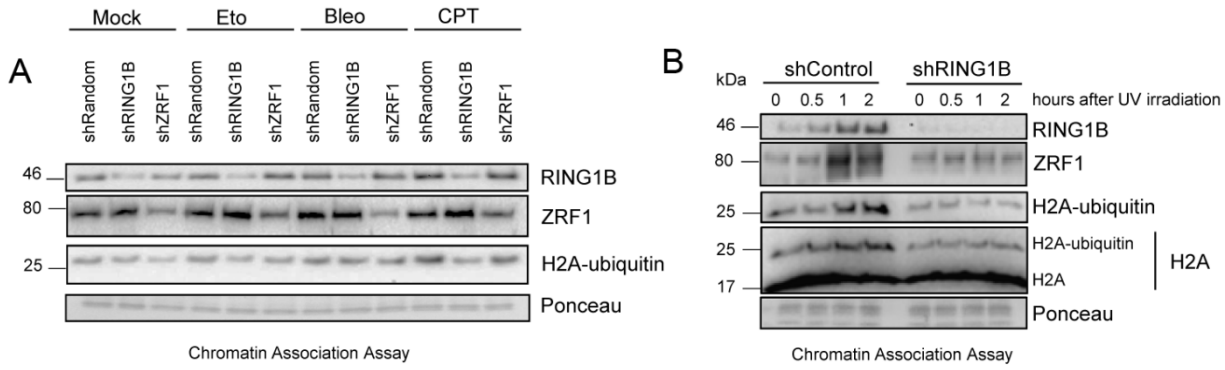


Figure 14

RING1B and ZRF1 are recruited to chromatin after induction of DNA damage

A) HEK293T cells stably expressing the respective shRNA (Control, RING1B, ZRF1) were treated with indicated genotoxic drug and subjected to chromatin association assay. Concentration and duration of treatment are indicated in Materials and methods. Protein enrichment at chromatin was analyzed by Western Blot. Results provided by Martha Taubert
B) HEK293T cells stably expressing the respective shRNA (Control, RING1B) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.

[1] The majority of results presented in these thesis are published as:

Gracheva, E., Chitale, S., Wilhelm, T., Rapp, A., Byrne, J., Stadler, J., Medina, R., Cardoso, M.C., and Richly, H. (2016). ZRF1 mediates remodeling of E3 ligases at DNA lesion sites during nucleotide excision repair. The Journal of cell biology 213, 185-200.

RING1B was previously described to be important for H2A monoubiquitination after UV-irradiation (Bergink et al., 2006). Using chromatin association assay after irradiation of cells with UV we observe an accumulation of RING1B at chromatin. The accumulation appears already at the early timepoints with the peak at 1h after UV-irradiation (Figure 14B). This observation complements previous observations, which were restricting the activity of RING1B to the post-incision steps of NER. To place recruitment of RING1B at the context of NER we have analyzed chromatin from the XPA affected patients, in which the steps after recognition of the damage are abolished. Indeed, analysis of chromatin reveals that recruitment of RING1B and accumulation of H2A-ubiquitin is not different from the control within the first 2 hours after UV-irradiation (Figure 15).

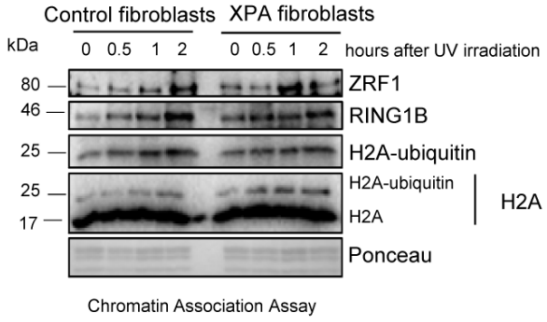


Figure 15

Recruitment of RING1B and ZRF1 are independent of XPA

XPA patient fibroblasts and XPA patient fibroblasts complemented with XPA cDNA were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.

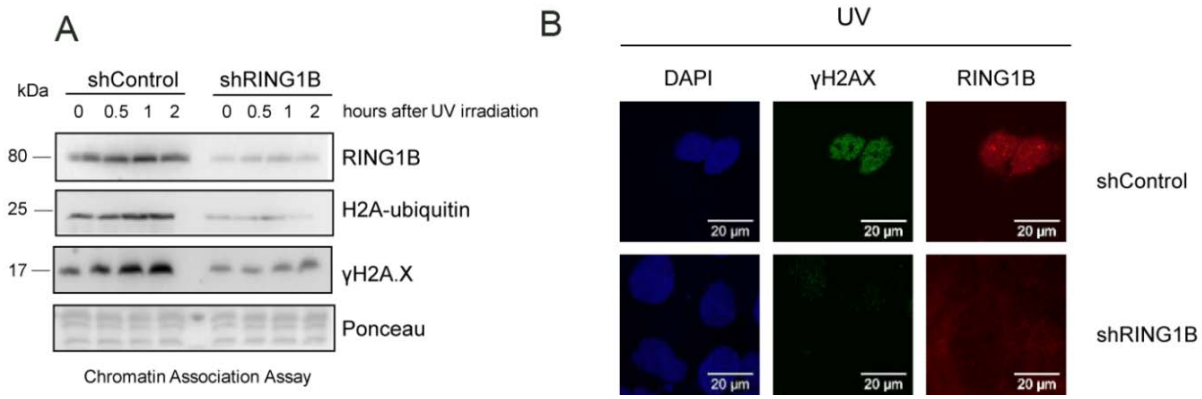


Figure 16

RING1B is important for induction of γH2A.X after UV-irradiation

A) HEK293T cells stably expressing a respective shRNA (Control, RING1B) were irradiated with UV-C, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
B) U2OS cells stably expressing a respective shRNA (Control, RING1B) were irradiated with UV-C, fixed 1h after irradiation and stained with antibodies against RING1B and γH2A.X.

Postinsision steps of NER are characterized by formation of γH2A.X foci, which are regarded as a marker of DNA damage (Vrouwe et al., 2011). We have analyzed the effect of knockdown of RING1B on the accumulation of γH2A.X after UV-irradiaiton. In RING1B knockdown

cells no increase in H2A.X phosphorylation was observed (Figure 16A). Similarly, immunofluorescence microscopy in RING1B knockdown cells using γH2A.X and RING1B antibodies validates this finding (Figure 16B). These data suggests that RING1B is likely to be involved both in the early and the late steps of the repair of the UV-mediated DNA damage, and the latter activity could be related to the preformation of the DSB recognition machinery (Marteijn et al., 2009).

ZRF1 is recruited to chromatin in dependence of the enzymatic activity of RING1B and presence of H2A-ubiqutin (Richly et al., 2010). After induction of the DNA damage in wildtype HEK293T cells ZRF1 is recruited to chromatin (Figure 14A). However, in the RING1B knockdown cells we have observed two different variants, depending on the type of the DNA damage. After induction of DNA damage with etoposide, bleomycin and CPT, ZRF1 is recruited to damaged chromatin irrespectively of the presence of RING1B and consequent presence of H2A-ubiquitin. However, these types of DNA damage agents are inducing various ubiquitination signals, including ubiquitination of H2A by RNF168 and BRCA1/BARD complex, as well as mono-ubiquitination of PCNA by Rad6 (Hoege et al., 2002; Kalb et al., 2014; Mattiroli et al., 2012). These ubiquitin moieties could serve as a signal for the recruitment of ZRF1 by its ubiquitin-binding domain. Chromatin, purified from the UV-irradiated cells, is enriched by RING1B and ZRF1 and recruitment of ZRF1 is dependent on the presence of RING1B and H2A-ubiquitin (Figure 14B). These data is consistent with the previous observations, stating that recruitment of ZRF1 is dependent on RING1B as well as H2A-ubiquitin (Richly et al., 2010) . Furthermore, the ubiquitin-binding domain of ZRF1 is required for its association with chromatin after UV-irradiation, as the association of ZRF1 depleted of the UBD with damaged chromatin is diminished, compared to the control (Figure 17).

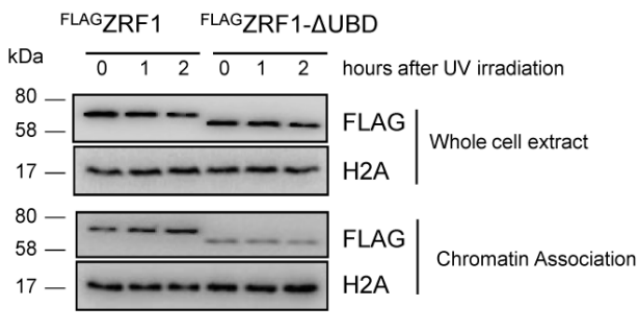


Figure 17

UBD domain of ZRF1 is required for association with chromatin after UV-irradiation

HEK293T cells were transfected with wildtype and mutant ^{FLAG}ZRF1 harvested at selected timepoints. ZRF1 enrichment at chromatin was analyzed by Western Blot.

Additionally we have addressed whether RING1B and ZRF1 are recruited to DNA damage sites by a live microscopy approach. After transfection of vectors encoding fluorescently tagged RING1B and ZRF1 in HeLa cells, cells were locally irradiated with a 405nm laser and the accumulation of RING1B and ZRF1 at the DNA damage site was examined (this experiment was done by Dr. Alexander Rapp, TU Darmstadt). Both RING1B and ZRF1 were recruited to the site of the local irradiation (Figure 18).

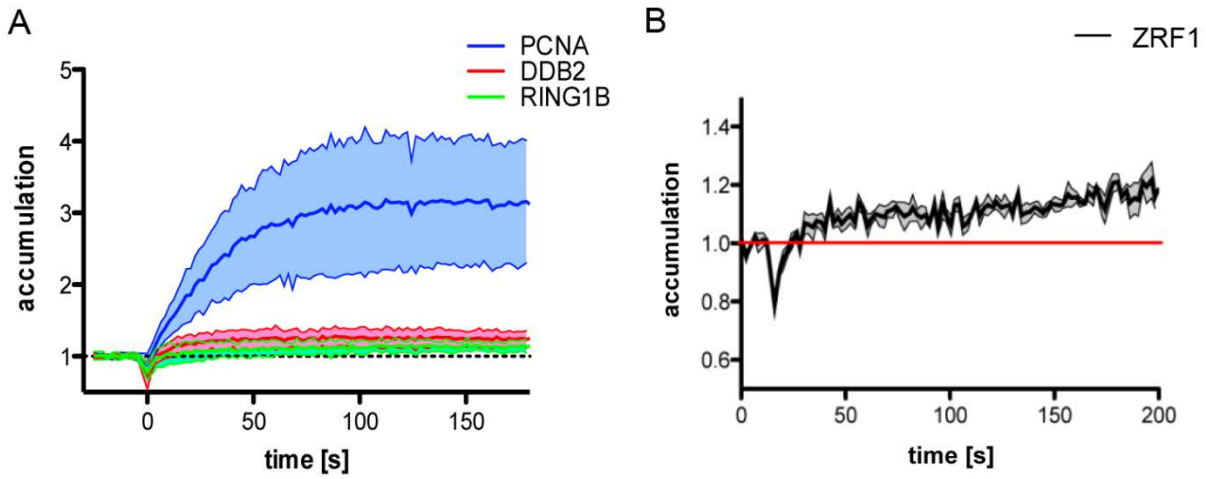


Figure 18

RING1B and ZRF1 are localizing to the DNA damage sites inflicted by irradiation with 405nm UV-laser

A) YFP-RING1B and DDB2-GFP were coexpressed in HeLa-Kyoto cells, stably expressing Cherry-PCNA. Live cells were irradiated with laser and the accumulation of the proteins was measured.
B) mCherry-ZRF1 was expressed in HeLa cells. Live cells were irradiated with laser and the accumulation of the protein was measured

405nm UV laser irradiation is reported to induce various types of DNA damage including formation of oxydated nucleotides, single and double strand breaks and formation of CPD, usually repaired by NER (Dinant et al., 2007). To associate ZRF1 and RING1B recruitment with NER, we have used local irradiation of cells with UV-C regarded as a 'gold standard' to investigate the behavior of NER-related proteins. Unlike in the experiments with laser irradiation RING1B is not recruited to the sites of UV-C local irradiation. Nevertheless, this observation is consistent with the previous reports, stating that RING1B is not accumulated at the sites of micropore irradiation (Zhu et al., 2009). This study suggests though, that RING1B might be a component of a yet not identified transient complex, acting early in NER. Nonetheless, ZRF1 is detected at the sites of local UV-irradiation, marked with NER components XPC, XPA and DDB2 (Figure 19 and 25C). Importantly, this association was abolished after administration of the inhibitor of PRC1 PRT4165, which diminishes the levels of H2A-ubiquitin (Figure 20) (Ismail et al., 2013).

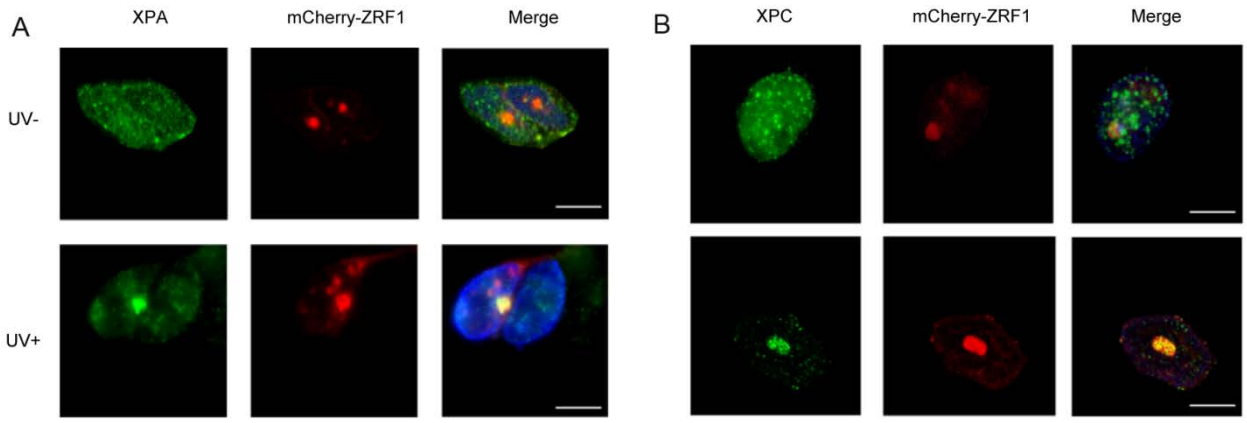


Figure 19

ZRF1 is localized to the site of local UV-C irradiation

MRC5 fibroblasts expressing mCherry-ZRF1 locally irradiated with UV-C. Cells were fixed 30 minutes after irradiation and stained with XPA (A) or XPC (B) antibody. Results provided by Rebeca Medina

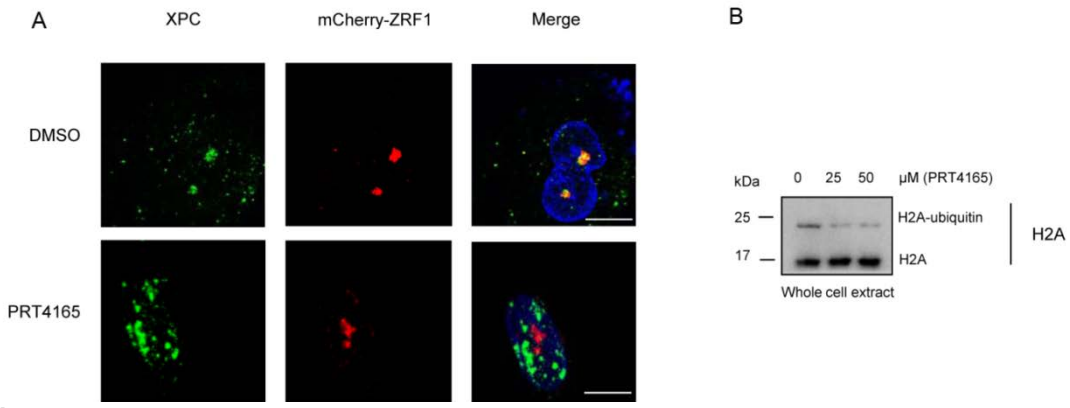


Figure 20

ZRF1 is recruited to the DNA damage sites in dependence of H2A-ubiquitination

A) MRC5 fibroblasts expressing mCherry-ZRF1 were pretreated with 50μM PRT4165 for 4h and locally irradiated with UV-C. Cells were fixed 30 minutes after irradiation and stained with XPC antibody.

B) HEK293T cells were pretreated with different concentrations of PRT4165 and lysed in Laemmli buffer. H2A-ubiquitination was analyzed by Western Blot.

Results provided by Rebeca Medina

Previous reports suggest that ZRF1 is recruited to chromatin to displace RING1B from PRC1-associated genes in differentiating cells (Richly et al., 2010; Papadopoulou et al., 2016). Therefore, we decided to confirm whether ZRF1 displaces RING1B recruited to chromatin after UV-irradiation. Using the cells depleted of ZRF1, we observe that knockdown of ZRF1 promotes retention of RING1B and subsequently elevated levels of H2A-ubiquitin (Figure 21). Collectively, these data show that ZRF1 and RING1B are recruited to chromatin after induction of DNA damage with UV, suggesting these proteins to be components of the NER pathway.

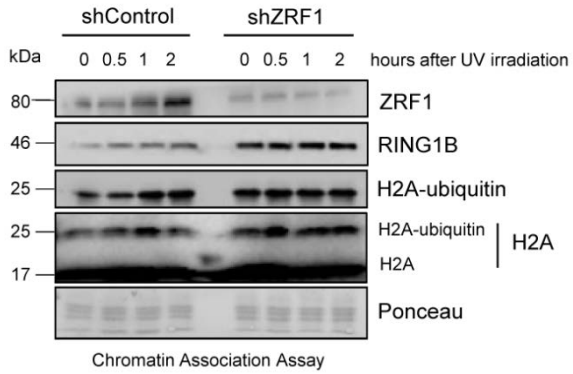


Figure 21

ZRF1 displaces RING1B after UV-irradiation

HEK293T cells stably expressing a respective shRNA (Control, ZRF1) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.

To functionally determine the impact of RING1B and ZRF1 on the UV-mediated DNA repair, we have used several approaches. One of them is unscheduled DNA synthesis, which allows measuring the amount of newly synthetized DNA, restored by DNA repair. We have measured the amount of the nucleotide analogue EdU incorporated in DNA in cells with different genetic background, irradiated with UV. Compared to the control cells, cells depleted of NER-related factors XPC and DDB2 are characterized by lower efficiency of DNA repair. Similarly, cells depleted of RING1B and ZRF1 exhibit low levels of DNA repair (Figure 22A). Moreover, we could recapitulate the effect of RING1B depletion on DNA repair efficiency by treating cells with PRT4165 inhibitor (Figure 22B).

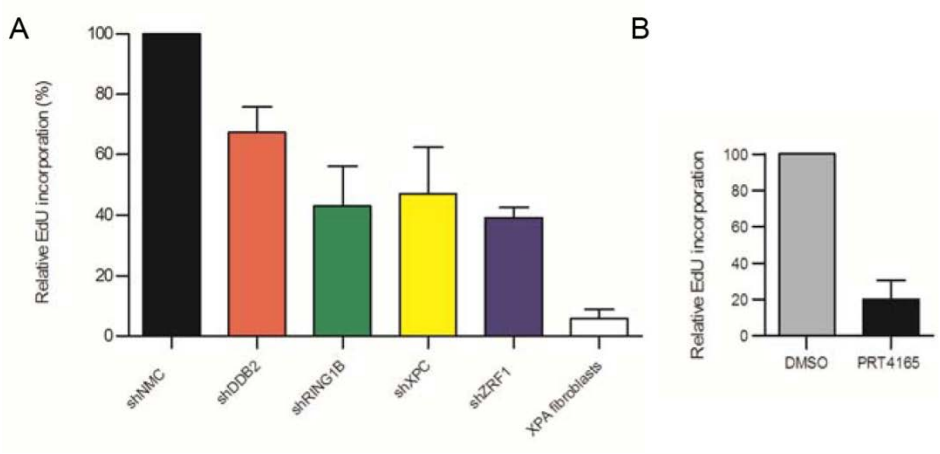


Figure 22

Knockdown of RING1B and ZRF1 reduces UDS after UV-irradiation

A) MRC-5 fibroblasts were transduced with the respective shRNA (control, DDB2, RING1B. XPC, ZRF1). XPA fibroblasts were used as a negative control. Cells were irradiated with UV and incubated with EdU for 2h. Amount of incorporated EdU was measured after fluorescent labelling by Click-reaction.

B) MRC-5 fibroblasts were pretreated with 50μM PRT4165 for 4h and irradiated with UV. Cells were irradiated with UV and incubated with EdU for 2h. Amount of incorporated EdU was measured after fluorescent labelling by Click-reaction.

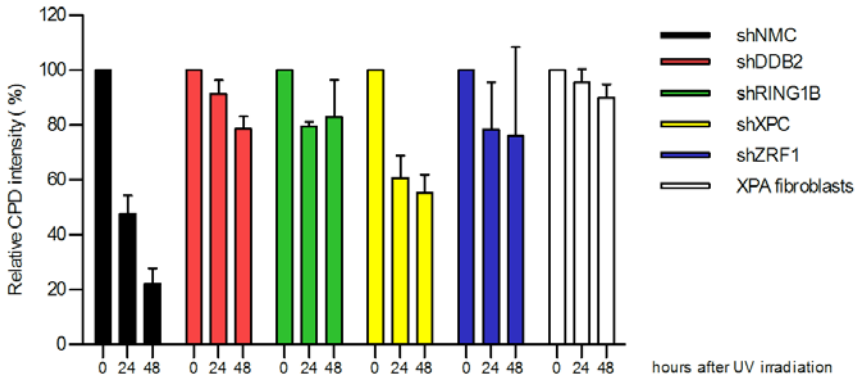


Figure 23

Knockdown of RING1B and ZRF1 reduces repair of CPD

MRC-5 fibroblasts transduced with the respective shRNA were irradiated with UV. Amount of residual CPD is measured as intensity of fluorescently labelled CPD in various timepoints after UV-irradiation. Results are provided by Rebeca Medina

Results of the UDS experiment suggest that RING1B and ZRF1 are not only recruited to the site of DNA damage, but actively involved in the repair process. However, this method doesn't discriminate between repair of different types of the damage, induced by UV-irradiation or the endogenous factors, such as base modifications. CPDs are regarded as the primary target of NER

machinery. Using the same cells as in UDS experiment, we irradiated them with UV, and measured the reduction of CPD intensity in selected timepoints. We have observed that knockdown of NER components as well as RING1B and ZRF1 reduces the effectivity of CPD removal (Figure 23). In sum, we conclude that RING1B and ZRF1 are implicated in the active repair of UV-mediated DNA damage.

Previous observations place recruitment of RING1B to the DNA damage sites in the context of PRC1 complex. We sought to explore whether UV-mediated recruitment of RING1B is related to the PRC1. For that, chromatin from cells, depleted of PRC1 component BMI-1 was analyzed. We have identified that BMI-1 is recruited to chromatin in response to UV-irradiation, suggesting that the full PRC1 complex might be recruited to chromatin. However, absence of BMI-1 does not change the recruitment of RING1B. Moreover, knockdown of BMI-1 is not altering the dynamics of H2A-ubiquitination after irradiation with UV, suggesting that it is not required for the activity of ubiquitin E3 ligases, ubiquitinating H2A after UV-irradiation (Figure 24A). Additionally, we have tested whether knockdown of RING1B or BMI-1 impacts cell survival after UV-irradiation. Consistently with the measurements of the UDS and CPD removal, RING1B cells exhibit a mild reduction of the colony formation potential. Similarly, we observe this repair defect in the BMI-1 knockdown cells. Interestingly, simultaneous knockdown of both proteins showed an additive reduction of the colony formation potential. This suggests that BMI-1 and RING1B likely have different functions in the repair of UV-mediated DNA lesions (Figure 24B)

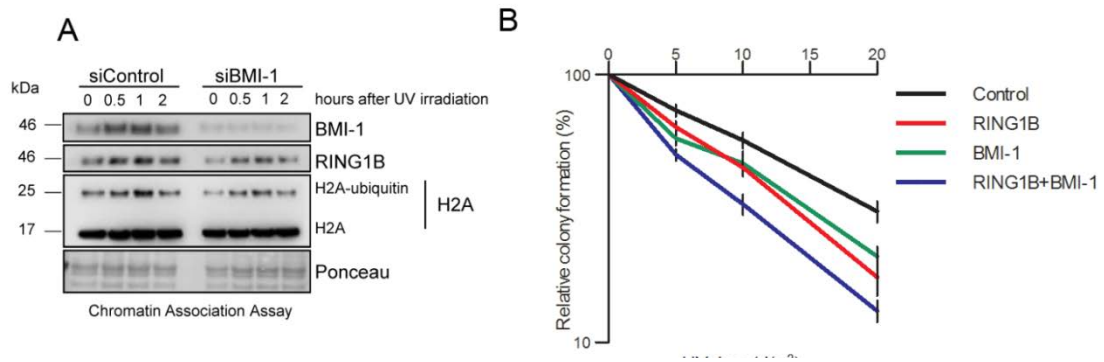


Figure 24

RING1B and BMI-1 act as components of different DNA repair pathways acting after UV-irradiation

A) HEK293T cells transfected with the respective siRNA (control, BMI-1) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
B) Relative colony formation in control or RING1B knockdown cell lines treated with siRNA at different UV doses. Control cells were transfected with either control siRNA (Control) or BMI-1 siRNA (BMI-1). RING1B knockdown cell lines were transfected with either control siRNA (RING1B) or BMI-1 siRNA (RING1B + BMI-1).

Binding partners of RING1B in NER

After identification of RING1B as a component of the NER pathway, we decided to explore the hypothesis that RING1B is recruited to the UV-damaged chromatin as a component of a novel PRC1-like complex. To identify possible interactors of RING1B after UV-irradiation ^{FLAG}RING1B was expressed in HEK293T cells to carry out affinity purifications with the FLAG-antibody. As expected, RING1B is co-immunoprecipitated with the PRC1 subunit BMI-1 (Wang et al., 2004). Interestingly, RING1B interacts with DNA damage binding protein DDB2 but not with other selected NER factors, such as XPA and XPF (Figure 25A). The RING1B-DDB2 interaction further was verified by immunoprecipitation of endogenous RING1B in UV-irradiated cells (Figure 25B). A reversed approach of purification of ^{FLAG}DDB2 confirmed strong binding of RING1B along with the interaction with its binding partners DDB1 and CUL4A (Figure 25C).

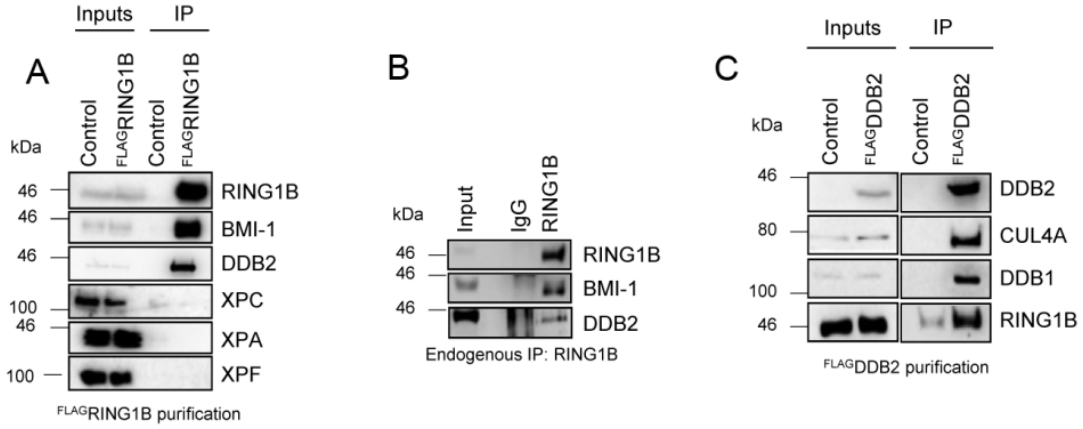


Figure 25

RING1B interacts with DNA damage binding protein DDB2

A) Control cells and cells expressing ^{FLAG}RING1B were irradiated with UV light. After immunoprecipitation with FLAG-M2-Agarose the purified material was analyzed by Western Blot.
B) Endogenous immunoprecipitations with RING1B antibodies after UV-irradiation were analyzed by Western Blot. Results provided by Shalaka Chitale.
C) Control cells and cells expressing ^{FLAG}RING1B were irradiated with UV light. After immunoprecipitation with FLAG-M2-Agarose the purified material was analyzed by Western Blot.

Next we examined whether RING1B interacts with DDB2 and BMI-1 in a mutually exclusive manner. Knockdown of RING1B does not alter the association of DDB2 and BMI-1 to chromatin, suggesting that the recruitment of DDB2 to the damaged DNA is a prerequisite to its association with RING1B (Figure 26A). Additionally, these data point to a divergent role of RING1B and BMI-1 in the repair of UV-mediated DNA damage. While immunoprecipitating BMI-1, we observed binding of RING1B, but not DDB2 (Figure 26B), proposing that RING1B might form a complex either with BMI-1 or with DDB2 after UV-irradiation. Indeed, overexpression of BMI-1 leads to a slight increase in RING1B-BMI-1 interaction, but a complete loss of RING1B-DDB2 binding (Figure 26C). These data suggest that the majority of RING1B is associated with BMI-1 rather than DDB2, which is in agreement with the function of PRC1 in gene silencing.

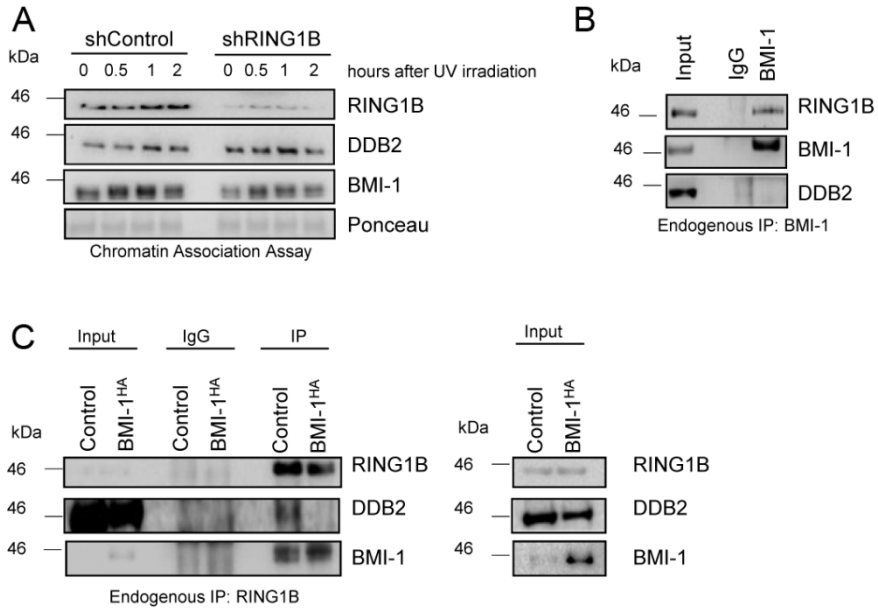


Figure 26

RING1B interacts with DDB2 or BMI-1 in a mutually exclusive manner

A) HEK293T cells stably expressing the respective shRNA (Control, RING1B) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
B) BMI-1 does not interact with DDB2 after UV-irradiation. Endogenous immunoprecipitations with BMI-1 antibodies. Endogenous immunoprecipitations with RING1B antibodies after UV-irradiation were analyzed by Western Blot. Results provided by Shalaka Chitale.
C) BMI-1 and DDB2 compete for binding to RING1B. HEK293T cells overexpressing BMI-1^{HA} were irradiated with UV. Endogenous immunoprecipitations with RING1B antibodies were analyzed by Western Blot.

To investigate a joint function of DDB2 and RING1B in DNA repair, we performed colony formation assays. Depletion of RING1B and DDB1 promotes reduced colony formation ability as

observed by us (Figure 24B) or in the experiments measuring the survival of the XPE patient fibroblasts after UV-irradiation (Rapic-Otrin et al., 2003). A simultaneous depletion of both proteins showed no further reduction of the colony formation potential supporting the idea that RING1B and DDB2 likely act in a common DNA repair pathway (Figure 27).

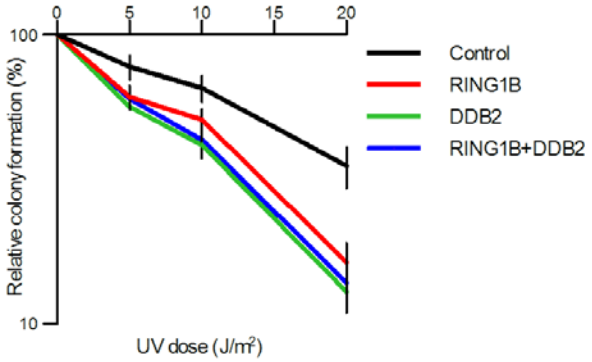


Figure 27

RING1B and DDB2 are epistatic in the repair of UV-mediated DNA damage

Relative colony formation in control or RING1B knockdown cell lines treated with siRNA at different UV doses. Control cells were transfected with either control siRNA (Control) or DDB2 siRNA (DDB2). RING1B knockdown cell lines were transfected with either control siRNA (RING1B) or BMI-1 siRNA (RING1B + DDB2).

As RING1B does not have a DNA binding domain, we hypothesized that DDB2 is responsible for recruitment of RING1B to UV-damaged DNA. Indeed, in the cells, depleted of DDB2 by siRNA treatment, as well as in the fibroblasts derived from the patients affected with XPE, RING1B is not recruited to chromatin (Figure 28). Consequently, these cells exhibit reduced H2A-ubiquitylation after irradiation with UV consistent with a previous report (Kapetanaki et al., 2006). Notably, knockdown of DDB2 did not impair BMI-1 recruitment to chromatin further uncoupling functions of BMI-1 and RING1B in NER (Figure 28A).

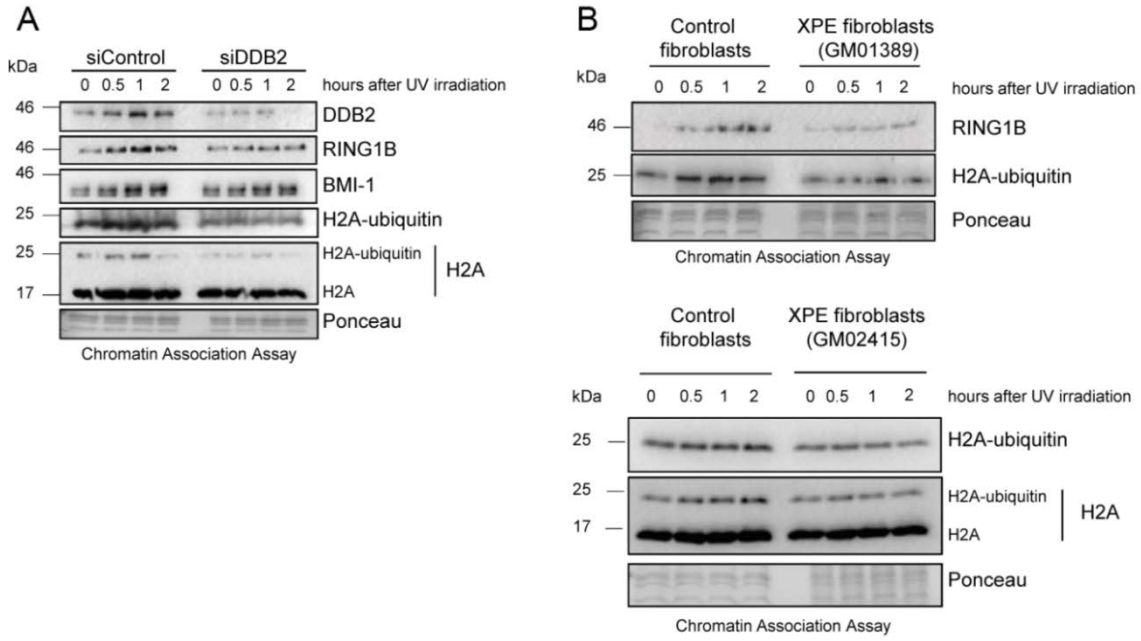
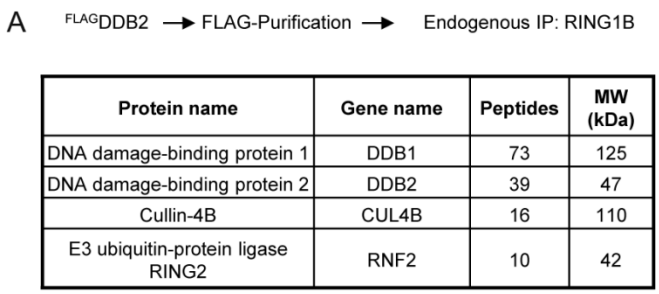


Figure 28

DDB2 is important for RING1B-mediated monoubiquitination of H2A after UV-irradiation

A) HEK293T cells transfected with the respective siRNA (control, DDB2) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
B) Control fibroblasts and fibroblasts from XPE patients were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.



Protein name	Gene name	Peptides	MW (kDa)
DNA damage-binding protein 1	DDB1	73	125
DNA damage-binding protein 2	DDB2	39	47
Cullin-4B	CUL4B	16	110
E3 ubiquitin-protein ligase RING2	RNF2	10	42

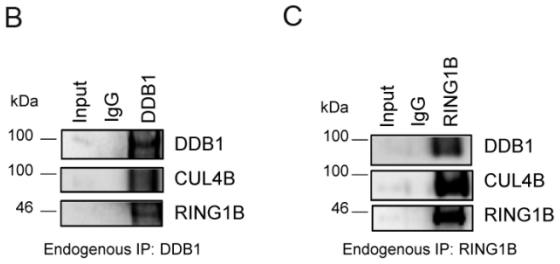


Figure 29

Identification of UV-RING1B complex

A) Protein interaction partners of RING1B and DDB2. Mass spectrometry analysis after sequential immunoprecipitations with FLAG and RING1B antibodies revealed DDB1 and CUL4B as main interaction partners of DDB2 and RING1B. IP performed by Shalaka Chitale, mass-spectrometry analysis is provided by IMB core facility Proteomics.
B) and C) UV-RING1B components interact with each other. Endogenous immunoprecipitations with DDB1 or RING1B antibodies were analyzed by Western Blot.

To further characterize the interaction of RING1B and DDB2, mass spectrometry approach was used. We expressed ^{FLAG}DDB2 in HEK293T cells and performed purifications in UV-irradiated and untreated cells. After elution of ^{FLAG}DDB2 containing protein complexes with FLAG peptide, we used the eluate in immunoprecipitations with RING1B antibodies to purify specifically RING1B-DDB2 containing protein complexes. The complexes were analyzed by mass-spectrometry, revealing previously described DDB2 interactors such as DDB1 and CUL4B as the main interactors of RING1B-DDB2 (Figure 29A). Interestingly, CUL4A – the other paralogue of the scaffold protein CUL4, as well as RBX1 – the ubiquitin E3 ligase commonly interacting with Cullin proteins, were not identified in this pulldown. The interactions between the subunits of the complex were further confirmed by immunoprecipitations with the antibodies against DDB1 and RING1B (Figures 29B and 29C). These data suggests existence of a novel Cullin-containing complex, comprised out of DDB2-DDB1-CUL4B-RING1B (later referred as UV-RING1B), which is probably formed at the DNA damage site after UV-irradiation.

UV-RING1B contains RING1B as the E3 ligase subunit, which is rather uncommon for the Cullin-based E3 ligases. We have reasoned that CUL4B is capable of interacting both with RING1B and RBX1 in a mutually exclusive manner. To test that, ^{FLAG}RING1B was overexpressed in HEK293T cells and the lysate from these cells was used for immunoprecipitation with the antibodies against RBX1. Excessive amounts of RING1B disrupted CUL4B-RBX1 binding, suggesting that indeed CUL4B is capable to interact with either RBX1 or RING1B (Figure 30).

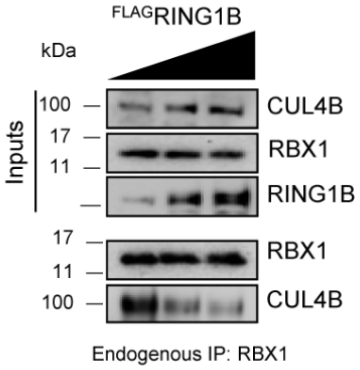


Figure 30

CUL4B interacts with RBX1 or RING1B in a mutually exclusive manner

HEK293T cells expressing increasing amounts of ^{FLAG}RING1B construct were UV-irradiated. Endogenous immunoprecipitations with RBX1 antibodies were analyzed by Western Blot.

As the UV-RING1B complex contains a ubiquitin E3 ligase subunit, we have examined whether the complex has a ubiquitin E3 ligase activity. For that we purified the complex and used it for the subsequent *in vitro* ubiquitination reactions. To enrich for the four subunits and reduce the co-purification of other E3 ligases potentially binding to the DDB2, we overexpressed FLAG-tagged DDB1, DDB2, RING1B and STREP-FLAG-tagged CUL4B in HEK293T cells. After enriching for the FLAG-tagged proteins we purified CUL4B containing complexes via the STREP tag (Figure 31A). We subjected the purified material to colloidal coomassie staining and mass spectrometry, which confirmed the assembly of the UV-RING1B complex (Figure 31B). No other E3 ligases or contamination with chromatin components were found in these purifications.

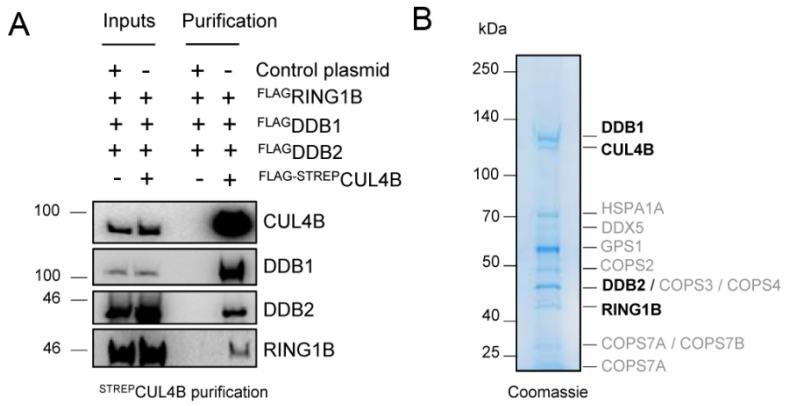


Figure 31

Purification of UV-RING1B complex for *in vitro* experiments

A) Assembly of the UV-RING1B complex. Plasmids expressing ^{FLAG}DDB1, ^{FLAG}DDB2 and ^{FLAG}RING1B were cotransfected in combination with either control plasmid or a plasmid encoding $^{FLAG\text{-}STREP}$CUL4B. After immunoprecipitation with STREP-Tactin® beads the purified material was analyzed by Western blot

B) Visualization of the UV-RING1B complex. Purified UV-RING1B complex was subjected to SDS gel-electrophoresis and colloidal coomassie staining. Mass spectrometry analysis revealed the presence of all four subunits (bold)

RING1B is a ubiquitin E3 ligase known to monoubiquitinate H2A. The activity of the purified complex towards histones was tested by the *in vitro* ubiquitylation assays, using recombinant histone H2A as well as recombinant nucleosomes. When compared to control reactions, the UV-RING1B complex strongly increased the monoubiquitination of histone H2A at lysine 119 as verified by the antibodies against H2A and H2A-ubiquitin. Similarly, the UV-RING1B complex caused monoubiquitination of nucleosomes at histone H2A in ubiquitylation assays (Figure 32A). This data is particularly important as it recapitulates the interaction of the purified complex with H2A in conditions similar to *in vivo* (Figure 32B).

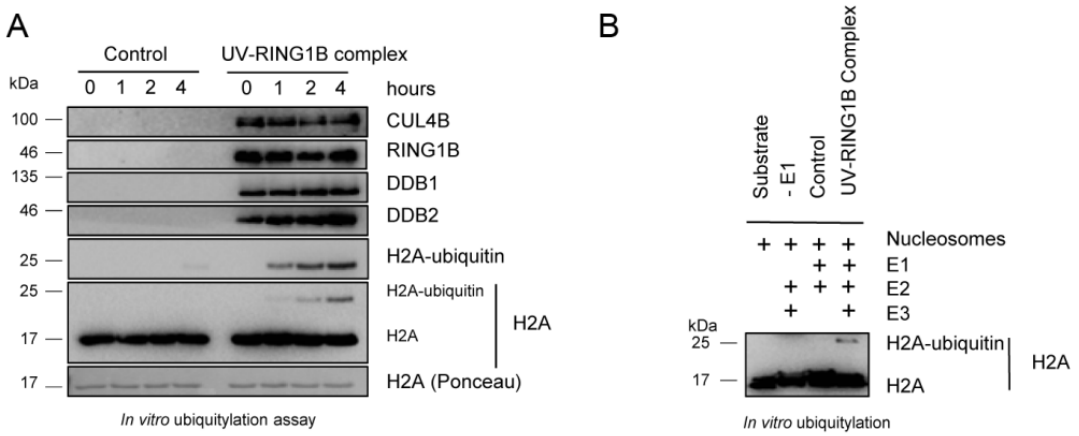


Figure 32

The UV-RING1B complex catalyzes ubiquitination of H2A *in vitro*

Ubiquitination assays were performed with recombinant H2A (A) or recombinant nucleosomes (B), E1 (UBA1), E2 (UBCH5) and either GST (control) or UV-RING1B complex. Reactions were performed at 37°C and samples were taken at the indicated time points. Material of the respective time points was analyzed by Western blot

Collectively, these data suggests that RING1B is a component of NER and it forms a novel ubiquitin E3 ligase complex, comprised of DDB2-DDB1-CUL4B-RING1B. This complex is able to ubiquitinate histone H2A after UV-irradiation.

Role of H2A-binding activity of ZRF1 in NER

Earlier we have identified that after irradiation with UV ZRF1 is recruited to the sites of the DNA damage and its recruitment depends on the presence of the H2A-ubiquitin (Figure. After identification of the UV-RING1B complex and its activity towards H2A, we sought to investigate the depletion of the components of the complex towards H2A-ubiquitination and recruitment of ZRF1. For that we have analyzed chromatin from the cells depleted of CUL4B and DDB2 using siRNA, or the fibroblasts from XPE patients (Figure 33). All mutant cells analyzed show reduced level of H2A-ubiquitin, consistent with the previous observations (Guerrero-Santoro et al., 2008; Kapetanaki et al., 2006). Consistently, enrichment of ZRF1 at chromatin was abolished in the absence of functional CUL4B and DDB2. These data suggests that H2A-ubiquitin is important for the recruitment of ZRF1 to chromatin after UV-irradiation.

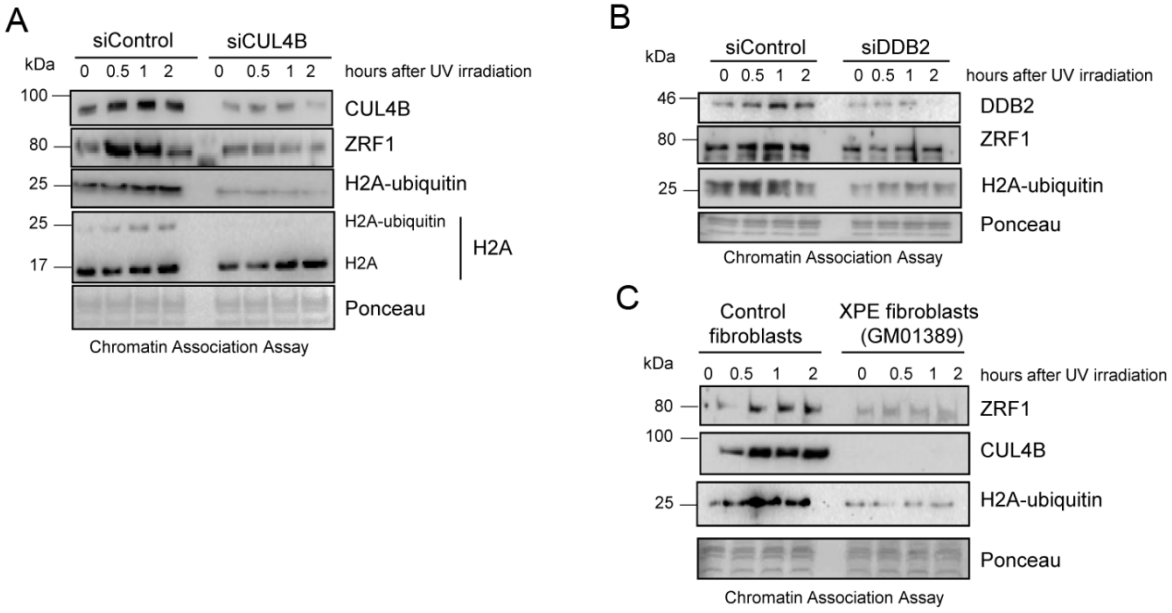


Figure 33

ZRF1 is recruited to chromatin in the presence UV-RING1B complex

A) HEK293T cells transfected with the respective siRNA (control, CUL4B) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
B) HEK293T cells transfected with the respective siRNA (control, DDB2) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
C) Control fibroblasts and fibroblasts from XPE patients were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.

As previously shown, the H2A-ubiquitin binding protein ZRF1 dislocates RING1B from chromatin during cellular differentiation of NT2 progenitor cells (Richly et al., 2010). We have reasoned that ZRF1 might function similarly towards the subunits of UV-RING1B complex. For that we have analyzed chromatin from the cells, depleted of ZRF1. Consistently with the observations of the RING1B dynamics (Figure 21), CUL4B – one of the components of UV-RING1B complex was enriched at chromatin after depletion of ZRF1 (Figure 34A). However, the amount of DDB2 associated with chromatin remained unaltered. This observation prompted us to investigate the effect of ZRF1 knockdown on recruitment of CUL4A – another protein associated with ubiquitination of H2A in NER (Kapetanaki et al., 2006). Interestingly, absence of ZRF1 abolished the recruitment of this protein to chromatin after UV-irradiation (Figure 34B). These observations were confirmed by analysis of the chromatin by purification of FLAG-tagged nucleosomes. We have reasoned that ZRF1 might have an effect on the downstream NER processes.

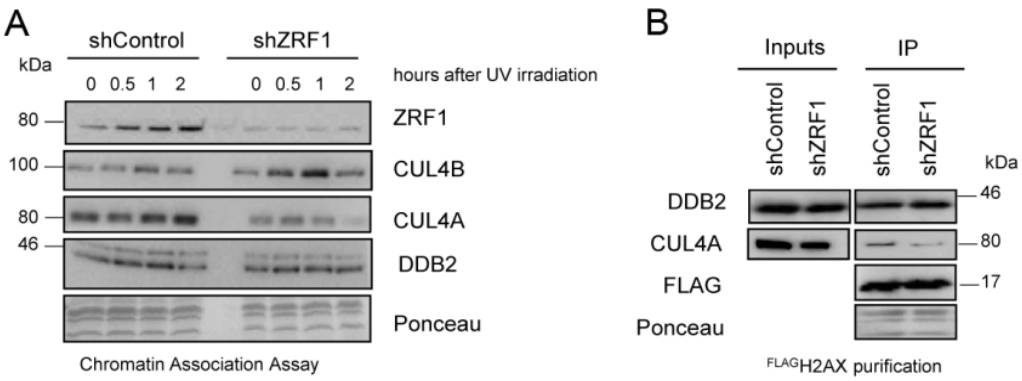


Figure 34

ZRF1 is important for the recruitment of CUL4A to chromatin

A) HEK293T cells expressing shRNA (control, ZRF1) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
B) HEK293T cells expressing shRNA (control, ZRF1) were transfected with ^{FLAG}H2A.X and irradiated with UV. After immunoprecipitation with FLAG-M2-Agarose the purified material was analyzed by Western Blot.

UV-CUL4A complex plays an integral role in the regulation of DNA damage recognition in NER (Sugasawa et al., 2005). Absence of ZRF1 restricts recruitment of CUL4A at chromatin, suggesting that ZRF1 plays a role in the formation of the UV-CUL4A complex. UV-RING1B and UV-CUL4 complexes are comprised out of two modules. The first module is similar in both complexes and comprised of the UV-DDB dimer containing DDB2 and DDB1. The second module is formed by a CUL4 scaffold and a ubiquitin E3 ligase and is different between UV-RING1B and UV-CUL4A. We have reasoned that ZRF1 might have a role in the reorganization of the UV-RING1B complex. In agreement with the previous findings, in purifications with ^{FLAG}DDB2 from ZRF1 knockdown cells we co-precipitated only reduced levels of CUL4A (Figure 35A). Next we analyzed whether the assembly of the UV-CUL4A complex was compromised in ZRF1 knockdown cells. We immunoprecipitated ^{HA}RBX1 from control and ZRF1 knockdown cells and observed diminished levels of DDB2 and DDB1 in the co-precipitate. Interestingly, CUL4A levels stayed unaltered (Figure 35B). This data is further supported by experiments *in vitro* with purified proteins (Gracheva et al., 2016). Taken together, these observations suggest that ZRF1 is likely involved in the assembly of the UV-CUL4A E3 ligase complex at chromatin.

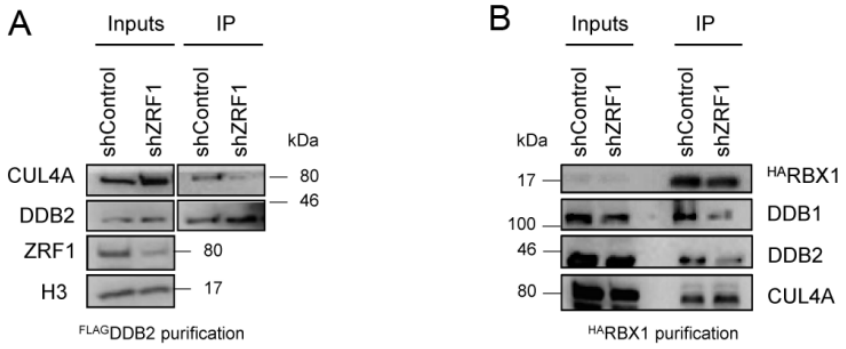


Figure 35

ZRF1 is important for the formation of UV-CUL4A complex after UV-irradiation

A) HEK293T cells expressing shRNA (control, ZRF1) were transfected with ^{FLAG}DDB2 and irradiated with UV. After immunoprecipitation with FLAG-M2-Agarose the purified material was analyzed by Western Blot.
B) HEK293T cells expressing shRNA (control, ZRF1) were transfected with ^{HA}RBX1 and irradiated with UV. After immunoprecipitation with FLAG-M2-Agarose the purified material was analyzed by Western Blot.

DNA damage binding protein XPC is the major target of UV-CUL4A mediated ubiquitination (Sugasawa et al., 2005). We have analyzed the effect of the knockdown of CUL4A on the ubiquitination status of XPC. As expected, depletion of CUL4A abolishes ubiquitination of XPC (Figure 36A). We have reasoned previously that ZRF1 is important for the formation of the UV-CUL4A complex, and therefore tested the effect of ZRF1 knockdown on the XPC-ubiquitination (Figure 36B). Consistent with the previous observations, XPC is not modified with ubiquitin in absence of CUL4A. Additionally, ^{HA}XPC, purified from the ZRF1 as well as RING1B knockdown cells, exhibits diminished ubiquitination status (Figure 36C).

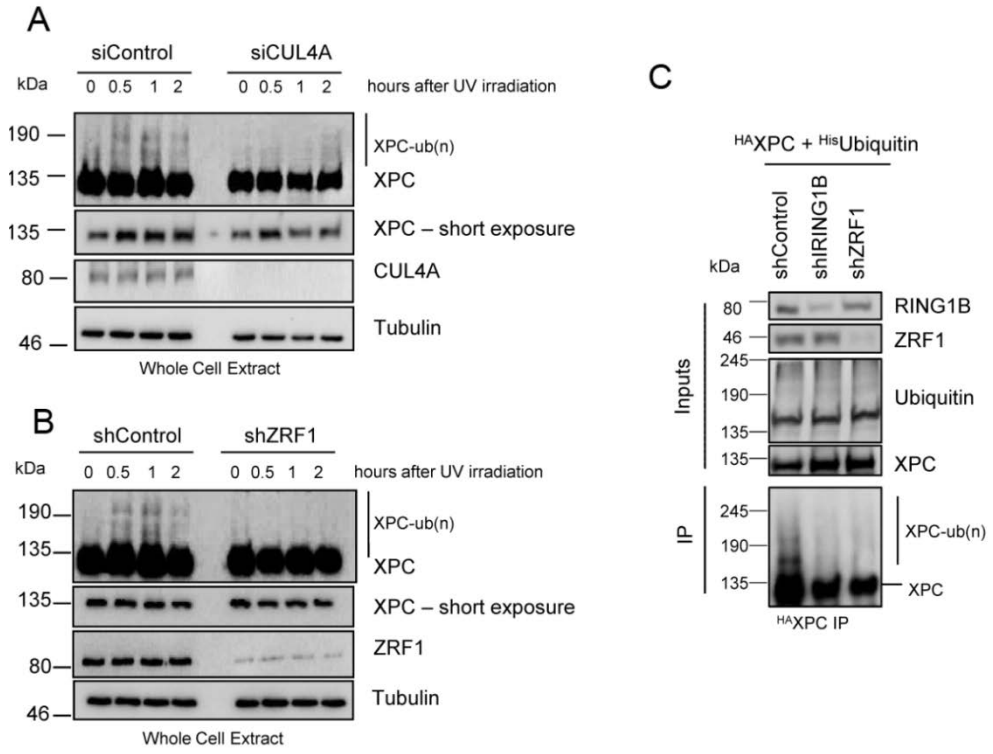


Figure 36

ZRF1 is important for the activity of UV-CUL4A complex

A) HEK293T cells transfected with the respective siRNA (control, CUL4A) were irradiated with UV, harvested at selected timepoints and lysed in Laemlli buffer. Protein amount and XPC-ubiquitination were analyzed by Western Blot.
B) HEK293T cells expressing shRNA (control, ZRF1) were irradiated with UV, harvested at selected timepoints and lysed in Laemlli buffer. Protein amount was analyzed by Western Blot. XPC ubiquitination status was analyzed by Western Blot.
C) HEK293T cells expressing shRNA (control, RING1B, ZRF1) were transfected with ^{HA}XPC and ^{HIS}Ubiquitin and irradiated with UV. After immunoprecipitation with HA antibody the purified material was analyzed by Western Blot. Results provided by Jens Stadler.

Interaction of ZRF1 with XPC complex

ZRF1 is binding to chromatin via the interaction of its ubiquitin binding domain with H2A-ubiquitin. We have previously shown that ZRF1 is recruited to the damaged chromatin after UV-irradiation. However, the mechanism how it is brought to the vicinity of the DNA damage site remains unclear. We have hypothesized that ZRF1 might interact with NER factors which facilitate its binding to the damaged chromatin. To explore if ZRF1 interacts with NER factors, we expressed ^{FLAG}ZRF1 in HEK293T cells and carried out affinity purifications. We found the DNA lesion recognition factor XPC interacting with ZRF1, but did not observe binding of other selected NER factors (Figure 37A). Likewise, we found XPC associated with ZRF1 in endogenous immunoprecipitations confirming the interaction of both proteins (Figure 37B). We did not observe alteration of XPC binding to chromatin after UV-irradiation in the absence of ZRF1 (Figure 37C), suggesting that these proteins are probably recruited to chromatin sequentially.

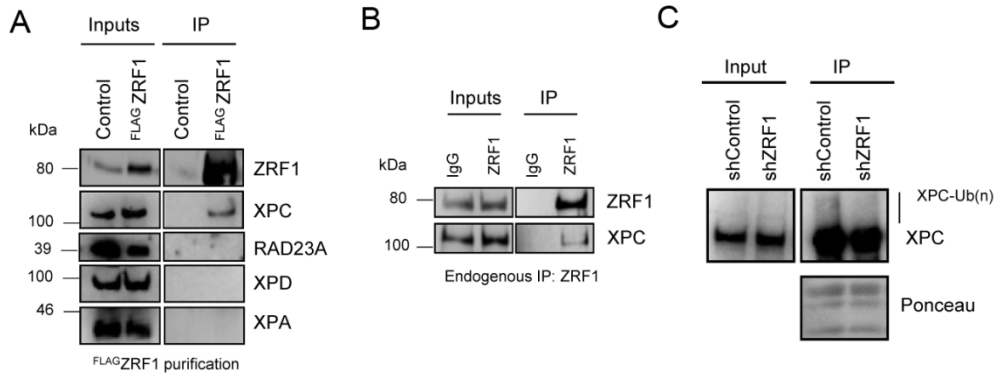


Figure 37

ZRF1 interacts with XPC

A) Control cells and cells expressing ^{FLAG}ZRF1 were irradiated with UV. After immunoprecipitation with FLAG-M2-Agarose the purified material was analyzed by Western Blot.
B) Endogenous immunoprecipitations with ZRF1 antibodies after UV-irradiation were analyzed by Western Blot. Results provided by Shalaka Chitale.
C) HEK293T cells expressing shRNA (control, ZRF1) were transfected with ^{FLAG}H2A.X were irradiated with UV. After immunoprecipitation with FLAG-M2-Agarose the purified material was analyzed by Western Blot.

To support the interaction of ZRF1 and XPC, we analyzed chromatin from XPC patient fibroblasts and control fibroblasts after UV-irradiation. Consistently we observed reduced levels of ZRF1 in XPC fibroblasts (Figure 38A). Accordingly, absence of XPC promotes enhanced RING1B and H2A-ubiquitin levels at chromatin, confirming that ZRF1 is recruited to chromatin after induction of H2A-ubiquitination. Additionally, siRNA mediated knockdown of XPC caused a drastic reduction of ZRF1 levels at chromatin after UV-irradiation (Figure 38B). To further investigate the interaction of XPC and ZRF1, we analyzed the localization of ZRF1 to lesions sites using DDB2 as a damage marker. In contrast to control fibroblasts, colocalization of DDB2 and ZRF1 was reduced in XPC patient fibroblasts (Figure 38C).

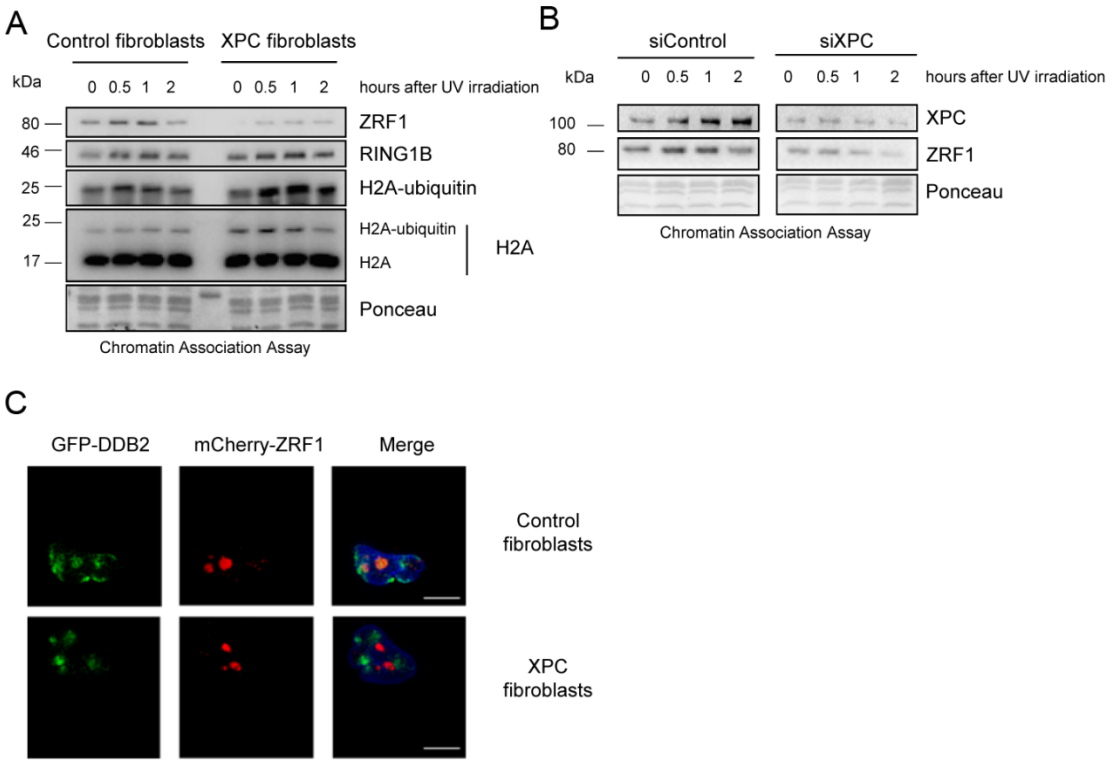


Figure 38

XPC is required for recruitment of ZRF1

A) Control fibroblasts and fibroblasts from XPE patients were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot.
B) HEK293T cells transfected with the respective siRNA (control, XPC) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot
C) Control and XPC fibroblasts expressing mCherry-ZRF1 and GFP-DDB2 were locally irradiated with UV-C. Cells were fixed 30 minutes after irradiation. Images provided by Rebeca Medina.

Next, we carried out an epistasis analysis to address the common functions of ZRF1 and XPC in NER. We observed a strong reduction in colony formation potential after irradiating ZRF1 knockdown cells or cells treated with siRNA directed against XPC, consistent with previous observations in XPC patient fibroblasts (Bohr et al., 1986). Simultaneous knockdown of both factors did not alter the colony formation potential compared to a single knockdown. These data suggests that ZRF1 and XPC are likely epistatic in human cells (Figure 39).

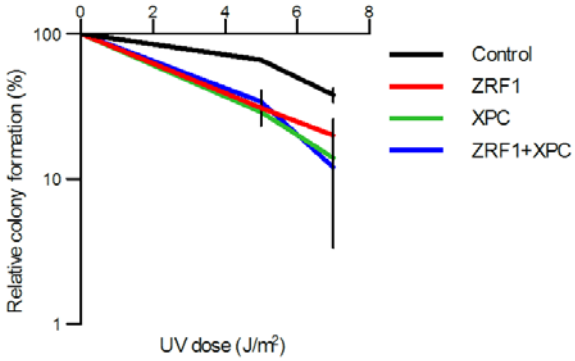


Figure 39

ZRF1 and XPC are epistatic in the repair of UV-mediated DNA damage

Relative colony formation in control or ZRF1 knockdown cell lines treated with siRNA at different UV doses. Control cells were transfected with either control siRNA (Control) or XPC siRNA (XPC). ZRF1 knockdown cell lines were transfected with either control siRNA (ZRF1) or XPC siRNA (ZRF1 + XPC).

Influence of ZRF1 on proteasomal degradation of NER proteins

Ubiquitin chain adaptor protein RAD23 is known to associate with XPC, and this association was described to be transient, as RAD23 dissociates from the complex after XPC is recruited to the damaged chromatin (Bergink et al., 2012). Absence of RAD23A doesn't alter recruitment of XPC or ZRF1 to chromatin (Figure 40A). This data suggests that ZRF1 and XPC associate independently of RAD23A. Our data points to the fact that ZRF1 associates with XPC, but not RAD23, suggesting that interaction of ZRF1 and XPC is independent of RAD23. Interestingly, after immunoprecipitating of ^{HA}RAD23A in control and RING1B and ZRF1 knockdown cells, we observe that the interaction of XPC and RAD23 depends on the presence of both RING1B and ZRF1 (Figure 40B). This data suggests that ZRF1 might affect the presence of RAD23 at the sites of the DNA damage.

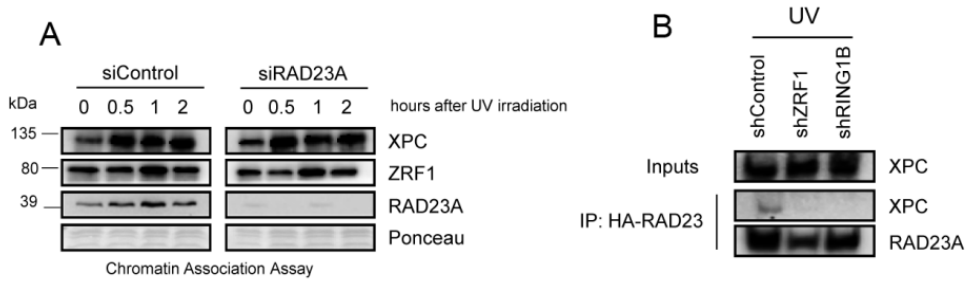


Figure 40

ZRF1 is important for the association of XPC and RAD23

A) HEK293T cells transfected with the respective siRNA (control, RAD23A) were irradiated with UV, harvested at selected timepoints and subjected to chromatin association assay. Protein enrichment at chromatin was analyzed by Western Blot
B) HEK293T cells expressing shRNA (control, RING1B, ZRF1) were transfected with ^{HA}RAD23 and were irradiated with UV. After immunoprecipitation with HA antibody the purified material was analyzed by Western Blot.

The role of RAD23 in NER remains unclear. To directly test whether RAD23 has a role in proteasomal degradation during NER we measured the decay of DNA repair factors after supplementing the cells with cycloheximide (Figure 41). This drug abolishes protein synthesis and allows measuring the amount of a protein retained in the cells. Cells, treated with siRNA against RAD23 show a stabilization of NER-associated proteins (DDB2 and ZRF1). To test the hypothesis that ZRF1 might control the function of RAD23 we performed protein degradation experiments with control and ZRF1 knockdown cell lines and observed stabilization of DDB2 in dependence of ZRF1 protein levels. This data supports that ZRF1 probably affects the activity of RAD23 and the proteasome degradation events in NER.

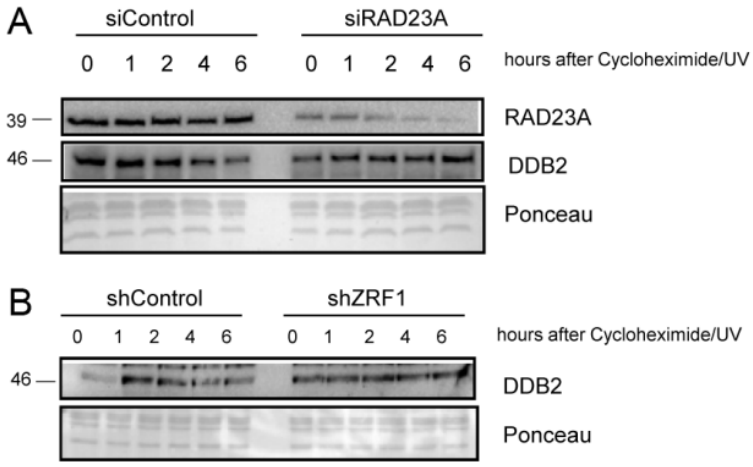


Figure 41

RAD23 and ZRF1 are important for the proteasomal degradation of DDB2

A) HEK293T cells transfected with the respective siRNA (control, RAD23A) were irradiated with UV-C and supplemented with a medium with 50µM cycloheximide, harvested at selected timepoints and lysed in Laemlli buffer. Protein amount was analyzed by Western Blot
B) HEK293T cells expressing shRNA (control, ZRF1) were irradiated with UV and supplemented with a medium with 50µM cycloheximide, harvested at selected timepoints and lysed in Laemlli buffer. Protein amount was analyzed by Western Blot.

Discussion

In the present study we have identified the role of proteins RING1B and ZRF1 in the repair of UV-mediated DNA damage. Up to now the interplay of these proteins is mainly characterized in the regulation of PRC1-mediated transcriptional repression and derepression, where H2A-ubiquitin, deposited by the ubiquitin E3 ligase activity of RING1B is recognized by ZRF1. The binding of ZRF1 to the ubiquitin moiety promotes displacement of RING1B, subsequent removal of H2A-ubiquitin and activation of transcription. Although RING1B was previously associated with UV-mediated H2A-ubiquitination, little is known about its activity at the DNA damage site. Consequently, ZRF1 potentially can bind newly deposited ubiquitin at chromatin and have a function in the DNA repair.

Timing of RING1B recruitment to chromatin after UV-irradiation

Several publications report recruitment of the PRC1 components to DNA damage sites (Ginjala et al., 2011; Ismail et al., 2010; Ui et al., 2015). Recent data shows that PRC1 subunit BMI-1 is recruited to the damaged chromatin via interaction with the transcription elongation factor ENL1 to induce local transcriptional repression. We investigated the recruitment of the PRC1 subunit RING1B to chromatin after induction of DNA damage. Corresponding with other observations, we observed a moderate recruitment of RING1B to chromatin after exposure of cells to etoposide, bleomycine and CPT. These chemicals are known to induce DSBs in replication-dependent (etoposide, CPT) and -independent (bleomycine) manners. Recruitment of RING1B is presumably mediating local H2A-ubiquitination, as judged by probing the Western blot membranes with an antibody specific for H2A-ubiquitin (Figure 14A).

Similarly we analyzed chromatin from the cells exposed with UV in a setup which activates the NER pathway. Irradiation with UV mediates recruitment of RING1B to chromatin, consistent with the observations made by Bergink and colleagues (Bergink et al., 2006). Interestingly, in this publication RING1B was not shown to localize at the sites of the UV-mediated DNA damage. The experimental setup used in this publication links RING1B to UV-mediated H2A-ubiquitination specifically at the post-incision steps of NER. In this setup accumulation of H2A-ubiquitin in the cells was dependent on the presence of XPA and hence late stages of NER. These data correspond with the further observations of Marteijn and colleagues, where recruitment of the classical DSB proteins such as RNF8 and MDC1 was identified at the post-incision stages of NER (Marteijn et al., 2009). Presumably this preassembly of the DSB repair machinery serves as a back-up mechanism in case of a failure of NER machinery and formation of a secondary DSB. Hence these observations mostly link the role of RING1B to the processing of a potential DSB.

We decided to explore the role of RING1B at late stages of NER. Protein kinase ATR is activated to phosphorylate histone variant H2A.X at the post-incision steps of NER (Vrouwe et al., 2011). ATR activity is also important for the recruitment of RING1B to the UV-mediated DNA damaged sites (Bergink et al., 2006). Analyzing the presence of γH2A.X in cells depleted of RING1B, we observe a strong reduction of H2A.X phosphorylation compared to control (Figure 15). Previous reports on the interplay of H2A-ubiquitination and γH2A.X in the repair of DSB show that knockdown of RING1B reduces the level of γH2A.X. Interestingly, these observations are linked to the activity of ATM, phosphorylating H2A.X after induction of the DSB, but not at the late stages of NER. Our data expands the possible relationship of phosphorylation and ubiquitination of histones at the site of the DNA damage. Further observations should be made regarding the regulation of the late stages of NER through the recruitment of RING1B.

Although several lines of evidence link RING1B mainly to the late stages of NER, analysis of chromatin from UV-irradiated cells and the microscopy data reveals rather early recruitment of RING1B to the DNA damage site (Figures 14 and 17A). We have hypothesized that RING1B indeed can be recruited to chromatin not only in the post-incision steps. To support this, we analyzed chromatin from the fibroblasts of an XPA patient, similarly to the approach of Bergink and colleagues. The direct assessment of chromatin-bound RING1B levels and H2A-ubiquitin shows no difference between control and patient cells. Hence we can speculate that the role of RING1B is not only confined to the DNA damage response at the late stages of NER.

We have tested whether cells depleted of RING1B are defect in UV-mediated DNA repair. Firstly, we measured the levels of unscheduled DNA synthesis in RING1B knockdown MRC-5 fibroblasts. This method is specifically designed to address the incorporation of the nucleotides after NER is complete. Indeed we observe that this ability is compromised in RING1B knockdown cells as well as in the cells depleted of DDB2 and XPC operating early in NER (Figure 22). This data supports the role of RING1B as a direct component of NER. In agreement, depletion of RNF8, linked to the preassembled DSB recognition machinery, does not lead to the reduction of the UDS (Marteijn et al., 2009). In agreement with the direct role of RING1B in NER, repair of UV-mediated CPDs in RING1B knockdown cells is compromised (Figure 23). Collectively, these data shows that RING1B is linked to the early stages of NER as well as in the NER-mediated preassembly of the DSB detection machinery.

RING1B functions independently of the PRC1 in NER

Recruitment of RING1B to the DNA damage sites is classically studied in the context of the PRC1 complex. BMI-1 is regarded as one of the principal binding partners of RING1B stimulating

its activity. Interestingly, reports of Ginjala and colleagues show that RING1B can be recruited to the DSB sites independently of BMI-1 (Ginjala et al., 2011). To investigate the interaction of BMI-1 and RING1B after UV-irradiation, we have analyzed chromatin of UV-irradiated control HEK293T cells and cells depleted of BMI-1 (Figure 24A). Interestingly, in absence of BMI-1 we similarly observe an enrichment of chromatin with RING1B, reminiscent of the observations of Ginjala and colleagues. These data proposes a presence of a complex, which contains RING1B devoid of BMI-1. It remains unclear however, whether this complex is linked to the transcriptional repression as the canonical PRC1 complex.

Given the link between RING1B and the NER machinery we sought to investigate the binding partners of RING1B after UV-irradiation. To this end, we immunoprecipitated RING1B and performed an initial screening by detecting the possible NER interactors with antibodies. For this screening this approach is superior to a mass-spectrometry approach, as the mass-spectrometry analysis is prone to produce false-positive and false-negative results. Publically available data of RING1B interactors mainly show Polycomb proteins and the proteins related to transcription (Sanchez et al., 2007). However, the high level of the interaction enrichment may hinder the low-enriched interactions. Focusing only on the NER-related interactions, we have identified an interaction of RING1B with DNA damage recognition protein DDB2, confirmed by immunoprecipitation with antibodies against tagged and endogenous RING1B as well as tagged-DDB2 (Figure 25).

We reasoned that the association of DDB2 with RING1B might be mediated by the PRC1 complex. To test this hypothesis, we immunoprecipitated BMI-1 and observed no co-precipitation of DDB2. Therefore, we suggest that RING1B and DDB2 are associated with each other, but this interaction does not involve BMI-1. However, in the presence of DDB2 RING1B may interact with it. Our data predicts presence of a complex, where RING1B is associated with NER components, but not with the classical Polycomb proteins. This hypothesis expands the roles of this ubiquitin E3 ligase beyond the classical transcriptional inactivation.

UV-RING1B complex and its regulation

To further characterize the interaction between RING1B and DDB2 we specifically purified the putative RING1B-DDB2 complex using a double immunoprecipitation approach and analyzed it by mass-spectrometry (Figure 29). Among the top interactors of RING1B-DDB2 were DDB1 – a common interactor of DDB2, and the scaffold protein CUL4B. Interestingly, we couldn't identify the enrichment with RBX1, the E3 ligase characteristic for the classical Cullin-RING ubiquitin E3 ligases.

Among all currently characterized PRC1-like complexes, BCOR is known as probably one of the most unusual. This complex was independently identified in the works of Sanchez and Gearhart and colleagues (Gearhart et al., 2006; Sanchez et al., 2007). It contains a large BCOR subunit, described previously as a transcriptional activator, PRC1 components, including RING1B, and a module, containing SKP1 and FBXL10 subunits. SKP1 serves as the adaptor protein in the CUL1 E3 ligase complex, and is reminiscent of DDB1 in CUL4 complexes. FBXL10 interacts with SKP1 as a substrate recognition module for FBX-SKP-CUL1 E3 ligase. Recent data point to the fact that FBXL10 and SKP1 are important for the recruitment of the PRC1 modules to the genomic loci, controlled by the BCOR complex to induce local ubiquitination (Wu et al., 2013b). The newly identified UV-RING1B complex contains a substrate recognition module (DDB2) and an adaptor module (DDB1), which serve to recruit the complex to the damaged chromatin and promote local ubiquitination activity. This is supported by the fact that DDB2 is required for the recruitment of RING1B to chromatin (Figure 28A). Remarkably, unlike BCOR complex, UV-RING1B is not enriched in the PRC1 subunits, but contains a CUL4B scaffold protein. This relates it more with the modular Cullin-RING ubiquitin E3 ligases, however, devoid of RBX1. So far, there are no Cullin-RING complexes without the RBX ubiquitin E3 ligase described in human, however, in *C.elegans* the E3 ligase RPM-1 (MYCBP1 in human) interacts with CUL1 to promote ubiquitination and subsequent degradation of the protein kinase ALC1 (Liao et al., 2004).

Composition of the UV-RING1B complex suggests that CUL4B may interact with both RBX1 and RING1B. An overexpression of RING1B indeed weakens the interaction of RING1B and RBX1 (Figure 30). However, what determines the interaction of CUL4B either with RBX1 or RING1B is not understood. This is correspondent to the general question: what determines the assembly of the UV-RING1B complex. We suggest three possible scenarios:

1. The UV-RING1B complex may be preassembled in the nucleoplasm to be later recruited to the DNA damage site;
2. The DDB2-DDB1 module may interact directly with CUL4B and indirectly with PRC1 components before UV-irradiation at chromatin. After the UV-irradiation RING1B is transferred to the CUL4B module and the complex is stabilized;
3. After UV-irradiation the DDB2-DDB1 module is recruited to the damaged DNA and sequentially attracts CUL4B and RING1B.

The first scenario is supported by the evidence that the components of UV-RING1B complex interact with each other *in vitro* and in absence of UV-irradiation *in vivo* (Gracheva et al., 2016; Appendices 2 and 3). However, this does not exclude the possibility that the complex is

assembled at chromatin and UV-irradiation triggers the stabilization of the UV-RING1B complex (scenario 2). Stabilization of large portions of DDB2 at chromatin after UV-irradiation mediates the association of RING1B as reflected in the accumulation of RING1B at the UV-damaged chromatin (Figure 28). Whether PRC1 serves as a donor of RING1B to facilitate the assembly of the UV-RING1B complex is not understood. This scenario also implies that UV-RING1B complex is not formed uniformly in the genome, but primary at the heterochromatic regions. Indeed, Polycomb-silenced sites are usually heterochromatic and are repaired by GG-NER. The third scenario reflects the overall modular structure and the dynamics of the subunits of the Cullin-RING E3 ligases, however how the DDB2-DDB1 module attracts CUL4B and RING1B is not understood.

The possible mechanisms of regulation of the UV-RING1B complex assembly and function may be explained after analysis of the proteins co-precipitated with the complex. PARP1 is purified together with the UV-RING1B complex in the presence of UV-irradiation (Appendices 2 and 3). Although mass-spectrometry analysis often reports PARP1 as a contaminant in immunoprecipitations, PARylation of the NER proteins including DDB2 is characteristic for the early steps of NER. PARylation was shown to be important for the functionality of UV-CUL4 E3 ligases (Robu et al., 2013). Interestingly, reports of Chou and colleagues show that the Polycomb components are recruited to the damaged chromatin in a PARP-dependent manner (Chou et al., 2010). This can contribute to the formation of the UV-RING1B complex. Additionally, other uncharacterized proteins can be PARylated in response to UV-irradiation, facilitating this process.

Cullin-RING ligases are regulated by the components of the COP9 signalosome. In the active condition (in case of UV-CUL4 – after UV-irradiation), the COP9 signalosome is disassociated from the CUL4 subunit followed by the NEDDylation of CUL4. This stimulates recruitment of the E2 ligase to the RBX1 subunit, enhancing its ubiquitin E3 ligase activity (Furukawa, 2000). Interestingly, we found an association of the subunits of the COP9 signalosome with UV-RING1B complex even after irradiation with UV (Appendices 2 and 3; Figure 31B). Unlike UV-CUL4 complexes, UV-RING1B does not contain RBX1. It remains unclear whether the COP9 signalosome may contribute to the activity of the UV-RING1B complex. To elucidate this question, the activity of UV-RING1B complex with NEDDylated and deNEDDylated form of CUL4B should be tested.

Role of H2A-ubiquitination in NER

As UV-RING1B contains the RING1B protein, we have studied its impact on UV-mediated ubiquitination of H2A. Knockdown of RING1B, DDB2 and CUL4B indeed reduces ubiquitination of H2A after UV-irradiation, suggesting that the functional role of RING1B in NER is formation of H2A-

ubiquitin at the DNA damage site (Figure 28). We confirmed this data by *in vitro* ubiquitination reactions, using recombinant H2A and nucleosomes (Figure 32). These observations do not exclude though that RING1B may ubiquitinate other substrates, as RING1B ubiquitinates some other proteins, such as geminin (Ohtsubo et al., 2008). Nevertheless, the main ubiquitination substrate in NER is histone H2A.

H2A is known to be ubiquitinated by several ubiquitin E3 ligases after UV-irradiation. Apart from the newly identified UV-RING1B, it was shown that UV-CUL4A and UV-CUL4B have the same function (Guerrero-Santoro et al., 2008; Kapetanaki et al., 2006). Several lines of data also show that these complexes are not redundant and UV-CUL4B ubiquitinates H2A with higher efficiency. Additionally, it was shown that UV-CUL4B ubiquitinates H2A at lysine 119, similarly to the UV-RING1B and the PRC1 complex (Guerrero-Santoro et al., 2008). Nevertheless, the function H2A ubiquitination remains unclear. Lan and colleagues propose that monoubiquitination of histone H2A, mediated by UV-CUL4B, promotes eviction of histones from the nucleosomes at the damage site (Lan et al., 2012). Subsequently, this would lead to the release of the UV-CUL4 complexes from chromatin. However, this hypothesis lacks *in vivo* evidence. Moreover, these observations do not explain how ubiquitination of the same site of H2A by UV-CUL4B leads to a probable histone eviction, whereas ubiquitination of H2A by RING1B prevents release of the nucleosomes by inhibiting the FACT complex (Zhou et al., 2008). One of the explanations could be the impact of other histone modifications, such as polyubiquitination of H3 and H4 mediated by UV-CUL4, previously reported by Wang and colleagues (Wang et al., 2006), or attraction of chromatin remodelers, mediating sliding of nucleosomes and formation of the open chromatin structures. Chromatin remodeling complex INO80 as well as histone acetylase STAGA are known to interact with DDB1 to promote local chromatin relaxation (Martinez et al., 2001), however the direct role of H2A-ubiquitination in this process is not understood.

Ubiquitination of H2A in the repair of DSBs is performed by a series of ubiquitin E3 ligases, which include RNF168 and RNF8 (mono- and polyubiquitination of lysines 13-15, respectively) and a parallel modification of lysines 119 and 127-129 by RING1B and the BRCA1/BARD complex, respectively (Kalb et al., 2014; Mattiroli et al., 2012; Ui et al., 2015). The sequence of H2A-ubiquitination in the NER remains unclear. We propose, that the initial ubiquitination of H2A can be performed either by UV-RING1B or by UV-CUL4B complexes, which is possibly linked to the chromatin remodeling at the DNA damage site. Additionally, at the late stages of NER the H2A-ubiquitin landscape at the lesion site can be expanded by the PRC1 complex and RNF8, possibly elongating the monoubiquitin moieties to the polyubiquitin chains. Although there are several E3

ligases implicated in ubiquitination of H2A after UV-irradiation, the direct role of this histone modification in NER is a subject of the further investigation.

Function of ZRF1 in the remodeling of the UV-RING1B complex

Up to now, the ubiquitin binding protein ZRF1 remains the only known reader of H2A-ubiquitin (Richly, 2010). We have hypothesized that ZRF1 is recruited to chromatin after UV-irradiation to bind newly deposited H2A-ubiquitin. Indeed, in UV-irradiated cells chromatin is enriched with ZRF1. Recruitment of ZRF1 depends on the activity of RING1B and the presence of H2A-ubiquitin, deposited by the UV-RING1B complex (Figure 33). To confirm that the recruitment of ZRF1 is a part of the NER repair pathway, we have analyzed the repair efficiency of ZRF1-depleted cells. Similar to NER-compromised cells and RING1B knockdown cells, they exhibit reduced UDS as well as a defect in the repair of CPD (Figures 22 and 23). This links ZRF1 to the NER pathway as a probable effector of H2A-ubiquitination.

Recruitment of ZRF1 to chromatin promotes displacement of RING1B and other PRC1 subunits to promote local transcriptional activation, at least during cellular differentiation. Our data show that the recruitment of ZRF1 mediates the same effect towards the two subunits of the UV-RING1B complex (RING1B and CUL4B) (Figures 21 and 34). Interestingly, chromatin levels of DDB2, in the HEK293T cells depleted of ZRF1, remain unaltered, whereas the presence of CUL4A – the paralogue of CUL4B – is decreased. These data suggests that ZRF takes part in the remodeling of the UV-RING1B complex by exchanging the CUL4B-RING1B module with the CUL4A-RBX1 module. Possibly it has the same effect towards recruitment of the CUL4B-RBX module to form UV-CUL4B E3 ligase complex.

The association with the COP9 signalosome might explain a possible mechanism of the UV-RING1B remodelling. The COP9 complex prevents NEDDylation of the Cullin-RING E3 ligases. Association of the UV-RING1B complex with the COP9 signalosome suggests that CUL4B in this complex is not NEDDylated. A protein CAND1 is known to bind unNEDDylated forms of Cullin. This interaction is due to the occupation of the binding site of Cullin and masking of the NEDDylation site (Goldenberg et al., 2004). Association of CAND1 with Cullins, including CUL4B, promotes the exchange of the adaptor module and the Cullin-RING module (Wu et al., 2013a). It is possible that ZRF1 exerts a similar function in the exchange the CUL4B-RING module of the UV-RING1B. It is not clear whether ZRF1 requires NEDD to promote the exchange of the subunits. Interestingly, association of Cullins with the COP9 complex does not necessarily lead to COP9-mediated deNEDDylation. The biochemical mechanism that limits the deNEDDylation activity of COP9 is unknown; however, approximately half of the COP9 associated cullins seem to be resistant to

deNEDDylation (Lyapina et al., 2001). In this case, the binding of ZRF1 or an alternative factor, recruited by ZRF1, to an exposed NEDD moiety might facilitate the remodeling of the UV-RING1B complex. Further studies should be performed to identify the NEDDylation status of CUL4B in the UV-RING1B complex and its possible impact on the remodeling.

An alternative mechanism of ZRF1-mediated UV-RING1B remodeling is the association with chaperone proteins. ZRF1 (also known as MPP1) was first described as a component of the ribosome associated complex together with the chaperone HSP70L1 and possibly other HSP70 homologues (Otto, 2005). ZRF1 acts as a co-chaperone, stimulating the ATP-dependent activity of the chaperones and facilitating the folding, translocation and degradation of the protein substrates. HSP70 chaperones are known to bind nascent polypeptide chains and provide their proper folding (reviewed in Mayer and Bukau, 2010). Additionally, they can interact with the HSP90 family of chaperones. This group of proteins facilitates the final maturation of proteins, promoting their stabilization and activation. The function of HSP70 is mainly attributed to their cytoplasmic function in association with ribosomes, nevertheless, HSP90 and HSP70 chaperone systems were described to interact with the DNA repair machinery. For example, HSP70 was detected at the sites of the DNA damage, induced with benzo[a]pyrene (Yang et al., 2009). Similarly, HSP90 is reported to interact with ATM, BRACA1/2 and MRN complex, mainly contributing to their degradation (ATM) or localization of the MRN complex at the DNA damage site (Dote et al., 2006; Noguchi et al., 2006). Whether ZRF1 acts as a co-chaperone for both HSP70 and HSP90 chaperone systems remains unclear. Analyzing the mass-spectrometry results of the purified UV-RING1B complex, we detect an interaction with HSP70 proteins as well as HSP90 and ZRF1 (Appendices 2 and 3). ZRF1 may target HSP70 and/or HSP90 to the UV-RING1B complex and promote its further remodeling. This could be tested by analyzing the remodeling of the complex in the absence of the J-domain of ZRF1, which is responsible for its co-chaperone function.

Interaction of ZRF1 and XPC

H2A-ubiquitin is a prerequisite for the binding of ZRF1 to chromatin. Nevertheless, other factors may contribute to the binding of ZRF1 to the DNA damage sites. We have analyzed the possible interactions of ZRF1 with the NER machinery using an immunoprecipitation approach similar to what we used for RING1B. We found a strong interaction of ZRF1 with DNA damage binding protein XPC (Figure 37). This interaction is apparently chromatin mediated, as the absence of ZRF1 does not alter the interaction of XPC with the damaged DNA, whereas in absence of XPC the tethering of ZRF1 is abolished. It is still unclear, what the function of the XPC-ZRF1 interaction is. One possible explanation is that XPC prolongs the half-life of ZRF1 at chromatin. This corresponds to the ability of XPC to bind damaged DNA. As ZRF1 does not have specificity to the

damaged DNA, its docking by both XPC and H2A-ubiquitin locks it at the DNA damage site to prolong its residence time. In turn, ZRF1 contributes to the indirect stabilization of XPC at chromatin promoting its polyubiquitination by UV-CUL4A. The additional mechanism of the ZRF1-dependent stabilisation of XPC is through its possible association with the ubiquitin specific proteases. In the process of ZRF1-dependent removal of H2A-ubiquitin, ZRF1 promotes the recruitment of the deubiquitinase USP21. In NER, USP7 stimulates stabilisation of XPC by protecting it from the proteasomal degradation (He et al., 2014). It remains unclear whether ZRF1 promotes recruitment of the specific DUB to the NER site.

Another aspect of the presence of XPC at chromatin is its binding to RAD23. These proteins are recruited to the damaged DNA as a complex, but after binding to the DNA the complex is rapidly dissociated (Bergink et al., 2012). Interestingly, immunoprecipitation of RAD23A in absence of ZRF1 shows no interaction between XPC and RAD23A (Figure 40). Given the function of ZRF1 in the remodeling of the UV-RING1B complex, it is possible that ZRF1 regulates the association of XPC and RAD23A. RAD23A is characterized by a presence of two ubiquitin-associated (UbA) and one ubiquitin-like domain (UbL). The UbA domains interact with the ubiquitinated proteins and the UbL-domain is responsible for the interaction of RAD23 with the proteasome (reviewed in Dantuma et al., 2009). The direct interaction of the ubiquitinated NER proteins with RAD23 was not shown directly, but the fact that the knockdown of the RAD23 seems to stabilize the ubiquitinated proteins to prevent their degradation, proves this theory (Figure 41).

An additional member of the machinery processing of ubiquitinated proteins in NER is VCP/p97. This protein in cooperation with the ubiquitin-binding adaptors was identified to interact with ubiquitinated proteins and separate them from their binding partners (Rape et al., 2001). Importantly, association of the substrate protein with VCP/p97 is a prerequisite for its association with RAD23 (at least in the yeast model), as was shown by Richly and colleagues (Richly et al., 2005). VCP/p97 extracts ubiquitinated XPC and DDB2 from chromatin to ensure proper progression of NER (Puumalainen et al., 2014). It is still unclear, what the later fate of these proteins is. In case of proteasomal degradation they further may associate with RAD23. Given the possible role of ZRF1 in the mediation of this process, ZRF1 might act as a cofactor of VCP/p97 and mediate a possible shuttling of the ubiquitinated substrate to the RAD23 and further handoff to the proteasome.

Collectively, these observations suggest a role of ZRF1 in the regulation of the proteasomal degradation of the NER proteins. Furthermore, it should be verified, whether ZRF1 is indispensable for the functions of VCP/p97 or RAD23 in NER.

Other functions of ZRF1 in DNA repair

In this project we have mainly focused on the functions of ZRF1 in NER. However, we cannot exclude that ZRF1 is a component of other DNA repair pathways, where ubiquitination of various substrates plays an important role in their regulation. We have tested the recruitment of ZRF1 to chromatin after induction of the DNA damage by various components. Interestingly, ZRF1 is recruited to damaged chromatin after induction of the DNA damage by etoposide, bleomycin and CPT independently of the presence of RING1B. This presumably means that ZRF1 might have alternative binding substrates. Indeed, formation of the double strand breaks by exposure of cells to these agents is accompanied by the monoubiquitination of H2A by RNF168 and BRCA1/BARD complex (Mattirolli et al., 2012, Kalb et al., 2014). Whether ZRF1 can bind H2A ubiquitinated at the lysines other than lysine 119 or the other ubiquitinated substrates remains unknown, however, in this case it can contribute either in the regulation of the H2A-ubiquitination by recruiting additional factors or by processing of the ubiquitin moiety via deubiquitination.

Monoubiquitination of PCNA by Rad6 potentially serves as potential substrate for the recruitment of ZRF1 (Hoege et al., 2002). Indeed, ZRF1 is recruited to chromatin in response to the treatment of cells with hydroxyurea or etoposide, which are known to cause the stalling of the replication forks (Figure 14; Marta Taubert, bachelor thesis, JGU, 2015). We speculate that a possible binding of ZRF1 to ubiquitinated PCNA may contribute in the resolving of stalling replication forks. Indeed, it can contribute to this process by the interaction with the VCP/p97 segregase, as suggested to be one of the mechanisms of this process (Davis et al., 2012; Mosbech et al., 2012).

Although in cells treated with etoposide, bleomycin and CPT ZRF1 is recruited to the chromatin irrespectively of the RING1B presence, ZRF1 still can bind the products of its activity. It is known that the activity of the PRC1 complex mediates local transcriptional repression at the DSB sites. The possible role of the ZRF1 recruitment is transcriptional reactivation of the sites, marked with H2A-ubiquitin after DNA damage. We cannot exclude that ZRF1 might play a similar role in the processing local transcriptional repression as it has in the differentiation.

Finally, the role of ZRF1 might be mediation of the chromatin reorganization as it interacts with several chromatin remodelers. Mass-spectrometry analysis of the ZRF1 interactors shows that ZRF1 interacts with SMARCA5 and BRG1 previously implicated in the repair of the UV-mediated DNA damage (Chitale et al, unpublished; (Aydin et al., 2014; Luijsterburg et al., 2012; Zhang et al., 2009). These chromatin remodelers were displayed to have functions in the transcriptional reactivation (SMARCA5) or local chromatin relaxation (BRG1). Additionally, BRG1 is prerequisite

for the loading of XPC at the site of UV-induced DNA damage and the possible handover of the DNA lesion from DDB2 to XPC. One of the conceivable functions of ZRF1 is either the recruitment of the chromatin remodelers to the DNA damage site, or mediation their function. As the presence of H2A-ubiquitin is essential for the recruitment of ZRF1, ZRF1 may contribute to the H2A-ubiquitin mediated relaxation of chromatin as was previously proposed by Lan and colleagues (Lan et al., 2012).

Collectively, the presence of ZRF1 at the UV-mediated DNA damage site may elicit different functions, through its complex-remodeling function in cooperation with HSP70 or VCP/p97 chaperones or the interaction with the chromatin remodelers. It remains unclear, what the molecular mechanisms of these interactions are, and further investigations must be performed to deepen our knowledge in the role of ZRF1 in NER.

Conclusion

In this project we identified chromatin factors RING1B and ZRF1 as essential components of NER. In NER RING1B associates with the DNA damage binding proteins DDB2 and DDB1 and the scaffold protein CUL4B to monoubiquitinate histone H2A. This complex has a new type of composition atypical of Cullin-RING based E3 ligases.

Ubiquitin-binding protein ZRF1 is the first known reader of ubiquitination of H2A in NER and has an impact on the progression of the downstream NER events. Recruitment of ZRF1 to damaged chromatin is facilitated by the presence of H2A-ubiquitin and XPC. ZRF1 facilitates remodeling of the UV-RING1B complex and formation of the downstream UV-CUL4A complex, to mediate the ubiquitination of the NER factors such as XPC and DDB2

Importantly, ZRF1 plays a role in the regulation of the proteosomal degradation of the NER factors by its possible impact on the activity of RAD23A.

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Appendix 1

Antibodies used in this study

Abbreviations: WB – Western Blot, IP – immunoprecipitation, IF – immunofluorescence microscopy

Protein name/clone	Host / company / catalog number	Application
Primary antibodies		
Histone H2A	Rabbit, Abcam, ab18255	WB
H2A-Ubiquitin (H2AK119ub) clone (D27C4)	Rabbit, Cell Signaling, # 8240	WB
γH2A.X (clone JBW301)	Mouse, Millipore, 05-636	WB, IF
RING1B	Rabbit, selfmade (Richly et al., 2010)	WB
RING1B (clone D22F2)	Rabbit, Cell Signaling, #5694	WB, IP, IF
CUL4A	Rabbit, Cell Signaling, #2699	WB
XPC (clone [3.26])	Mouse, Abcam, ab6264	IF
XPC (clone D1M5Y)	Rabbit, Cell signaling, #14768	WB
BMI-1 (clone D20B7)	Rabbit, Cell Signaling, #6964	WB, IP
αTubulin	Mouse, GeneTEX, GT114	WB
DDB2 (clone D4C4)	Rabbit, Cell Signaling, #5416	WB
DDB1	Rabbit, Bethyl, A300-462A	WB
XPA	Mouse, Novus Biologicals, 25500002	WB, IF
XPF (clone 3F2/3)	Mouse, Santa Cruz, sc-398032	WB
CUL4B	Rabbit, Atlas Antibodies, HPA011880	WB
RBX-1 (clone D3J5I)	Rabbit, Cell Signaling, #11922	WB, IP

RAD23A (clone [EPR4818])	Mouse, Abcam, ab108592	WB
FLAG-tag (clone M2)	Mouse, Sigma, F3165	WB, IP
HA-tag (clone Y-11)	Rabbit, Santa Cruz, sc-805	WB, IP
HIS-tag	Rabbit, Cell Signaling, #2365	WB
ZRF1	Rabbit, selfmade, (Richly et al, 2010)	WB
XPD (clone 184.7)	Mouse, Santa Cruz, sc-101174	WB
CPD (clone TDM-2)	Mouse, Cosmo Bio or Kamiya	IF
Secondary antibodies		
Anti-rabbit IgG, HRP-conjugated	Goat, Cell signaling, 7074	WB
Anti-mouse IgG, HRP-conjugated	Goat, Cell signaling, 7076	WB
Anti-Rabbit IgG, Alexa Fluor® 488-conjugated	Goat, Life technologies, A-11008	IF
Anti-Mouse IgG, Alexa Fluor® 594-conjugated	Goat, Life technologies, A-11005	IF

Appendix 2 and Appendix 3

Appendices 2 and 3 are showing peptide numbers and protein names for all proteins identified in the mass spectrometry analysis after sequential immunoprecipitations with FLAG and RING1B antibodies (2) and of the purified UV–RING1B complex (3).

The full tables are available at:
http://jcb.rupress.org/content/suppl/2016/04/14/jcb.201506099.DC1/JCB_201506099_Tables.zip

Curriculum Vitae

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2011 – present
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Project “Functions of RING1B and ZRF1 in ubiquitin-mediated regulation of nucleotide excision repair”.

2009-2011
Student / Research assistant
Institute of Cytology and Genetics, SB RAS. Laboratory of Developmental Epigenetics.
Project “Epigenetic status of X-chromosomes in several lines of human induced pluripotent stem cells”

Education

2011-present
Institute of Molecular Biology, Mainz / Johannes Gutenberg University Mainz, Germany
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Conference participations

Gracheva E. et al., “RING1B and ZRF1 regulate DNA lesion recognition in Nucleotide Excision Repair” – DNA repair and genome stability in a chromatin environment, IMB conference, Mainz, Germany (presented in June 2015, poster report)

Gracheva E. and Richly H. “Role of RING1B ubiquitin ligase activity in UV-mediated DNA repair” - 8th epigenetics course – Institute Curie, Paris, France (presented in March 2012, poster report)

Gracheva E.A “Epigenetic status of X-chromosomes in several lines of human induced pluripotent stem cells” - XLIX International Scientific Student Conference, Novosibirsk State University, 2011 (presented in April 2011, oral presentation)

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Publications

Gracheva, E., Chitale, S., Wilhelm, T., Rapp, A., Byrne, J., Stadler, J., Medina, R., Cardoso, M.C., and Richly, H. (2016). ZRF1 mediates remodeling of E3 ligases at DNA lesion sites during nucleotide excision repair. The Journal of cell biology 213, 185-200.

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