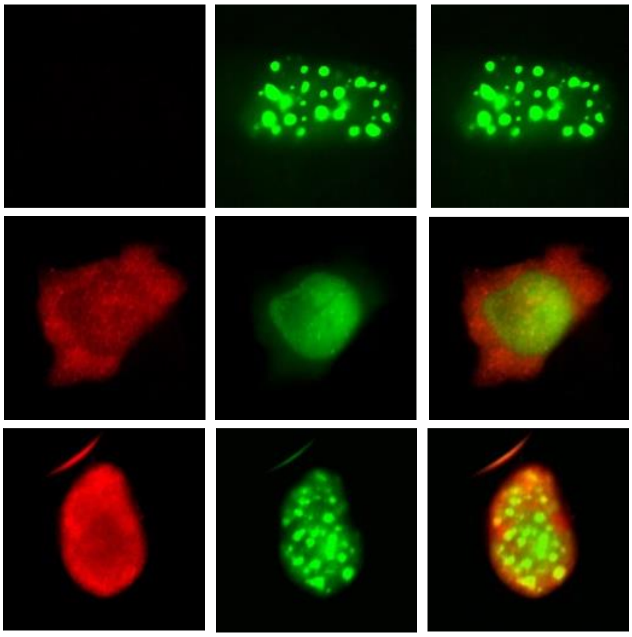
Universidad de La Laguna

# NOVEL REGULATORS OF POST-TRANSLATIONAL MODIFICATIONS IN THE DNA DAMAGE RESPONSE



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Certifica que:

La memoria presentada por la Licenciada en Biología Dña. Mª del Rocío Delgado Díaz Pérez titulada “Novel regulators of post-translational modifications in the DNA damage response” ha sido realizada bajo mi dirección en la Unidad de Investigación del Hospital Universitario de Canarias y, considerando que reúne las condiciones de calidad y rigor científico, autorizo para que pueda ser presentada y defendida ante la comisión nombrada al efecto para optar al grado de Doctor con Mención Internacional por la Universidad de La Laguna.

La Laguna, junio de 2016

V. Smits

Fdo: **Dra. Veronique AJ Smits**

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*To my parents and sister*

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# ABBREVIATIONS

| | |
|---|---|
| 53BP1 | p53-binding protein 1 |
| 9-1-1 | RAD9/HUS1/RAD1 |
| A-T | Ataxia Telangiectasia |
| ATM | Ataxia Telangiectasia mutated |
| ATR | Ataxia Telangiectasia- and Rad3- related |
| BLM | bloom syndrome helicase |
| BRCA1 | breast cancer type 1 susceptibility protein |
| BRCA2 | breast cancer type 2 susceptibility protein |
| CAK | CDK-activating kinase |
| Chk1 | checkpoint kinase 1 |
| Chk2 | checkpoint kinase 2 |
| CI | catalytic inactive |
| CDK | cyclin dependent kinase |
| CtIP | CtBP-interacting protein |
| Cyc | cyclin |
| DDR | DNA damage response |
| D loop | displacement loop |
| DNA-PKcs | DNA-dependent protein kinase catalytic subunit |
| Dox | doxorubicin |
| DSB | double-strand break |
| DUB | deubiquitin enzyme or deubiquitinase |
| ETP | etoposide |
| EV | empty vector |
| EXO1 | DNA exonuclease I |
| FA | Fanconi anemia |
| FHA | forkhead associated |
| HATs | histone acetyltransferases |
| HMTs | histone methyltransferases |
| HR | homologous recombination |
| HU | hydroxyurea |
| ICLs | interstrand cross-links |
| IDLs | insertions/deletions |
| IR | ionizing radiation |
| IRIF | ionizing radiation-induced foci |
| LIG1 | DNA ligase I |
| LIG3 | DNA ligase III |
| LIG4 | DNA ligase IV |

| | |
|---|---|
| MIU | motif interacting with ubiquitin |
| MMS | methyl methanesulfonate |
| MPF | M phase promoting factor |
| MRN | MRE11/RAD50/NBS1 |
| NBS | Nijmegen breakage syndrome |
| NEM | N-ethylmaleimide |
| NHEJ | non-homologous end joining |
| PARI | PCNA-associated recombination inhibitor |
| PCNA | proliferating cell nuclear antigen |
| Phleo | phleomycin |
| PIKKs | phosphatidylinositol 3-kinase-like kinase family |
| PIP | PCNA-interacting protein |
| Pre-RC | pre-replication complex |
| Rb | retinoblastoma protein |
| ROS | reactive oxygen species |
| RPA | replication protein A |
| SSB1 | single-stranded DNA-binding protein 1 |
| SSB2 | single-stranded DNA-binding protein 2 |
| SSBs | single-stranded breaks |
| ssDNA | single stranded DNA |
| SUMO | small ubiquitin-like modifier |
| TLS | translesion synthesis |
| Ub | ubiquitin |
| UBD | ubiquitin binding domain |
| UBM | ubiquitin binding motif |
| UDR | ubiquitination-dependent recruitment domain |
| UV | ultraviolet light |
| WRN | werner syndrome RecQ helicase-like |
| WT | wild type |
| XP | Xeroderma pigmentosum |

# I. INTRODUCTION

## 1. CELL CYCLE CONTROL

The cell cycle is an extensively studied biological process that covers a range of events leading to cell division into two daughter cells. A crucial aspect of the cell cycle is the correct replication and transmission of the DNA to daughter cells. DNA replication is a meticulously planned process that takes place during the S phase of the cell cycle. It presents a carefully orchestrated program, starting at specific sites called replication origins and following a temporal progression. Progression through the cell cycle is regulated by cyclin-dependent protein kinases (CDKs), whose activity is controlled by molecular modulators, including associating proteins Cyclins and other activators and inhibitors. As the expression of CDKs is largely unchanged during the cell cycle, it is the timed expression and degradation of the Cyclins that is important for the scheduled activity of Cyclin/CDK complexes. Transition from one cell cycle phase to the other is controlled by timed activation of different Cyclin/CDK complexes. CDKs phosphorylate a range of substrates, most of them proteins involved in cell cycle progression (1) .

Most cells in adult mammals remain in a quiescent state or G0 phase, from which they can restart their mitotic cell cycle through the action of Cdk4/Cdk6, upon transcriptional induction of Cyclin D in response to mitogenic stimuli (Figure 1). These kinases phosphorylate and thereby inactivate the retinoblastoma protein (Rb), an adaptor protein that represses transcription. In human cells, Rb contains 13 conserved sites that are phosphorylated by CDKs in proliferating cells. CDK-dependent inactivation of Rb results in de-repression of multiple genes encoding proteins required for DNA synthesis (S phase) or mitosis (2). The next Cyclin-CDK complex, Cyclin E/Cdk2, then drives cells from G1 phase into S phase, when the Cyclin A/Cdk2 complex takes over. After completion of DNA replication, Cdk1 becomes activated by Cyclin A, and subsequent activation of the Cyclin B/Cdk1 complex triggers entry into mitosis (Figure 1). During S and M phase, the Cyclin/CDK complexes also phosphorylate and thereby inhibit proteins required to initiate DNA replication (1).

The Cyclin/CDK complexes itself are also regulated by phosphorylation and dephosphorylation. The CDK-Activating Kinase (CAK) is formed by Cdk7 associated with Cyclin H and activates CDKs through phosphorylation of the Thr160 in the CDK activation loop (T-loop) (3). Phosphorylation of CDK Thr14 and Tyr15 residues by Wee1 and Myt1 kinases inhibits CDK activity, keeping the complex in a temporarily inactive state, also under conditions of DNA damage. Inhibitory phosphorylation at Thr14 and Tyr15 does not result in major changes in the CDK structure, but inhibits the CDK activity by reducing the affinity of the CDK for its substrates. Elimination of these phosphates by phosphatases of the Cdc25 family is then required for activation of CDKs and subsequent cell cycle progression (4). CDKs are also negatively regulated by binding to small proteins of the INK4 or Cip/Kip families of inhibitors. INK4 proteins (p16, p15, p18 and p19) are specific for the Cdk4/6 subfamily and interact with monomeric CDKs. They function by distorting the cyclin interface and the ATP-binding pocket, thus preventing activation of Cdk4 and Cdk6 by Cyclin D or by CAK (5).

Members of the Cip/Kip family of inhibitors (p21, p27 and p57) contact both the CDK and cyclin subunits and thereby inhibit CDK-cyclin heterodimers, giving additional levels of regulation once these complexes have already formed (6).

Chromosomal replication begins at discrete locations named DNA replication origins, in two consecutive steps. First, pre-replication complexes (pre-RCs) are formed at potential replication origins in an ATP dependent process, termed origin licensing. Second, selected pre-RCs are activated by kinases (CDKs and Dbf4-dependent kinases) to initiate DNA replication. Pre-RCs only form during the G1 period of the interphase, which is characterized by suppressed CDK activity (7; 8). The entry into mitosis or M phase is triggered subsequently by the activation of Cdk1/Cyclin B, which promotes mitosis by phosphorylating many downstream mitotic proteins, including other protein kinases such as Aurora and Polo-like kinase, in all eukaryotic cells (9).

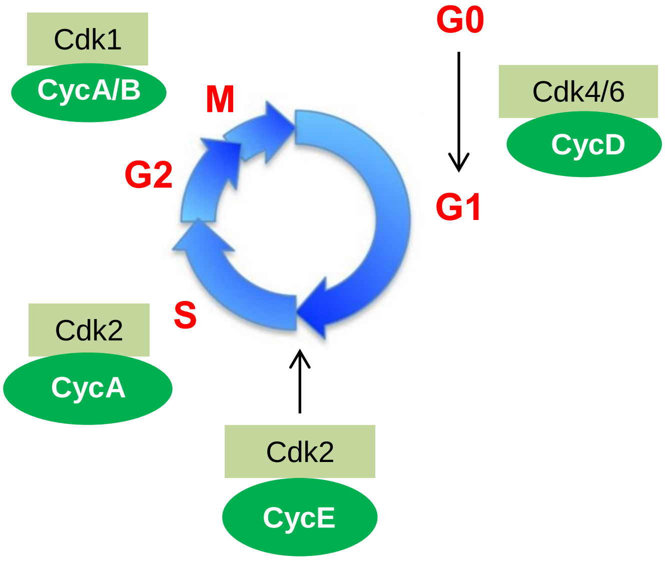


**Figure 1: Timed activation of Cyclin/Cdk complexes regulates cell cycle progression.** See text for details.

## 2. DNA DAMAGE CHECKPOINT RESPONSE

Cells are subjected to thousands of DNA lesions inflicted every day. These DNA damages can be caused exogenously by exposure to different types of radiation or genotoxic agents, or endogenously through, for example, base depurination and deamination or reactive products of cellular metabolism (10). Without proper care, the consequent DNA lesions can lead to alteration of the genomic structure, affecting the faithful transmission of genetic information. If unrepaired or wrongly repaired, such lesions may be lethal to the cell or give rise to mutations that can affect cell viability or induce aberrant cellular behaviour leading to the development of malignancies such as cancer. During evolution, organisms have acquired mechanisms responding to and repairing DNA damage, assuring the faithful transmission of genetic information and thereby the maintenance of genome stability. DNA damage checkpoints are important mechanisms that, in the event of DNA lesions, actively stop the cell cycle until DNA repair is complete or, under certain circumstances, trigger apoptosis. Both processes prevent passing on the damaged DNA to the daughter cells. During the last decade, complex connections between the key kinases ATM and ATR in the pathway and other checkpoint proteins have been demonstrated. A relationship between mechanisms of damage checkpoint pathways and DNA damage repair to maintain genomic stability were also discovered.

### 2.1. DNA damage signalling pathways

The complex network of signalling pathways that orchestrates the detection and repair of DNA damage with a cell cycle arrest is known as the DNA damage response (DDR) (11). The DDR signalling pathways are kinase cascades in which mediator proteins facilitate the phosphorylation events. Substrates of the DDR kinases are effector proteins that participate in numerous cellular processes important for genome stability, such as DNA replication, DNA repair and cell cycle control.

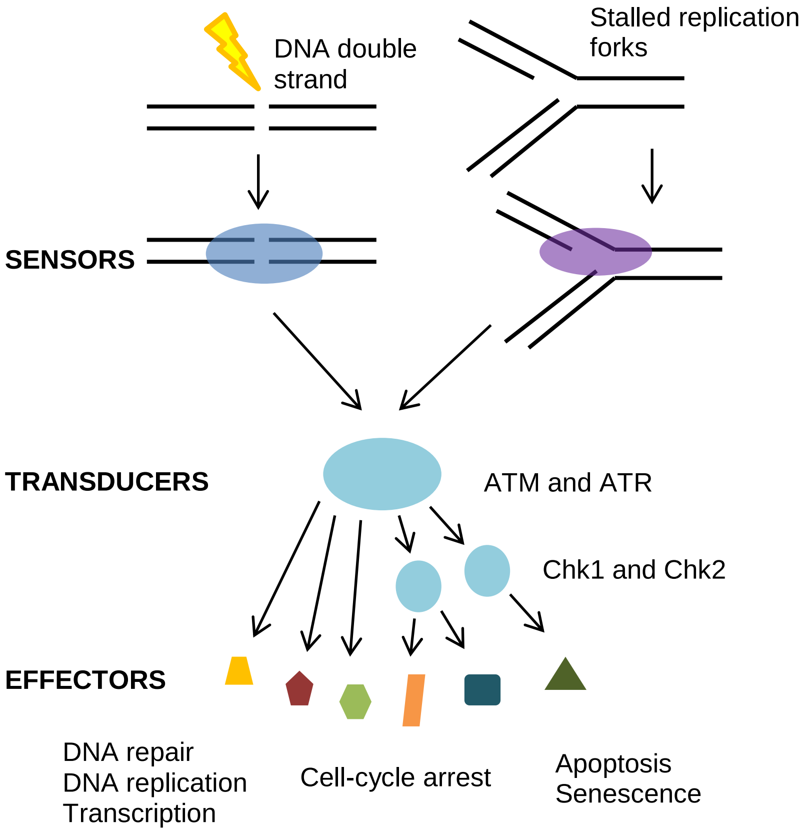

DNA double
strand
Stalled replication
forks
SENSORS
TRANSDUCERS
ATM and ATR
Chk1 and Chk2
EFFECTORS
DNA repair
DNA replication
Transcription
Cell-cycle arrest
Apoptosis
Senescence

**Figure 2: The framework of the DDR signalling pathway.** The DDR signalling pathway consists of signal sensor, transducer, and effector proteins. The sensors of this pathway are proteins that recognize DNA structures induced by DNA damage and DNA replication stress. The transducers of this pathway are the ATM and ATR kinases. The effectors of this pathway are substrates of ATM and ATR, for example Chk1 and Chk2, involved in a wide spectrum of cellular processes that are important for maintenance of genomic stability of organisms.

### 2.1.1. ATM-dependent pathway

Among the different types of DNA lesions, DNA double-strand breaks (DSBs) are the most harmful. Unrepaired or misrepaired DSBs can result in loss of genetic information, potentially harmful mutations or chromosomal rearrangements, which can lead to cancer development. DSBs can arise intrinsically through the collapse of stalled replication forks or extrinsically through exposure to ionizing radiation (13).

ATM is the central kinase involved in the response to DNA DSBs. The recruitment of ATM to sites of DNA DSBs is mediated through the MRE11/RAD50/NBS1 (MRN) complex (Figure 3). The MRN complex rapidly assembles at sites of DSBs, where it acts as a damage sensor (14). ATM recruitment requires binding of ATM to the C-terminus of NBS1, an interaction that enhances the kinase activity of ATM (15). Irradiation triggers ATM autophosphorylation at Ser1981, which results in the dissociation of ATM dimers into monomers and starts ATM kinase activity. It is hypothesized that ATM activation is not the direct consequence of the DNA lesion but rather of chances in chromatin structure (16).

Interestingly, MRN complex components not only modulate the activity of ATM, but are also ATM targets. Phosphorylation of NBS1 and Rad50 by ATM was shown to be necessary for the intra-S phase checkpoint mediated by SMC1 and ATM-dependent phosphorylation of Mre11 initiates signalling to control the extent of resection during homologous recombination (HR) repair and provides the signal for Mre11 to dissociate from DNA at later time points after damage (17; 18; 19). This way, ATM and the MRN complex work together at the sites of DNA DSBs for a tight regulation of localization of DDR factors and effective DNA repair.

Immediately after its recruitment to sites of DNA DSBs, ATM phosphorylates histone H2AX on Ser139 (γH2AX) in the chromatin around the break (20). H2AX phosphorylation starts a cascade that assembles DDR components at the breakage site (21). The primary role of the phosphorylation of H2AX is to provide a high affinity binding site for protein MDC1 (Figure 3), responsible for the recruitment of many other factors to the damaged chromatin, which can be observed by immune-fluorescence microscopy as nuclear foci. MDC1 has two tandem repeats of BRCT domains (phospho-protein binding domains) at the C-terminus that bind phosphorylated H2AX. The MDC1-H2AX interaction also protects γH2AX from dephosphorylation (22). MDC1 also harbours a binding site for the NBS1 component of MRN complex, promoting additional MRN and ATM recruitment and thereby ATM kinase activation (23; 24). The capability of MDC1 to bind γH2AX and NBS1 simultaneously promotes positive feedback phosphorylation of H2AX by ATM and generates an enormous γH2AX response surrounding DSBs (25).

After its recruitment to the DSB, MDC1 is phosphorylated by ATM. The forkhead associated (FHA) domain (phospho-peptide binding motif) of the E3 ubiquitin ligase RNF8 binds phosphorylated MDC1 and is thereby recruited to the DSBs (Figure 4). RNF8 was shown to ubiquitinate phosphorylated H2AX, creating docking sites for ubiquitin-binding proteins (26; 27). RNF8 partners with the UBC13-Mms2 complex, an E2 that builds polyubiquitin chains via Lys63 linkage (28). An additional E3 ligase, RNF168, is recruited to RNF8-mediated ubiquitinated H2AX, and amplifies the accumulation of ubiquitin conjugates around the lesion (Figure 4) (29; 30). Among these ubiquitin-binding proteins are 53BP1 (p53-binding protein 1) and BRCA1 (breast cancer type 1 susceptibility protein), critical in DSB repair by non homologous end joining (NHEJ) and HR respectively. Multiple regulators were described to control RNF8 and RNF168-mediated ubiquitination of H2A(X). Six ubiquitin hydrolases were shown to counteract the action of RNF8 and RNF168 on H2A(X) ubiquitination: USP3, USP16, USP44, BRCC36, OTUB1 and DUB3 (31; 32; 33; 34; 35; 36). In addition, two E3 ubiquitin ligases (TRIP12 and UBR5) were demonstrated to target RNF168 for proteasome-mediated degradation (37).

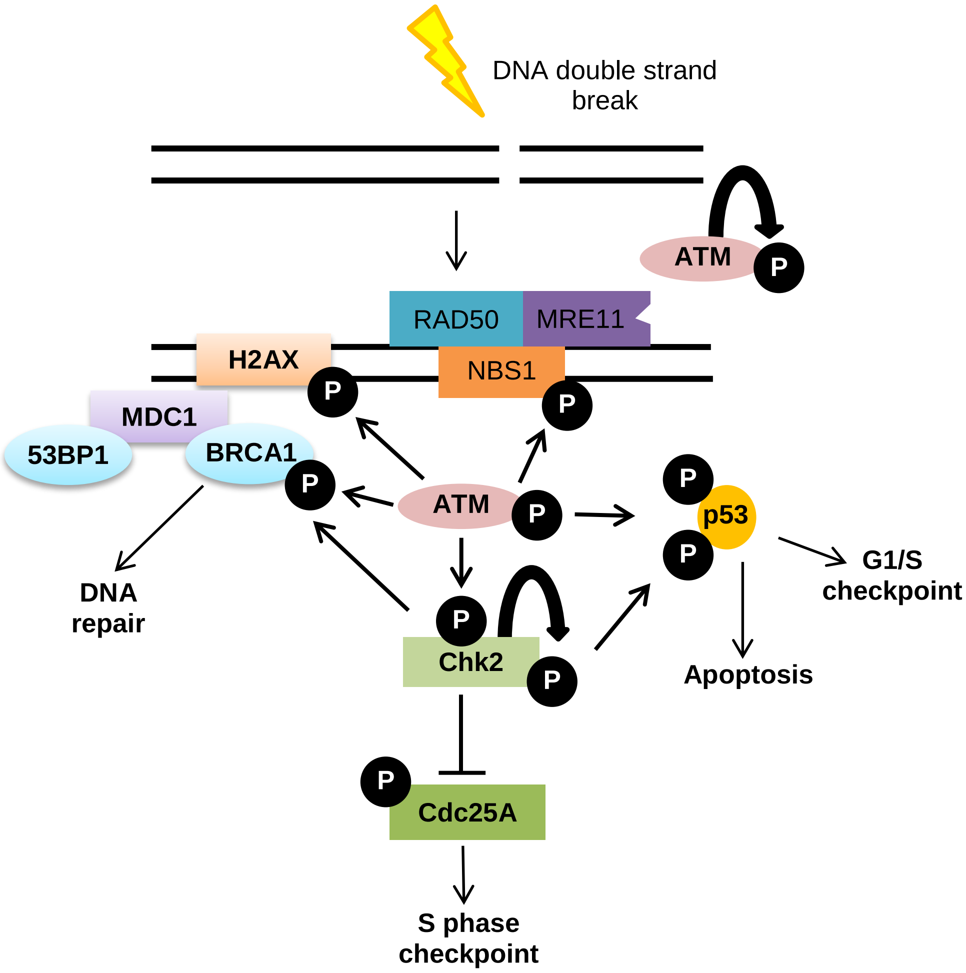

DNA double strand break
ATM
P
RAD50
MRE11
H2AX
NBS1
MDC1
53BP1
BRCA1
ATM
p53
G1/S checkpoint
DNA repair
Chk2
Apoptosis
Cdc25A
S phase checkpoint

**Figure 3: ATM-dependent pathway.** DSB formation stimulates ATM autophosphorylation but full activation additionally requires recruitment to sites of damage in conjunction with the MRN complex. ATM then phosphorylates multiple substrates including the downstream effector kinase Chk2.

In addition, HERC2, another E3 Ub ligase, was shown to bind RNF8 and facilitate binding of RNF8 with its E2 partner UBC13 and the accumulation of Ub chain formation (38). Interestingly, yet another E3 ligase, RNF169, has an unexpected negative role in regulating this pathway by directly binding to RNF168-modified chromatin, thereby physically limiting the recruitment of 53BP1 and BRCA1 (39). Both HERC2 and RNF168 are specifically modified by SUMO1 at DSB sites in a manner dependent on the SUMO E3 ligase PIAS4. SUMOylation of HERC2 was shown to be required for its DSB-induced association with RNF8 and for stabilizing the RNF8-UBC13 complex (40). PIAS4-mediated SUMOylation impacts directly on the activity of RNF168 ubiquitin ligase (41). Together these data show that several enzymes together constrain the signalling response around the damaged site, and may in this way terminate the signal after completion of repair.

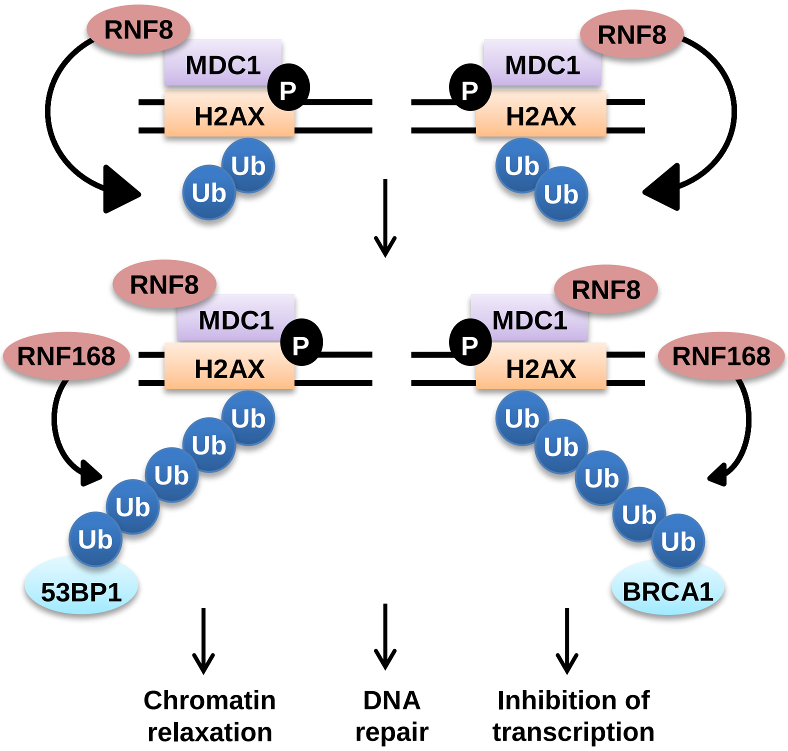


**Figure 4: Damage-dependent hierarchical localization of proteins in IR-induced foci.** DSBs trigger H2AX phosphorylation, leading to accumulation of MDC1 and other MDC1-binding proteins. These include the E3 ubiquitin ligases RNF8 and RNF168 that initiate ubiquitination of H2A(X) at sites of DNA damage. This chromatin modification allows a second wave of protein accumulation, including 53BP1 and BRCA1.

As mentioned before, ATM plays an essential role in the activation of the G1/S and intra-S phase cell cycle checkpoints, in which the ATM substrates are the cell cycle proteins that execute the checkpoint arrest. In the G1/S checkpoint this is the tumor suppressor p53, a transcription factor that drives the expression of p21, but also of several genes that are involved in the induction of apoptosis (42). ATM substrates in the intra-S phase checkpoint are NBS1, cohesion protein SMC1 and effector kinase Chk2. The latter triggers ubiquitin-dependent degradation of the S phase-promoting phosphatase Cdc25A (43). Important additional information about the extensive function of ATM came from screenings for the identification of ATM substrates by searching for ATM consensus phosphorylation motifs. In these screenings numerous novel DDR proteins were discovered, which underscore the complexity of the ATM-mediated DDR pathway (44).

### 2.1.2. ATR-dependent pathway

In contrast to ATM, ATR activity is mostly triggered by single-stranded DNA (ssDNA) structures, which appear at resected DNA DSBs, in response to ultraviolet (UV) light or at stalled replication forks.

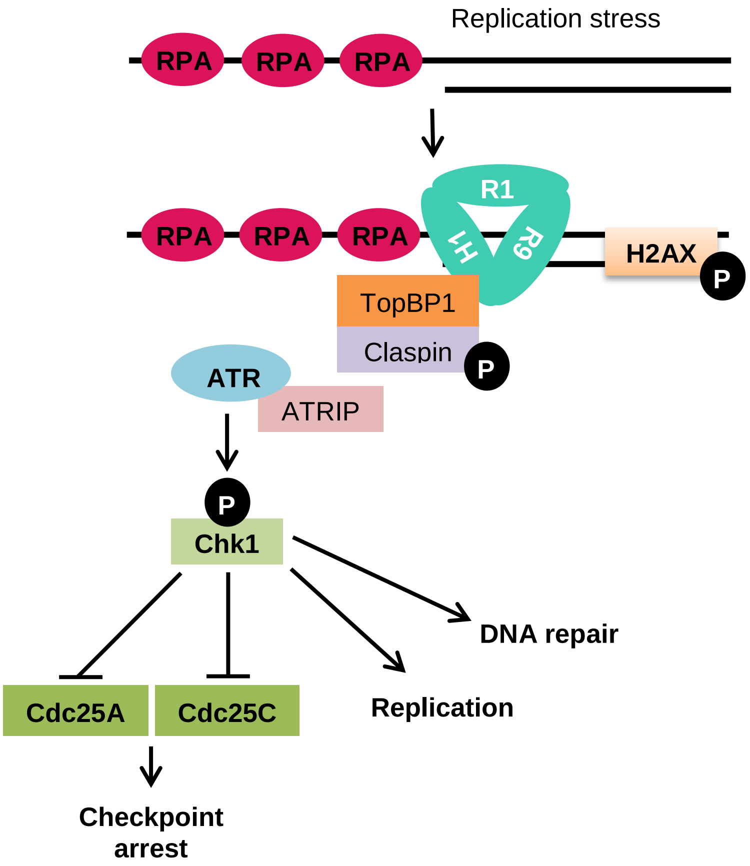

Replication stress
RPA
RPA
RPA
RPA
RPA
RPA
R1
H1
R9
H2AX
P
TopBP1
Claspin
P
ATR
ATRIP
P
Chk1
DNA repair
Replication
Cdc25A
Cdc25C
Checkpoint arrest

**Figure 5: ATR-dependent pathway.** ATR is recruited to tracts of ssDNA-RPA through its interacting partner, ATRIP, where it phosphorylates and activates Chk1 in conjunction with the TopBP1 and Claspin mediator proteins.

When DNA polymerases stall during DNA replication, the replicative helicases continue to unwind the DNA ahead of the replication fork, leading to the generation of long stretches of ssDNA, which are then bound by the single-strand binding protein complex RPA (45). ATR interacting partner (ATRIP) directly binds RPA-bound ssDNA and thereby recruits ATR to these sites of damage (46). Furthermore, RPA-ssDNA complexes promote the independent binding of the RAD17/RFC2-5 clamp loader complex to the damage sites, which is responsible for the loading of the RAD9/HUS1/RAD1 (9-1-1) heterotrimer onto the chromatin (47). Both Rad17 and Rad9 are ATR substrates (48). The 9-1-1 complex subsequently recruits TopBP1 via binding of the BRCT domains of TopBP1 to phosphorylated RAD9. The TopBP1 activation domain then binds ATR, thereby stimulating ATR kinase activity and facilitating ATR to recognize its substrates (49; 50). Once activated, ATR phosphorylates downstream targets to induce DNA repair, stabilisation and restart of stalled replication forks and cell cycle arrest (51; 52; 53). Many of these functions are regulated via the ATR downstream target kinase Chk1 (Figure 5) (54).

The activation of Chk1 by ATR is dependent on mediator protein Claspin, that, in a phosphorylated state, binds Chk1 and also mediates its activation (55). ATR phosphorylates Chk1 kinase on Ser317 and Ser345 and these phosphorylation events are required for the G2/M checkpoint arrest (56; 57). Active Chk1 phosphorylates the Cdc25A and Cdc25C phosphatases, leading to ubiquitin-mediated proteasomal degradation of Cdc25A and nuclear export and inactivation of Cdc25C (Figure 5) (58; 59; 54).

### 2.1.3. Interplay between ATM and ATR pathways

Although ATM and ATR are activated by different types of DNA lesions and act, in principle, in different pathways, their downstream targets and the mediated responses are partially overlapping (60). These kinases share substrate specificity as they both phosphorylate serine or threonine residues followed by glutamine (SQ/TQ motifs) (44; 61). As mentioned before, a proteomic study analysing proteins phosphorylated on consensus sites recognized by ATM and ATR in response to DNA damage identified over 700 putative targets (44). Some of these targets, like p53 and H2AX have been shown to be common targets of both kinases.

Also Chk1, considered the most specific ATR downstream target, can be phosphorylated by ATM on Ser317 and Ser345 in response to ionizing radiation (IR) (54; 60). Importantly, DNA DSBs, that trigger the ATM pathway, are processed leading to single-strand overhangs that, in turn, activate ATR (62). Such DNA lesions therefore activate both pathways: first and directly ATM and at later time points, after processing, ATR (Figure 6). In this way, ATM and ATR collaborate in mediating the cellular responses to a diverse range of DNA lesions.

ATM is considered the principal mediator of the G1/S checkpoint, while the intra-S phase and G2/M checkpoints are generally primarily executed by ATR. However, ATM also participates in the activation and maintenance of intra-S and G2 cell cycle arrest. These data suggest a functional overlap of ATM and ATR signalling in checkpoint activation (63).

In the intra-S phase checkpoint for example, ATM and ATR both target the Cdc25A phosphatase for ubiquitin-dependent degradation thereby regulating the timing of replication origin firing in response to DNA damage (43; 64). ATM has also been demonstrated to be involved in phosphorylation of Cdc25C via Chk2, thereby collaborating to the activation of the G2/M checkpoint (65). In conclusion, ATM and ATR signalling pathways are not overall redundant, but partially overlapping instead (Figure 6). Defects in one pathway can therefore, at least in part, be compensated by the other pathway.

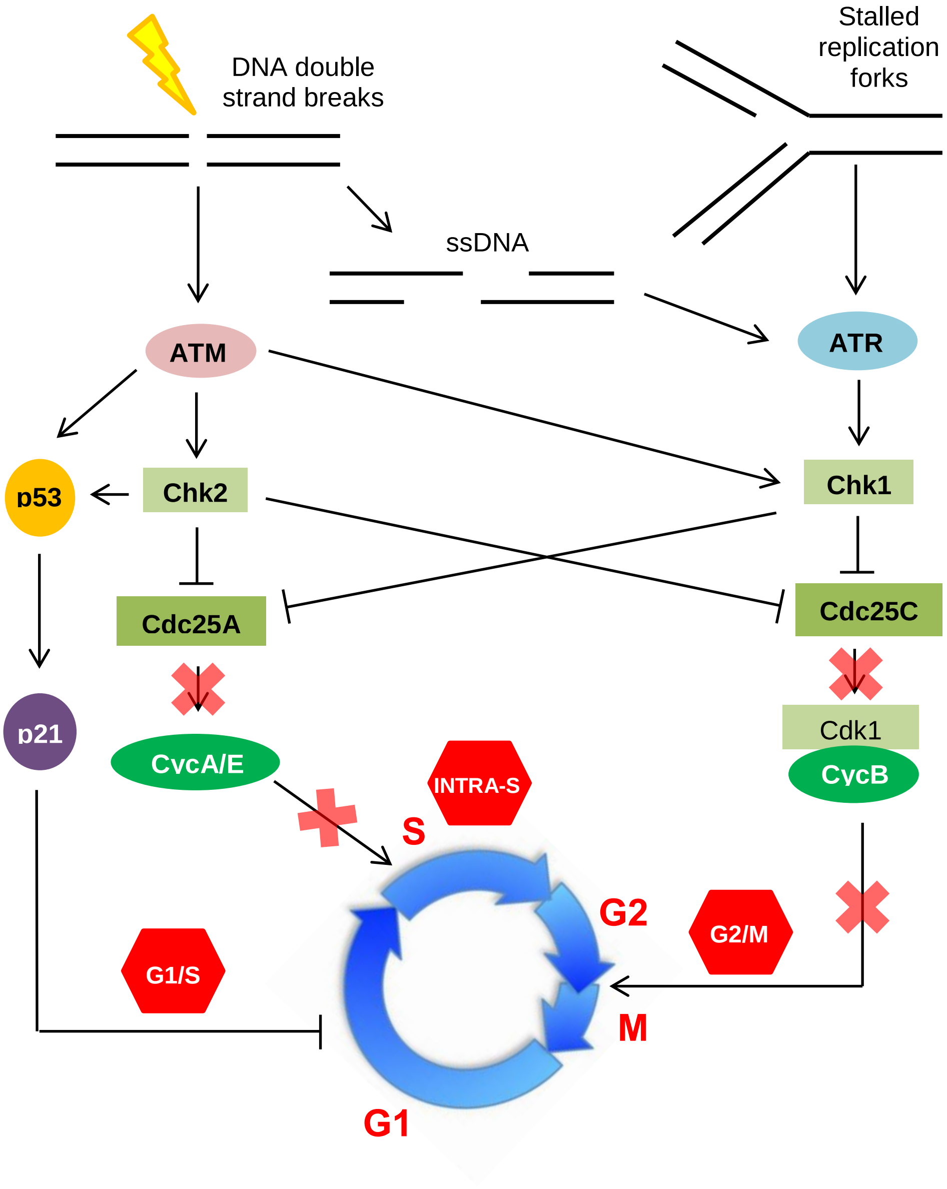

DNA double strand breaks
Stalled replication forks
ssDNA
ATM
ATR
p53
Chk2
Chk1
Cdc25A
Cdc25C
p21
CycA/E
Cdk1
CycB
INTRA-S
S
G2
G2/M
G1/S
M
G1

**Figure 6: ATM- and ATR-dependent checkpoint signalling.** ATM is activated by the presence of DSBs whereas ATR is activated by ssDNA after DSBs processing or in DNA replication stress. Cell cycle progression is subsequently inhibited by ATM- and ATR-dependent phosphorylation of p53, Chk1 and Chk2. The G1/S cell cycle arrest is primarily mediated through a p53-dependent increase in p21. Main targets of Chk1/2 include the Cdc25 phosphatases.

## 2.2. Cell cycle checkpoints

### 2.2.1. G1/S checkpoint

In response to DNA damage, the G1/S checkpoint avoids cells from entering in S phase by inhibiting the initiation of DNA replication. p53 is essential for the G1/S DNA damage checkpoint but has also been implicated in several other cellular pathways including apoptosis (66). Loss of p53 function has been widely implicated in genomic instability and tumorigenesis (67). p53 is modified by phosphorylation and acetylation, which control both p53 protein levels and transactivation activity, respectively (68).

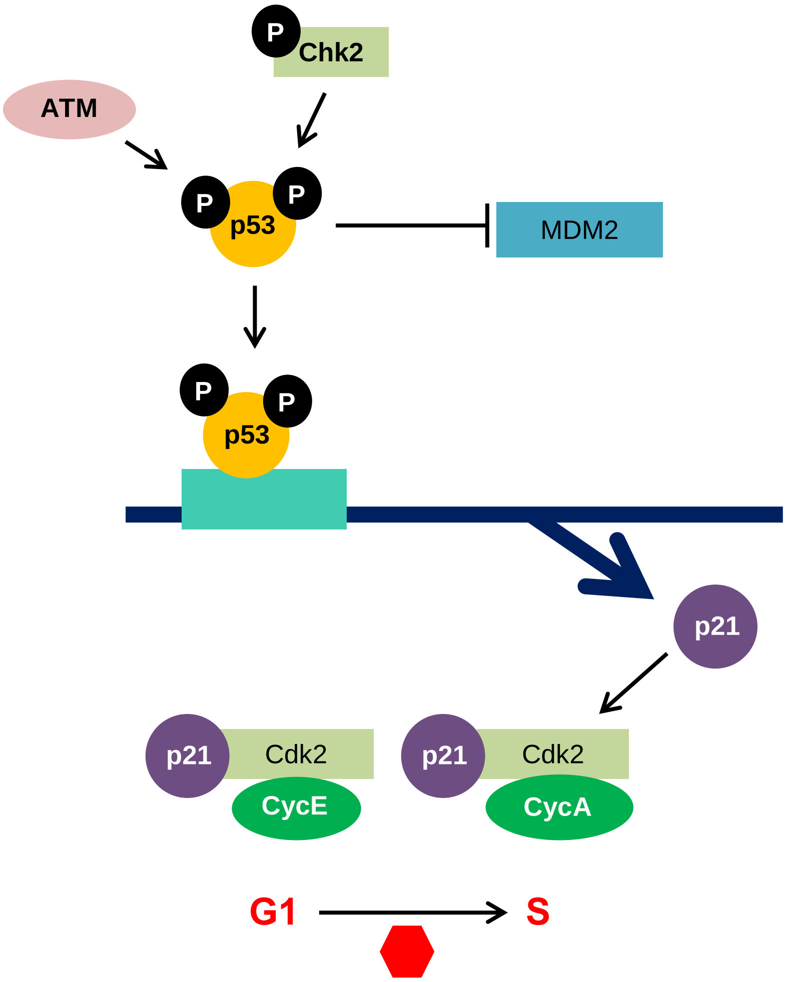


**Figure 7: G1/S DNA damage checkpoint mediated cell cycle arrest.** Following DNA damage, p53 is phosphorylated at Ser15 and Ser20 by ATM and Chk2, respectively. This results in the displacement of MDM2 and mediates both the stabilisation and accumulation of p53. p53 is free to activate transcription of its target genes, including p21, which inhibits Cyclin E/Cdk2 and Cyclin A/Cdk2, leading to cell cycle arrest and inhibition of DNA replication.

Checkpoint kinases ATM and Chk2 have essential roles in the regulation of p53. Under normal conditions, p53 is an unstable protein. p53 binds E3-ubiquitin ligase MDM2, which targets p53 for ubiquitin- and proteasome-mediated degradation (69). Upon DNA damage, p53 is phosphorylated on Ser20 by Chk2 and by ATM on Ser15 (Figure 7) (70). These phosphorylation events inhibit MDM2 binding, resulting in p53 stabilisation, accumulation in the nucleus, and activation of transcription of p53-responsive genes involved in the cell cycle checkpoint. Between these genes is MDM2 itself, ensuring the temporal nature of this response (71). For executing the G1/S DNA damage checkpoint, induction of the CDK inhibitor p21 by activated p53 is critical. p21 inhibits both Cyclin E/Cdk2 and CyclinA/Cdk2 complexes, required for entry into and pass through G1 and S phase, respectively. Triggering p21 expression in response to DNA damage thereby leads to a cell cycle arrest (Figure 7) (72).

### 2.2.2. Intra-S phase checkpoint

In the intra-S phase checkpoint, the presence of DNA lesions transiently slows DNA synthesis. Activation of ATM triggers two independent branches: the NBS1/SMC1 branch and the Chk2/Cdc25A branch (Figure 8). In the first pathway, NBS1 serves as an adapter protein helping ATM to phosphorylate SMC1, a protein required for sister chromatin cohesion that also is involved in the DDR, at Ser957 and Ser966 by ATM kinase. These phosphorylation events are required to the arrest of DNA replication and for cell survival after damage (73). In addition, ATM phosphorylates and thereby activates Chk2, which in turn phosphorylates Cdc25A on Ser123, Ser178 and Ser292. Phosphorylation of Cdc25A triggers proteasome-dependent degradation of the phosphatase, thereby preventing Cyclin E/CDK2 and Cyclin A/CDK2 activation and progression through S phase. (74).

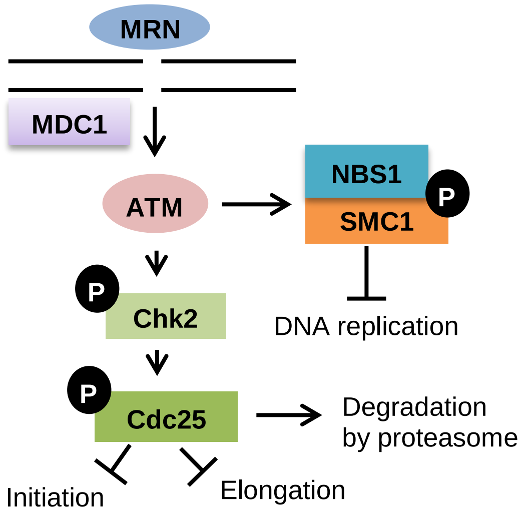


**Figure 8: Intra-S phase checkpoint signalling.** DNA lesions are detected by MRN complex and ATM is recruited and activated. ATM phosphorylates SMC1, inhibiting DNA replication. ATM also phosphorylates Chk2, an effector kinase that triggers Cdc25A phosphorylation and degradation and thereby inhibits DNA replication initiation and elongation.

### 2.2.3. G2/M checkpoint

Cells that suffer DNA damage in G2 phase, or have escaped from earlier checkpoints with DNA lesions are stopped at the G2/M checkpoint. As described before, the primary complex responsible for the transition from G2 to M is the Cyclin B/Cdk1 complex. Cdc25 phosphatases, that trigger Cyclin B/Cdk1 activation, are the primary target of this checkpoint. Activation of ATM/ATR leads to phosphorylation and activation of Chk1 and Chk2 effector kinases. Chk1 and Chk2 phosphorylate Cdc25 phosphatases that are then inhibited by different mechanisms. Cdc25A is phosphorylated on Ser123, Ser178 and Ser292 and then degraded by the proteasome, whereas Cdc25C is phosphorylated on Ser216 by Chk1, which creates a binding site for 14-3-3 proteins (58; 64; 75; 76). The binding of Cdc25C to 14-3-3 proteins facilitates its export from the nucleus and subsequent cytoplasmic sequestration, thereby inhibiting its ability to dephosphorylate and activate nuclear Cdk1, which in turn prevents entry into mitosis (Figure 9) (77; 78).

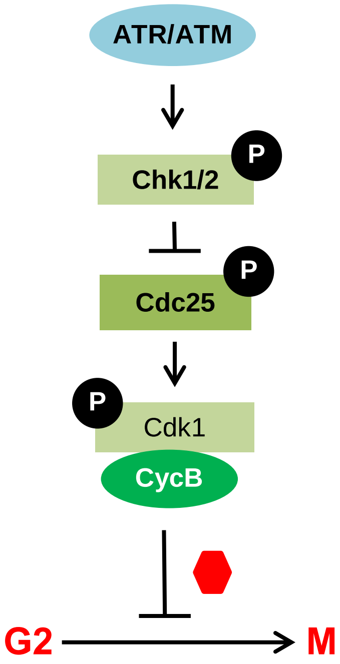


**Figure 9: G2/M DNA damage checkpoint.** Following DNA damage, Chk1/2 is phosphorylated by ATR/ATM. Chk1/2 in turn phosphorylates Cdc25, leading to the inactivation of the phosphatase, thereby prevention activation of the CycB/Cdk1 complex. This results in an arrest of the cell cycle prior to mitosis.

## 3. DNA REPAIR

Every cell experiences up to 105 spontaneous DNA lesions per day. Such DNA alterations can originate from dNTP misincorporation during DNA replication, interconversion between DNA bases caused by deamination, loss of DNA bases following DNA depurination and modification of DNA bases by alkylation (10). In addition, programmed genome alterations exist in vertebrates such as V(D)J recombination, class-switch recombination and somatic hyper-mutation (79). These occur in developing B and T lymphocytes to generate immunoglobulin and T-cell receptor diversity, allowing effective recognition of diverse pathogens and antigens.

Environmental DNA damage can be produced by physical agents, like IR and UV light or by chemicals, like those used in cancer chemotherapy (80). To protect against such DNA lesions, cells are equipped with repair mechanisms specific for many types of lesions. DNA repair is carried out by enzymatic activities that modify DNA to repair the lesions including nucleases, helicases, polymerases, topoisomerases, recombinases, ligases, glycosylases, demethylases, kinases and phosphatases. DNA repair is tightly regulated, to recruit and activate the right factors in the right place at the right time.

### 3.1. Double Strand Break Repair

As mentioned before, two pathways exist to repair DNA DSBs: HR and NHEJ. HR restores the original DNA sequence at DSB sites using a template sequence from a sister chromatid or a homologous chromosome to direct the error-free repair of DSBs, and is therefore restricted to the S and G2 phases of the cell cycle. In addition to DSB repair, HR is also involved in the resolution of stalled replication forks and in the generation of genetic diversity through mitotic and meiotic recombination (81). By contrast, NHEJ directly joins the two ends of a DSB, regardless of the sequence template at the exposed ends of the break, making it an error-prone pathway, but is available at all times during the cell cycle. NHEJ is also involved in the maturation of immune cells through V(D)J recombination and class-switch recombination (82).

#### 3.1.1. Homologous Recombination (HR)

As mentioned, DSB repair pathway choice is mainly controlled by the availability of a homologous template. DSB end resection, a process that involves 5’ to 3’ nucleolytic degradation of DSB ends to produce a 3’ tail, is an additional key determinant of DSB repair pathway choice, which compromises cells to HR and is additionally required for activation of the ATR-mediated checkpoint response (83; 84). DSB resection is therefore tightly regulated during the cell cycle. An early component for DNA resection is the MRN complex in conjunction with CtIP (Figure 10) (85; 62). The initial resection is carried out by the endonuclease activity of Mre11, followed by

Mre11 exonuclease activity (86). CtIP continues this resection through interaction with MRN and stimulation of Mre11, most likely its endonuclease activity (62).

CtIP is controlled in several ways, among which cell cycle-dependent regulation. The level of CtIP protein is suppressed by proteasome-mediated degradation in G1 phase, which is eased as cells enter S phase (87). During S and G2 phases, CtIP expresses well and is phosphorylated by CDK on multiple sites, promoting resection in distinct ways. Ser327 phosphorylation is necessary for the CtIP-BRCA1 interaction and Thr847 for the localization of CtIP to DSBs and end resection interaction (85; 88). Long-range resection is subsequently carried out by two alternative pathways that require either the exonuclease function of EXO1 (DNA exonuclease I) alone, or the helicase function of BLM (bloom syndrome helicase) in concert with the nuclease function of DNA2 (DNA replication helicase 2) (89; 90; 91). It has been demonstrated that CDK1/2 promotes long-range resection in mammalian cells by directly phosphorylating EXO1 on four different sites (92).

Cells deficient in BRCA1 are radiation sensitive and display a reduction in HR. Although the precise role of BRCA1 in HR is not known, it is thought to be involved in multiple steps of the repair pathway by forming at least three mutually exclusive complexes: Abraxas, BACH1 and CtIP (93). BRCA1 is located to DSBs sites in the form of the BRCA1-Abraxas complex (93). The BRCA1/BARD1 complex subsequently acts as an E3 ubiquitin ligase inducing the formation of conjugated ubiquitin structures during S phase and in response to genotoxic stress (94; 95). SUMOylation of BRCA1 mediated by PIAS1 and PIAS4 SUMO ligases promotes the recruitment of the BRCA1/Abraxas/RAP80 complex in response to genotoxic stress, and induces the ubiquitin ligase activity of BRCA1 (41; 96).

Additional regulation of DSB repair pathway choice comes from the actions of 53BP1 and RIF1. In G1 phase, RIF1 is located to DSB sites through ATM-dependent 53BP1 phosphorylation, and the 53BP1-RIF1 complex protects the DNA ends from 5´end resection and BRCA1 recruitment. In this way, NHEJ is stimulated. In addition, in S and G2 phases, CDK- and ATM-dependent phosphorylation of CtIP (CtBP-interacting protein) support the formation of the CtIP/MRN/BRCA1 complex which displaces RIF1 at break sites to promote DNA resection and DNA repair by HR (97; 98).

The 3’ tail formed by end resection is coated and stabilized by RPA, which prevents ssDNA from forming secondary structure (Figure 10). RPA is then displaced by the recombinase RAD51. This loading of RAD51 is a crucial step in HR, as it generates a nucleoprotein filament that searches for and invades a nearby homologous DNA template (usually a sister chromatid). The second strand of the sister chromatid is displaced and a transient structure known as D (displacement) loop is formed (83). The loading of RAD51 onto ssDNA is promoted and controlled by multiple mechanisms (Figure 10) (83). BRCA2 is the main recombination mediator that facilitates the loading of RAD51 onto ssDNA by overcoming the inhibitory activity of RPA (99). PALB2, a partner of BRCA2, acts as a molecular adaptor between BRCA1 and BRCA2 to form the BRCC complex (100; 101). In this complex, BRCA1 tightly regulates HR through its modulatory role in the PALB2-dependent loading of the BRCA2/RAD51 repair machinery at DNA breaks (100; 101).

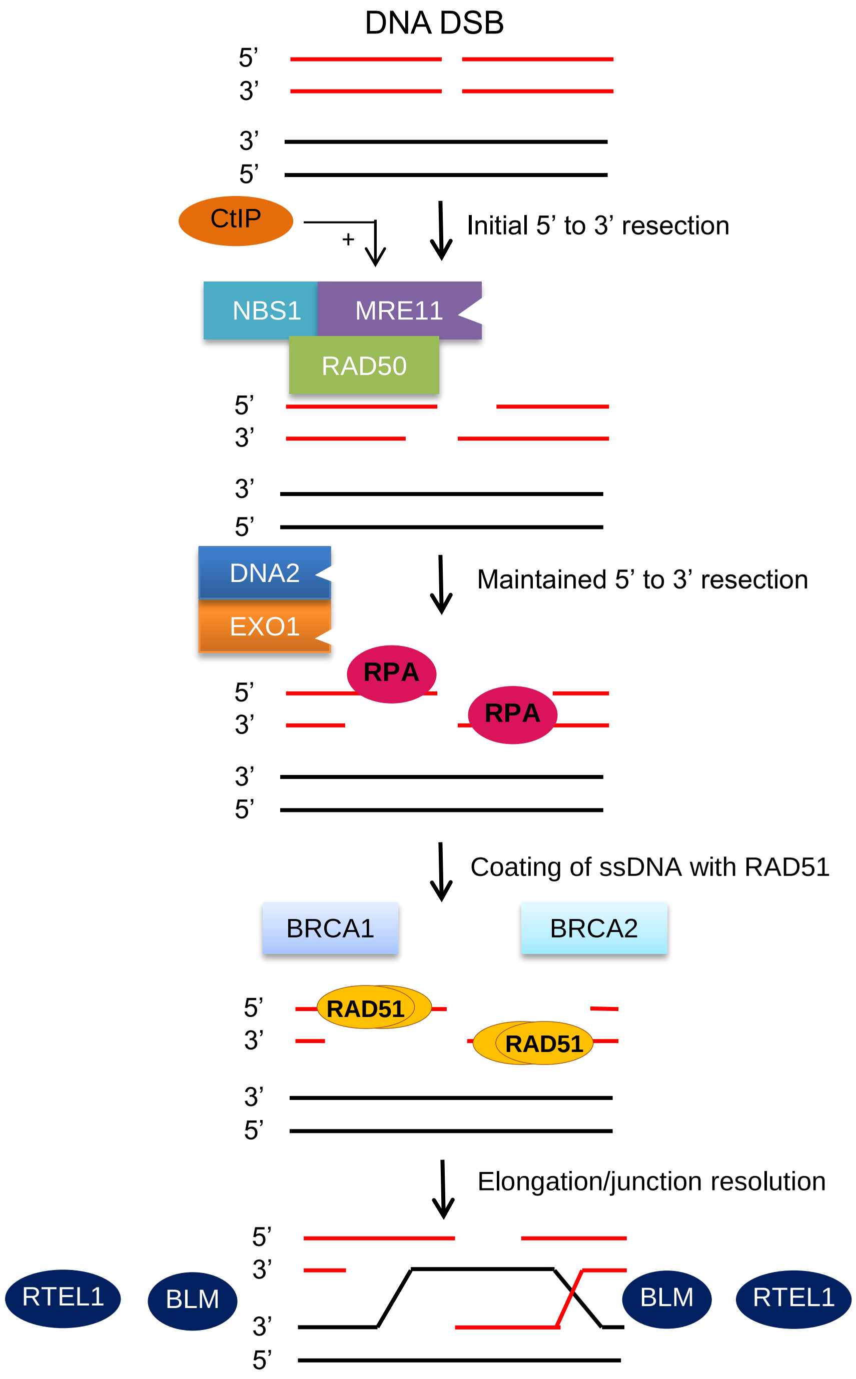

DNA DSB
5'
3'
3'
5'
CtIP
+
Initial 5' to 3' resection
NBS1
MRE11
RAD50
DNA2
EXO1
Maintained 5' to 3' resection
RPA
RPA
Coating of ssDNA with RAD51
BRCA1
BRCA2
RAD51
RAD51
Elongation/junction resolution
RTEL1
BLM
BLM
RTEL1

**Figure 10: HR pathway**. HR starts with 5'-3' end resection by MRN complex and CtIP. Further resection by the EXO1/DNA2 proteins is conducted to ensure maintained resection after which resected DNA-ends are bound by RPA. The actual recombination step within HR repair, termed strand exchange, is executed by the recombinase RAD51. RAD51 replaces RPA to eventually assemble nucleoprotein filaments. This process is facilitated by other HR components, including BRCA1 and BRCA2. Final step of junction resolution is executed by helicases including RTEL1 and BLM helicases.

The loading of RAD51 to ssDNA and formation of the D loop depend on the concerted action of other proteins, for example RAD52, RAD54 and RAD51AP1 (RAD51 associated protein 1). Rad52 supports Rad51-dependent HR, by mediating Rad51 strand invasion via physical association with the Rad51 protein and allowing for highly efficient reversal of RPA-imposed inhibition of the ssDNA-dependent ATPase and recombinase activity of Rad51 (102). Motor protein Rad54 translocates along dsDNA, interacts physically and functionally with Rad51 protein and strongly stimulates the Rad51 DNA strand exchange activity. Finally, RAD51 accessory protein RAD51AP1 stimulates joint molecule formation through the combination of structure-specific DNA binding and physical contact with RAD51 (103). After D loop formation, the 3’ end of the invading strand is used as a primer for elongation of this strand copying missing genetic information from the template (83). The replicative DNA polymerase δ subsequently extends the D-loop by synthesizing new DNA, restoring the strand on the homologous chromosome that was displaced during strand invasion (83). Finally, DNA helicases like BLM and RTEL1 have been described to resolve the D loops and thereby complete HR (Figure 10).

### 3.1.2. Non Homologous End Joining (NHEJ)

NHEJ is the main repair pathway in G1 phase, with little or no homology between the ends and HR therefore not active. Known are two forms of NHEJ: canonical and alternative. The canonical C-NHEJ pathway joins DSB ends in a Ku70/80 and XRCC4/DNA Ligase IV (Lig4)-dependent manner, but there is an alternative A-NHEJ that substitutes for C-NHEJ when this is defective. A-NHEJ is far from being fully characterized, but the common points are that it does not require extended sequence homologies, is independent of Ku70/80 or XRCC4, and joints often harbor local deletions with relatively long stretches of microhomology (104).

The most common amongst the two pathways is the canonical. C-NHEJ starts with the quick recognition and binding of the Ku heterodimer (Ku70/Ku80) to DSBs, which protects and stabilizes the DNA ends, and acts as a first scaffold where other NHEJ factors can dock (105; 106). Once Ku is bound to DSB ends, it directly recruits the DNA-PKcs kinase, a member of the ATM/ATR family of PI3KKs, to the sites of damage, leading to activation of its kinase activity (Figure 11) (107; 108). Activated DNA-PKcs can phosphorylate itself and a variety of other proteins, including NHEJ components (see below). Autophosphorylation appears to be essential for DNA-PK function in NHEJ (109). Ku also directly recruits, independent of the presence of DNA-PKcs, a complex composed of XRCC4, DNA ligase IV and XLF to ligate DNA ends (Figure 11) (110; 111). There is no known enzymatic activity for XRCC4 in NHEJ, and XRCC4 is therefore thought to act as a second scaffold for the recruitment of other DSB-processing enzymes in this pathway. In addition, XRCC4 and XLF form a filament that was hypothesized to bridge DSB ends (112).

In many cases the ends of a DSB are not liable to direct ligation. For example, the 5’ hydroxyls or 3’ phosphate termini of a DSB may be covalently modified or the ends may harbour 5’ or 3’ tails that must be resected or filled in prior to ligation. Several nucleases and helicases are involved in such processing of DNA ends, including PNKP, Aprataxin, Ku, APLF, Artemis and Werner protein (WRN) (113). After the removal of blocking end groups and DNA end resection, the resulting DNA gaps are filled by DNA polymerases μ and λ, and are later ligated by DNA Ligase IV in conjunction with XRCC4 and XLF to finish the repair (Figure 11) (114).

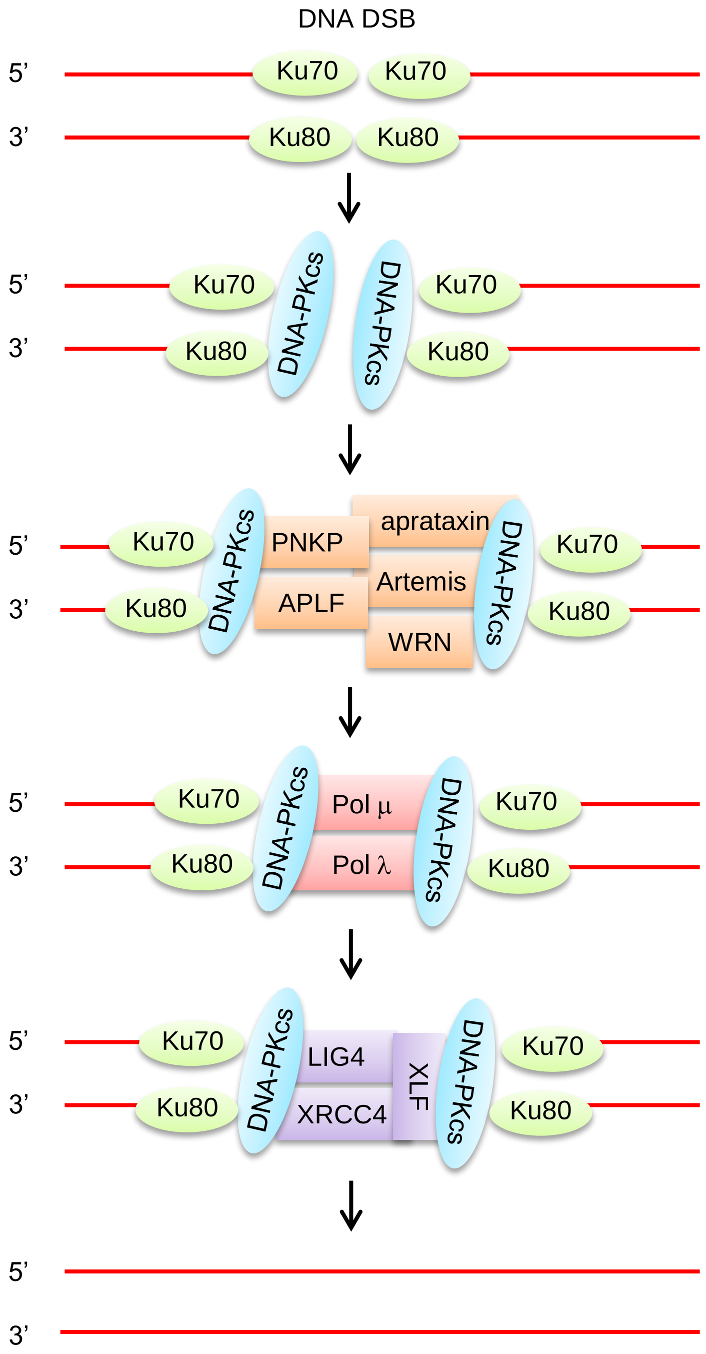


**Figure 11: Canonical NHEJ (C-NHEJ).** The C-NHEJ pathway is initiated by the Ku70/Ku80 heterodimer, which recruits DNA-PKcs. After DNA end processing, the synthesis step is catalysed by DNA polymerase μ and λ. The gap is subsequently ligated by the XRCC4/LIG4/XLF complex and the sequence continuity is restored.

In contrast, A-NHEJ has no mechanism to ensure the restoration of the original DNA sequence in the vicinity of DSBs. A-NHEJ often benefits from microhomology in the proximity of DSBs and has therefore sometimes been termed microhomology-mediated end-joining (MMEJ), although not all A-NHEJ requires microhomology for function (115). PARP1 initiates A-NHEJ and competes with Ku for binding to DSB ends. After this binding, MRN, CtIP and BRCA1 are located at damage sites for end resection, but this process can be blocked by 53BP1 to promote C-NHEJ to increase repair accuracy, for example during class switch recombination. The last step of A-NHEJ is ligation. In contrast to C-NHEJ, ligation in A-NHEJ is carried out by LIG3 forming a complex with XRCC1 or LIG1 (Figure 12) (116).

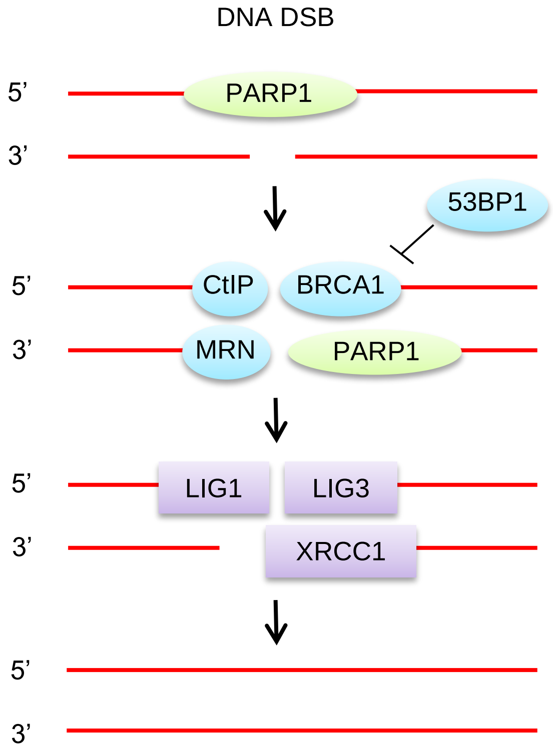


**Figure 12: Alternative NHEJ (A-NHEJ).** The broken ends are detected and bound by PARP1. This is followed by end-processing by MRN, CtIP and BRCA1, which can be inhibited by 53BP1. Final ligation is mediated by LIG3 in concert with XRCC1, or LIG1.

### 3.2. Stalled Replication Forks and HR

During DNA replication, the leading helicase unwinds the DNA allowing the accompanying polymerase to synthesize new strands of DNA. Encountering a DNA lesion will cause the replication forks to stall rapidly, and the helicase to separate from the polymerase, creating a DNA bubble that is covered with the ssDNA binding protein RPA (Figure 13). Eventually, the fork will regress because of torsion created by the supercoiled DNA ahead of the helicase. The regressed fork creates a fourth branch in which the two daughter strands are annealed (often termed as 'chicken foot') (117). Replication fork stalling leads to gaps resulting from reinitiation by DNA polymerases on the leading and lagging strands. Stalled forks and gaps can be recovered by different pathways, including HR, translesion synthesis (TLS) and template switching by fork regression (118).

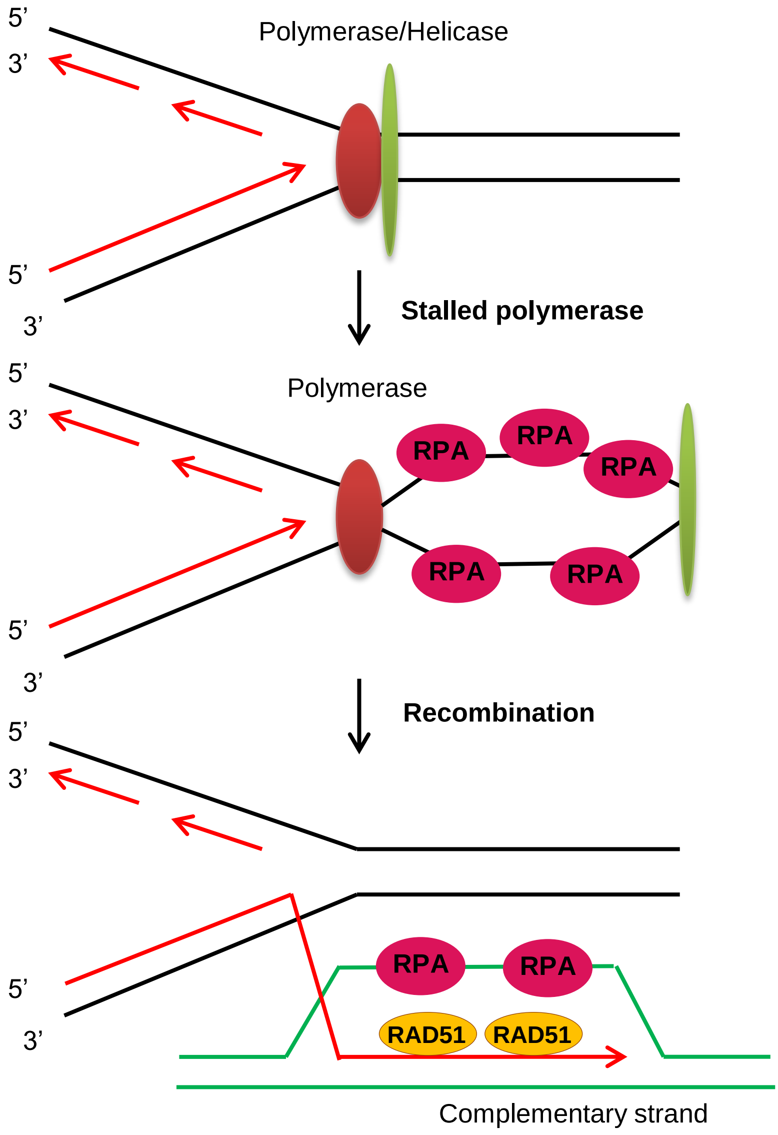

5'
3'
Polymerase/Helicase
5'
3'
Stalled polymerase
5'
3'
Polymerase
RPA
RPA
RPA
RPA
RPA
5'
3'
Recombination
5'
3'
RPA
RPA
RAD51
RAD51
5'
3'
Complementary strand

**Figure 13: HR at stalled replication fork.** During replication, the polymerase stalls and the helicase continues opening the strand. RPA coats the ssDNA and there is an invasion of the complementary strand to continue replication.

When a replication fork stalls due to depletion of deoxyribonucleotides or by encountering a DNA lesion, HR basically follows similar steps as after a DSB induced by for example IR. However, the initial substrate is different, namely a regressed replication fork that already contains ssDNA. If both leading and lagging strands of the replication fork stall simultaneously, the leading strand will always be longer such that a regressed fork could have an extended 3′ end which is required for HR. In this case, MRE11 nuclease is dispensable for the creation of ssDNA. However, the nuclease activity is required to remove any blocking lesions from the 3′ end (119). Completion of recombination appears critical for recovery of replication as in the continued absence of deoxyribonucleotides the stalled recombination obviously cannot be resolved.

### 3.3. Translesion Synthesis as an alternative repair

Translesion synthesis (TLS) is a DNA damage tolerance pathway that enables cells to cope with replication-blocking DNA lesions. TLS permits continuation of DNA replication on damaged templates, thus preventing replication fork collapse. This repair pathway depends on specialized DNA polymerases that are able to replicate bulky DNA lesions as well as non-Watson-Crick base pairing in their flexible active sites. TLS polymerases belong to the so-called Y-family and are capable of incorporating nucleotides opposite certain DNA lesions and extending from mismatched primer termini. These TLS polymerases lack the 3’ to 5’ exonuclease activity associated with the proofreading ability of replicative polymerases. Together, these characteristics make TLS polymerases intrinsically error-prone in replicating across DNA lesions. TLS polymerase activity is controlled by five domains: a domain that mediates interaction with the TLS regulator Rev1, acting as a scaffold protein, a PCNA-interacting protein (PIP) domain, two ubiquitin-binding zinc finger domains to bind ubiquitinated PCNA and a nuclear localization domain (120).

The homotrimeric DNA clamp PCNA, which normally acts as a processivity factor for replicative DNA polymerases, is a central player in controlling TLS polymerase activity (121). Upon stalling of the replication machinery at a DNA lesion, PCNA is monoubiquitinated (122). This event initiates a polymerase switch from a replicative polymerase to a TLS polymerase binding to PCNA and increases the affinity of TLS polymerases for binding to PCNA through their ubiquitin binding domains (UBD) (123; 124; 125). The TLS process requires the exchange of a replicative DNA polymerase stalled at the DNA damage site with a TLS polymerase, which thought to occur in a multi-step process. In the initial step, one of the Y-family polymerases is recruited to the stalled replication fork or the damage site for replication over the DNA lesion. As mentioned above, the recruitment of TLS polymerases is facilitated by DNA damage-induced PCNA monoubiquitination. Following incorporation of nucleotide opposite the damage site, the insertion TLS polymerase is replaced by extension polymerase that extends the TLS patch by around 18 nucleotides. This extension step allows the lesion to escape detection by the 3′-5′ exonuclease proofreading activity of a replicative DNA polymerase. Following extension past the DNA lesion, the TLS polymerase is switched back to the high fidelity DNA polymerase for resuming processive DNA replication (Figure 14) (120).

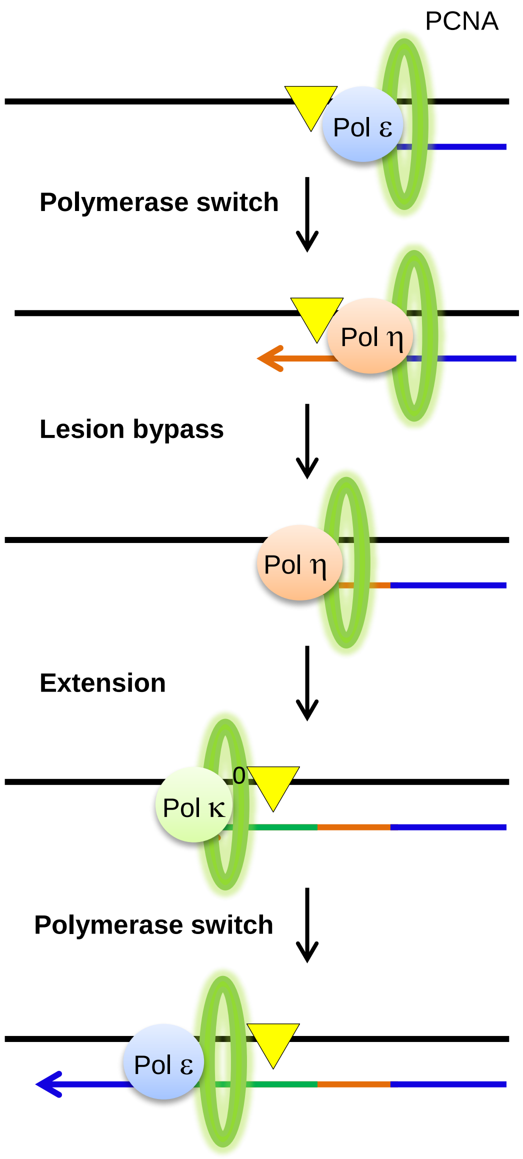

PCNA
Pol ε
Polymerase switch
Pol η
Lesion bypass
Pol η
Extension
Pol κ
0
Polymerase switch
Pol ε

**Figure 14: Model for polymerase switching during TLS.** Replicative DNA polymerase Pol ε stalls at DNA lesion in the DNA template. In the first polymerase switch, TLS specialized Pol η is recruited to the sliding clamp PCNA at the stalled fork and replicates over the lesion. This TLS "patch" is then extended by the same or another TLS polymerase, for example Pol κ. The final switch restores the replicative DNA polymerase to the template, and processive DNA synthesis continues.

## 3.4. Other types of repair

Three distinct pathways are involved in the repair of different lesions in single-stranded DNA: base excision repair, nucleotide excision repair and mismatch repair.

### 3.4.1. Base Excision Repair (BER)

BER is primarily responsible for removing small, non-helix-distorting base lesions from the genome, induced by ionizing radiation, alkylating drugs, and antimetabolites (126). Endogenous base damage entails oxidative base modifications from reactive nitrogen and oxygen species generated in cells during normal cellular respiration or after oxidative stress from ischemia or chronic inflammation (127). Deletion mutations in BER genes lead to breaks during DNA replication and have shown to result in a higher mutation rate in a variety of organisms, implying that loss of BER can contribute to the development of cancer (128).

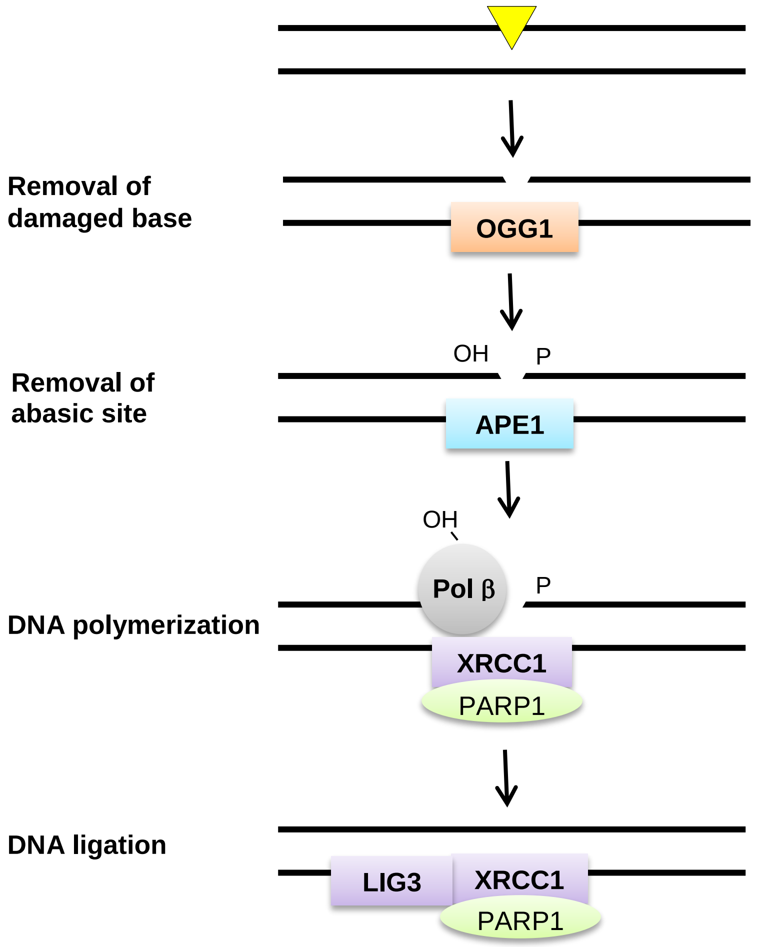

Removal of damaged base
OGG1
Removal of abasic site
OH
P
APE1
OH
Pol β
P
DNA polymerization
XRCC1
PARP1
DNA ligation
LIG3
XRCC1
PARP1

**Figure 15: Base excision repair.** DNA damage is detected and excised by OGG1, a specific glycosylase leaving an abasic site. The abasic site is processed by an apurinic/apyrimidinic endonuclease, APE1. DNA polymerase β fills in the missing DNA base. DNA ligase III (LIG3) seals the remaining DNA nick, completing the repair.

During the activity of BER, DNA glycosylases, for example OGG1, are responsible for recognition of the lesion. This enzyme then removes the damaged base, leaving an AP site. The phosphodiester backbone is then cleaved by an apyrimidinic/apurinic (AP) endonuclease or glycosylase/lyase, such as APE1, and a complementary nucleotide is inserted by Pol β, a polymerase from the X-family that only insert a single nucleotide. PARP1 recognizes the single-strand break and interacts with XRCC1 to form the protein scaffold upon which the repair complex is built. Finally, ligation of the DNA backbone by DNA ligase III restores the native structure and sequence (Figure 15) (126).

### 3.4.2. Nucleotide Excision Repair (NER)

Nucleotide Excision Repair (NER) repairs various types of DNA lesions including bulky helix-distorting lesions caused by chemicals such as aromatic amines as acetyl-aminofluorene, nitrosamines such as MNNG, and crosslinking agents. NER also effectively repairs thymidine dimers and 6-4 photoproducts that are induced by UV light and oxidative DNA damage and can recognize reactive products of lipid peroxidation (126).

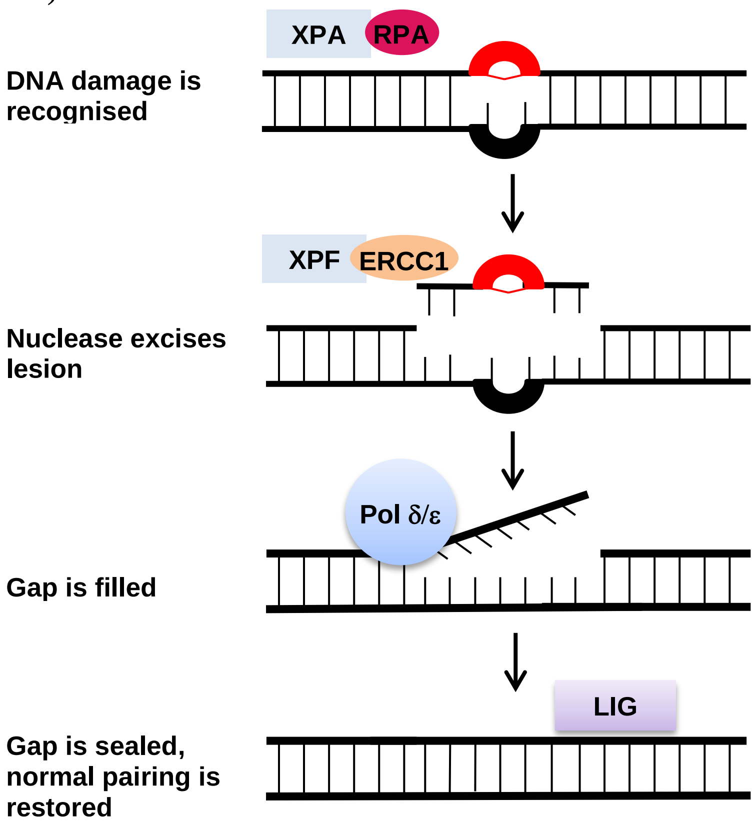


**Figure 16: Nucleotide excision repair.** After XPA/RPA complex detects the damage, a short ssDNA segment containing the lesion is removed by the nuclease ERCC1/XPF. The undamaged ssDNA remains and DNA polymerases δ and ε uses it as a template to synthesize a short complementary sequence. Final ligation to complete repair and form a double-stranded DNA is carried out by DNA ligase.

### 3.4.3. DNA Mismatch Repair (MMR)

Mismatch Repair (MMR) is the main pathway for the repair of base-base mismatches and insertion and/or deletion loops that are formed during DNA replication or recombination (130). The MMR system is highly important for the fidelity of DNA replication, whose impairment predisposes to the development and progression of many types of cancers. The human MMR pathway consists of two major components: MutS for lesion recognition and MutL for excision.

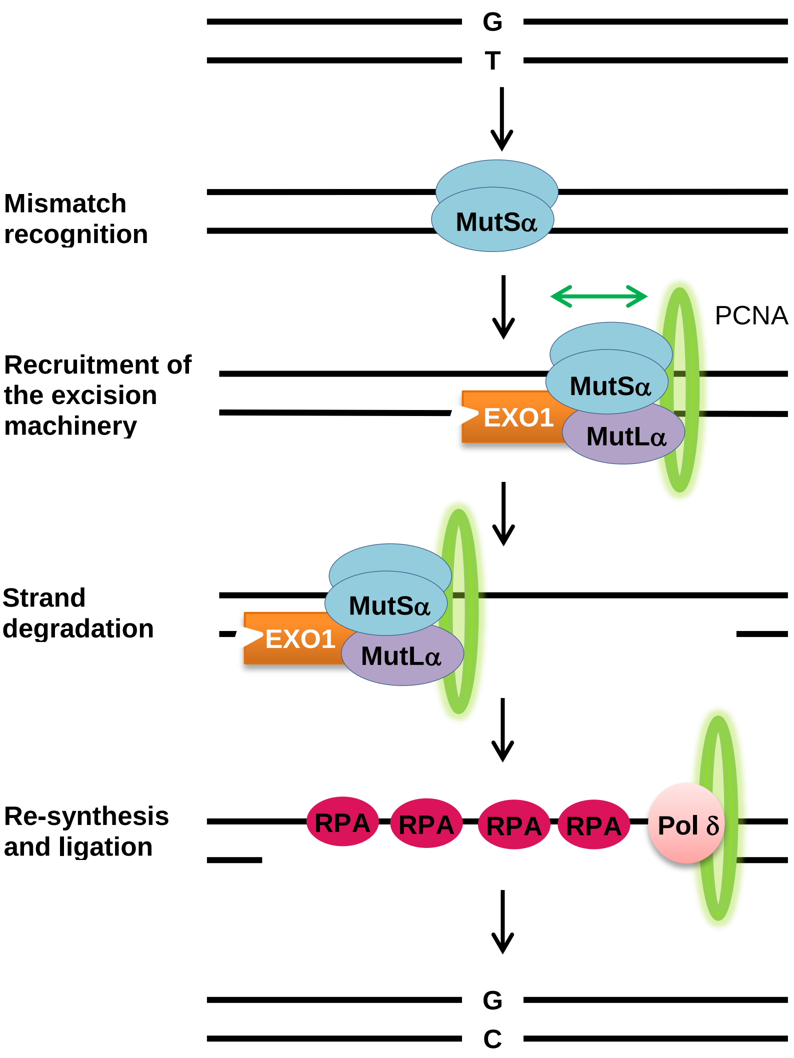

G
T
Mismatch recognition
MutSα
PCNA
Recruitment of the excision machinery
MutSα
EXO1
MutLα
Strand degradation
MutSα
EXO1
MutLα
Re-synthesis and ligation
RPA
RPA
RPA
RPA
Pol δ
G
C

**Figure 17: Mismatch repair.** MutSα heterodimer binds to a mismatch and recruits the MutLα heterodimer. This complex can translocate in either direction along the DNA (green arrows). When it encounters a strand discontinuity PCNA binding and loading of EXO1 initiate degradation of the nicked strand. RPA stabilizes the single-strand and the gap is then filled in by the replicative polymerase and the remaining nick is sealed.

There are two MutS homologs: MutSα (Msh2/Msh6), recognizing single base mismatches and 1-2 nucleotide insertions/deletions and MutSβ (Msh2/Msh3), which recognizes larger loops. After MutSα finds a strand discontinuity (such as a gap between Okazaki fragments) bound by PCNA, MutS undergoes an ADP–ATP exchange driven conformational change into a sliding clamp and recruits the MutL heterodimer. The complex formed by MutS/MutL can translocate in either direction along the DNA in search of a strand discontinuity. Loading of the exonuclease EXO1 then initiates degradation of the nicked strand that will terminate past the mismatch (Figure 17).

MutL homologs include MutLα, MutLβ and MutLγ and although its role in MMR is still largely unclear, MutLα was shown to have latent endonuclease activity that provides a place for EXO1-dependent excision or for polymerase-dependent strand displacement reactions (131). The resulting single-stranded gap is stabilized by RPA and then filled in by polymerase δ. The remaining nick is sealed by DNA ligase I (Figure 17).

## 4. POST-TRANSLATIONAL MODIFICATIONS AFTER DNA DAMAGE

The DDR exists of an elaborate signalling pathway starting on chromatin near the DNA lesion and coordinating ordered recruitment of specific factors that promote DNA repair and cell cycle arrest. This response relies on post-translational modifications of both chromatin components and non-chromatin proteins.

The nucleosome is the fundamental unit of chromatin, containing eight histone subunits, consisting of two dimers of histones H2A and H2B and a tetramer of histones H3 and H4. This octameric core of histones is combined with 146 base pairs of DNA to form the nucleosome core. Each core histone has a similar structure, consisting of a globular, hydrophobic internal region that forms the histone fold, and N- and C-terminal extensions that are relatively unstructured (132). These extensions, particularly those on the N-terminus, are sites for numerous post-translational modifications, which play essential roles in the regulation of DNA template-dependent processes, including DNA repair (133). Such post-translational modifications can alter the structure of the nucleosome directly by opening up the chromatin and making it accessible for the localization of DNA damage proteins. In addition, such modifications can act as signalling platforms for the recruitment of protein complexes that carry recognition domains for specific modifications (133). Aberrations in the post-translational modifications in the DDR are associated with a large number of human diseases, including cancer and neurological diseases (134).

### 4.1. Phosphorylation

Phosphorylation and its counterpart, dephosphorylation, turn many protein enzymes on and off by altering their function and activity. Protein phosphorylation is carried out by kinases and dephosphorylation by phosphatases. Many kinases and phosphatases have been identified in the DDR.

H2AX phosphorylation by master kinases ATM/ATR on Ser139 (γH2AX) is an early step in a cascade of chromatin modifications, including nucleosome remodelling and other post-translational histone modifications, which allows recruitment and retention of repair factors. H2AX knockout mice develop high chromosomal instability and display repair defects, and impaired recruitment of specific DNA-repair proteins on damaged DNA, pointing to the importance of H2AX phosphorylation in DDR (135). However, migration of repair and signalling proteins to DSBs is not abrogated in $H2AX^{-/-}$ cells, or in H2AX-deficient cells that have been reconstituted with H2AX mutants that eliminate phosphorylation. Despite their initial localization at DSBs, numerous factors, including NBS1, 53BP1 and BRCA1, subsequently cannot form IR-induced foci (IRIF). H2AX phosphorylation therefore does not seem the primary signal required for the redistribution of repair complexes to damaged chromatin, but functions to concentrate proteins in the vicinity of DNA lesions (136).

Many other factors are phosphorylated by ATM and ATR. These kinases share substrate specificity as they both phosphorylate serine or threonine residues followed by glutamine (SQ/TQ motifs) (44; 61). Downstream effector kinases Chk1 and Chk2, which have been mentioned before, phosphorylate cell cycle proteins and repair factors. These kinases are also serine/threonine kinases and their substrates present a RXXS/T consensus sequence (137).

Interestingly, phosphatases are also implicated in this response, counteracting the function of kinases. Several phosphatases such as PP2A, PP4, PP1, PP6, and Wip1 participate in the dephosphorylation of γH2AX. PP2A and Wip1 can also suppress ATM activation and PP5 plays an important role in the DDR through regulation of ATR activation (138).

### 4.2. Ubiquitination and SUMOylation

Protein ubiquitination, the addition of ubiquitin (Ub) to a substrate protein, is a reversible post-translational modification carried out by the coordinated activities of an E1 ubiquitin-activating enzyme, an E2 ubiquitin conjugase, and an E3 ubiquitin ligase. So far, 9 E1 Ubiquitin-activating enzymes, 35 E2 ubiquitin-conjugating enzymes and around 1000 E3 ligases have been identified in humans.

The initial step involves producing an intermediate product, adenylyl ubiquitin. Then the ubiquitin is transferred to the E1 active site specifically binding to the cysteine residue, with release of AMP. This step is performed by a thioester linkage between the C-terminal carboxyl group of ubiquitin and the sulfhydryl group of the cysteine or thiol E1. Transfer of ubiquitin to the active site of E1 to E2 ubiquitin conjugating enzyme through a transthioesterification reaction occurs. The final step in the ubiquitination cascade generates an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. In general, this step requires the activity of an E3 ubiquitin ligase. E3 enzymes, of which hundreds exist, function as main substrate recognition system and are capable of interacting with both E2 and substrate (139). In general, the E3 enzymes have a HECT or RING domain. Transfer of the ubiquitin and interaction with the substrate can occur in two ways: directly from the E2 enzyme catalysed by the RING domain or via an E3 enzyme catalysed HECT domain. In the last case, a covalent intermediate E3-ubiquitin is formed before transfer of ubiquitin to the protein substrate (139).

One or more ubiquitin molecules can be attached to the protein, and this conjugation occurs via lysine residues. In the case of polyubiquitination, the next ubiquitin molecules can be linked using one of 7 different lysine residues, of which linkage via Lys48 and Lys63 is most common. The functional consequence of ubiquitination is determined by the length of the ubiquitin chain as well as the linkage type. Generally, Lys48-linked polyubiquitin chains target proteins for degradation by the proteasome, whereas Lys63-linked polyubiquitination directs signalling in for example DNA repair (140). Monoubiquitination has been shown to regulate lysosomal degradation of proteins and also was shown to have signalling functions for example by mediating protein-protein interactions (124; 141).

Although the DDR was initially described as a kinase cascade, numerous studies now described the involvement of modification by ubiquitin in this response. The first evidence came from the observation that the post-replication repair protein Rad6 is actually an E2 ubiquitin enzyme that, in association with the E3 Rad18, mono-ubiquitinates the replication factor PCNA on Lys164 in response to DNA damage (122; 142). Other examples of the involvement of ubiquitination in the DDR are recent studies showing that DNA damage sites are enriched with Lys63 ubiquitin chains and the E3 ligases RNF8 and RNF168 provide a critical link between phosphorylation and ubiquitination of H2AX in the DDR (30; 29). In addition, the levels of many DDR proteins are regulated by ubiquitination to direct localization, activity or degradation by the proteasome.

Ubiquitination can be reversed by ubiquitin hydrolases (deubiquitinases, DUBs). These cysteine or metallo-proteases cleave ubiquitin from linear ubiquitin polypeptides or from specific mono- or polyubiquitinated substrates and are responsible for processing inactive ubiquitin precursors, proofreading ubiquitin-protein conjugates, and removing and recycling ubiquitin from cellular adducts (143). Cysteine protease DUBs are organized into four subclasses based on their ubiquitin-protease domains: ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolase (UCH), Otubain protease (OTU), and Machado-Joseph disease protease (MJD). The metallo-protease DUBs belong to the Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) (JAMM) domain superfamily. Several of the around 100 known ubiquitin hydrolases in human have been implicated in the DDR (143).

Several ubiquitin-like molecules have been identified, such as SUMO (small ubiquitin-like modifier) and NEDD8. Like ubiquitination, SUMOylation is catalyzed by a cascade of enzymes initiated by E1 SUMO-activating enzymes (SAE1 and SAE2), a single E2 conjugating enzyme (Ubc9), and various E3 SUMO ligases (PIAS), which are important for target selection (144). SUMO hydrolases also exist, around 10 in humans, but relatively little is known about them. Examples of SUMOylation regulating ubiquitination pathways have been described. In addition, the direct involvement of SUMOylation in the DDR has recently been described by demonstrating that all three SUMO isoforms (SUMO1, and the closely related SUMO2 and SUMO3) are highly enriched at sites of DNA damage in human cells (41; 96). Upon DNA damage, the SUMO E2 ligase Ubc9 and the PIAS family of SUMO E3 ligases rapidly mobilize and localize to DNA damage foci (41; 96). Knockdown of certain SUMO E3 ligases, particularly PIAS4, resulted in loss of SUMOylation at damage sites, as well as reduced accumulation of 53BP1, BRCA1, and Lys63-linked ubiquitin conjugated at damage sites without affecting MDC1 or RNF8 recruitment. Loss of MDC1 or RNF8 affected accumulation of all SUMO isoforms at damage sites, while loss of 53BP1 or BRCA1 selectively affected SUMO1 and SUMO2/3, respectively (41; 96). These results strongly suggest that SUMOylation acts at multiple steps in the DSB response.

#### 4.2.1. H2A(X) ubiquitination

Histone ubiquitination has been implicated in several steps of the DDR (145). H2AX-Lys119 and Lys13/15 ubiquitination is induced upon DSB induction and is, among others required for histone turnover at the site of damage (26; 146; 27; 147). H2AX ubiquitination is mediated by several E3 ubiquitin ligases. DNA damage-induced phosphorylation of the N-terminus of MDC1 by ATM/ATR recruits the RNF8 ligase via interaction with RNF8 FHA domain (27). RNF8 mediates Lys63-linked ubiquitination of H2A(X) at DNA damage sites in association with the E2 UBC13. The ubiquitination signal required for DDR signalling is not sufficiently maintained by RNF8 alone, and is highly dependent on the activity of a second RING E3 ubiquitin ligase, RNF168, which promotes ubiquitination of histone H2A(X)-Lys13/15 (148). H2A(X) ubiquitination by RNF8 and RNF168 is required for accumulation and retention of 53BP1 and BRCA1 at the break, two key effectors of the DSB response that promote NHEJ and HR, respectively (26; 146; 27; 29). Moreover, RNF168 also induces Lys27-linked ubiquitination, which is directly recognized by 53BP1, Rap80, RNF169, and RNF168 itself (149).

#### 4.2.2. PCNA ubiquitination and SUMOylation

Upon UV damage or at stalled replication forks, the uncoupling of the stalled replicative polymerase and the MCM helicase results in production of single-stranded DNA exposed in the vicinity of the stalled fork. This single-stranded DNA is coated by RPA, which interacts with E3 ubiquitin ligase Rad18 and thereby directs the E2 conjugating enzyme Rad6/Rad18 complex to the site of DNA damage (150; 151). These enzymes process triggers monoubiquitination of PCNA on Lys164 (122).

Once monoubiquitinated, PCNA recruits members of the Y-family TLS polymerases (Polη, Polκ, Polι, and REV1) as well as the B-family TLS polymerase Polζ to the site of DNA damage. The TLS polymerases interact with monoubiquitinated PCNA through their UBM or UBZ ubiquitin-binding domains as well as through their PIP motifs. The preferential binding of the TLS polymerases to monoubiquitinated PCNA enables the TLS polymerases to replace the stalled replicative DNA polymerase (Polδ or Polε) at the blocked sites of the DNA replication fork (124; 152; 123; 153; 154).

PCNA is also polyubiquitinated, although it occurs at much lower levels than monoubiquitination. In yeast, after PCNA monoubiquitination, Rad5 recruits the E2 complex UBC13/MMS2, which mediates Lys63-linked PCNA polyubiquitination. In mammalian cells, polyubiquitination of PCNA is only detectible after over-expression of the HLTF and SHPRH E3 ubiquitin ligases (155; 156). HLTF and SHPRH interact with RAD6/RAD18 and UBC13/MMS2 and thereby promote PCNA polyubiquitination (155; 156; 157). What triggers the switch from PCNA monoubiquitination to polyubiquitination is not totally clear, although the level of DNA damage is thought to play a role, since poly-ubiquitination is observed following treatment with increasing amounts of DNA damaging agents (156).

In addition to ubiquitin, PCNA is covalently modified by SUMO on Lys164, the same residue that is ubiquitinated, and Lys127, following replication stress or during unperturbed S phase progression. In yeast, enzymes involved in PCNA SUMOylation are the E2 SUMO conjugating UBC9 and the E3 Siz1 (122; 125). SUMOylated PCNA recruits the helicase Srs2 through a SUMO-interaction motif of Srs2 (158). This recruitment of Srs2 disrupts RAD51 single-stranded filaments, preventing recombination (159; 160). At a stalled replication fork, the inhibition of recombination by Srs2 allows for lesion processing by TLS or template switching (161; 162).

As SUMOylation and ubiquitination target the same residue in PCNA, SUMO seems to have an antagonistic action on ubiquitin-dependent DNA damage tolerance. However, the negative effect of SUMO on lesion bypass is not due to competition with ubiquitination but is rather mediated by the helicase Srs2, which affects genome stability by suppressing unscheduled HR. PCNA SUMO and ubiquitin therefore cooperatively control the choice of pathway for the processing of DNA lesions during replication (163).

### 4.3. Acetylation

During acetylation an acetyl functional group is introduced into a target protein, for example a histone. Histone acetylation alters the structure and charge of lysine residues and therefore regulates chromatin structure and function by modifying histone-DNA and histone-protein interactions, making histone acetylation directly involved in for example the regulation of transcription. Histone H3 and H4 acetylation plays an important role in the regulation of chromatin structure and histone acetyltransferases (HATs) therefore play key roles in gene expression and the DDR facilitating the accessibility for repair proteins (164).

One major HAT in the DDR is Tip60 and ectopic expression of mutated Tip60 lacking histone acetylase activity resulted in delaying the kinetics of DNA repair. The resulting cells lost their apoptotic competence against IR, suggesting a defect in the ability of the cells to signal the existence of DNA damage to the apoptotic machinery (165). A major target of Tip60, in complex with its cofactor TRRAP, is histone H4-Lys16 near sites of DNA DSBs and this acetylation was shown to promote chromatin relaxation (165; 166; 167). Cells lacking TRRAP cannot recruit Tip60 and have reduced histone acetylation near these damage sites. As a result, these cells demonstrate an impaired efficiency to repair DNA, particularly through HR (166). Tip60 has also been suggested to play a direct role in the activation of the ATM, by acetylating Lys3016, a residue adjacent to the ATM kinase domain. Mutation of this residue abrogates kinase activity upon DNA damage, resulting in decreased phosphorylation of downstream ATM targets and reduced survival (168). Regulation of Tip60 itself is dependent on post-translational modifications. Tip60 recognizes histone H3 methylated on Lys9 via its chromodomain (169). The binding of Tip60 to this modification triggers its activity, thereby facilitating the activation of ATM.

*Another* highly conserved HAT *involved in ATM activation is MOF*, which acetylates histone H4 at Lys16. Depletion of MOF in human cells reduced acetylated histone H4-Lys16 levels and these cells have reduced levels of ATM activation as well as defective appearance of IR-induced γH2AX foci (170; 171). INO80, SWR1 and RSC ATP-dependent chromatin remodelling complexes are additionally involved histone H3 and H4 acetylation (172). CBP and p300 acetyltransferases cooperate with the SW1/SNF remodelling complex to facilitate recruitment of NHEJ proteins such as Ku70/80 by inducing chromatin relaxation (173). Other acetylation events for the recruitment of HR factors occur on histones H3 and H4 with GCN5, NuA4 and HAT1 implicated in the modification (174; 175). GCN5 also interacts with BRCA1 through a mechanism that is dependent upon its HAT activity implicating a role in HR repair of DNA DSBs (176).In conclusion, histone acetylation is important during early stages of repair to facilitate the chromatin opening and subsequent access of repair proteins to the sites of DNA lesions.

### 4.4 Methylation

Methylation is the process of addition of a methyl group to a lysine in a protein substrate, for example a histone. While histone methylation is canonically associated with transcriptional regulation, histone H3 and H4 methylation has been described as a main participant in the DDR, mediated by histone methyltransferases (HMTs). Histone H4 can be mono-, di- or trimethylated, which results in distinct biological outcomes. Examples are trimethylation on Lys20 (H4K20me3) being a hallmark of silenced heterochromatic regions, whereas mono- and dimethylated Lys20 (H4K20me1 and H4K20me2) is involved in DNA replication and DNA damage repair in both yeast and mammalian cells (177; 178). Upon induction of DSBs, the HMT MMSET is recruited to sites of DNA damage by the γH2AX-ATM pathway, resulting in a local increase of H4K20me1/2. H4K20me2, then acts as a loading platform for 53BP1, via binding of the 53BP1 tandem Tudor domains to this methylation mark (179). As described earlier, 53BP1 is subsequently is required for efficient NHEJ repair (177; 178).

## 5. DDR-RELATED DISEASES

The importance of DDR mechanisms is demonstrated by the existence of several human syndromes, immunodeficiencies and cancers, in which the cells are defective in DNA damage signalling and repair processes, exacerbating the clinical effects of the unrepaired DNA damage. Such human diseases have been very helpful in elucidating the networks involved in the complex cellular response to DNA damage.

### 5.1. Ataxia Telangiectasia

Ataxia Telangiectasia (A-T) is an autosomal recessive disorder with a mutation in ATM (Ataxia Telangiectasia mutated) protein and characterised by immunodeficiency, cancer predisposition and neurodegeneration. Cells from A-T patients display chromosomal instability, radiosensitivity and defective cell cycle checkpoint activation (180). As described earlier, as the primary transducer of DSB-induced signalling, ATM is a central kinase in the DDR. After the recognition of DSBs by the MRN complex, the ATM kinase is rapidly activated. ATM autophosphorylation promotes its monomerization and kinase activity (16). This leads to a cascade of post-translational modifications on many substrate proteins and remodelling of chromatin structure around the break sites.

### 5.2. Seckel Syndrome

Seckel syndrome is an extremely rare form of primordial dwarfism characterized by growth delay, proportionate extreme short stature, a prominent beak-like nose, hypoplasia of the malar area, small chin, microcephaly, and skeletal malformations (181; 182). Seckel syndrome is a genetically heterogeneous disorder in which rare autosomal recessive mutations in eight different genes, all involved in the DDR mechanisms, have been identified to date. These include mutations in the ataxia-telangiectasia and Rad3-related (*ATR*) gene, *ATRIP*, *CENPJ*, *RBBP8*, *NIN*, *DNA2*, *CEP63* and *CEP152* (183).

### 5.3. RIDDLE Syndrome

RIDDLE syndrome is a human hereditary disease clinically characterized by immunodeficiency, dysmorphic features and learning difficulties. RIDDLE syndrome shares overlapping clinical features with A-T. Mutation of RNF168 has been implicated in this disorder (30). As mentioned, RNF168 functions as an E3 ligase in the DRR. Knockdown of RNF168 in human cells significantly impaired the recruitment of 53BP1, RAP80 and BRCA1 to sites of DNA lesions. Indeed, cells derived from a RIDDLE patient exhibit impaired localization of 53BP1 and BRCA1 to DSBs, while MDC1 and NBS1 remain unaffected (184). In addition, these cells are radiosensitive. *Therefore, RNF168* acts as a tumor suppressor gene and is thought to play a role in the development of sporadic malignancies, particularly those of lymphoid origin.

### 5.4. Nijmegen Breakage Syndrome

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive DNA repair disorder characterized by microcephaly, immunodeficiency and cancer (185; 186). In NBS, congenital osteoarticular malformations commonly occur in half of the patients. Hydronephrosis, hypoplastic kidney, anal atresia/stenosis or gonadal failure are less commonly observed (186). NBS is caused by hypomorphic mutations in the NBS1 gene, thereby expressing less functional NBS1. NBS1, together with MRE11 and RAD50, forms the trimeric MRN complex involved in the response to DNA DSBs (187). The MRN complex is a primary sensor of such lesions and is required for the effective activation of ATM. The accumulation of MRN at damaged chromatin requires the MDC1 protein with which it interacts via the FHA domain of NBS1 (14). NBS1 is required not only for ATM activation but also for Chk1 phosphorylation by the kinase ATR (188).

### 5.5. Xeroderma Pigmentosum

Xeroderma pigmentosum (XP) is an autosomal recessive disorder. XP results from mutations in any one of eight genes (*XPA-XPG* and *POLH*) (189). The products of *XPA* through *XPG* are involved in the repair of UV-induced photoproducts by the process of NER. Upon UV damage, the cell uses DNA polymerase $\eta$, encoded by the gene *POLH* and it is this gene that is mutated in XP-V patients (190). The main clinical symptoms of XP patients include the high frequency of tumors in the exposed areas of skin, indicating a clear causative relationship between unrepaired DNA lesions, genetic instability (XP cells are highly mutable) and cancer. Some XP patients, however, present more severe clinical phenotypes, including developmental problems, neurodegeneration and premature aging (191). XP cells display increased frequency of mutagenesis after exposure to UV light that is correlated with molecular defects in DNA damage processing, leading to mutagenic consequences in the cells and increased tumorigenesis.

### 5.6. Lynch Syndrome

Lynch syndrome, also known as hereditary non-polyposis colorectal cancer, is an autosomal dominant disorder caused by germline mutations in one of the MMR genes *MLH1*, *MSH2*, *MSH6* or *PMS2*. During DNA replication, mismatch mistakes commonly occur on the newly synthesized strand of DNA. In MMR-deficient cells these mismatches are not corrected, leading to the accumulation of mutations and, ultimately, neoplasia. MMR gene mutation carriers are at increased risk of developing cancers of the colorectum and endometrium, as well as cancers of the ovary, kidney, pancreas, stomach, and urinary bladder. The risk of colorectal cancer to age 70 years for MMR gene mutation carriers is between 12 and 50% (192).

# II. OBJECTIVES

Post-translational modifications are critical for DNA damage signalling, by regulating levels, activity and localization of key proteins in this response. The first type of post-translational modification described in this response was phosphorylation, but recently it was demonstrated that modifications by small molecules such as ubiquitin also play an important role. Examples are the DNA damage-induced monoubiquitination of H2AX and PCNA. Immediately after triggering DNA damage, phosphorylation and subsequent ubiquitination of histone H2AX is initiated, required for the recruitment of numerous mediator proteins in the DNA damage response to the sites of DNA lesions, thereby regulating cell cycle arrest and DNA repair. PCNA is an important element in both DNA replication and repair, in particular for the recruitment of specialized polymerases, responsible for translesion synthesis. DNA damage-induced monoubiquitination of PCNA increases its affinity such polymerases.

Although various E3 ubiquitin ligases have been identified to regulate H2AX and PCNA ubiquitination, less was known about what ubiquitin hydrolases are responsible for removing ubiquitin from these proteins.

The hypothesis was that deubiquitinases (DUBs) regulate these processes in the DNA damage response.

The main goal of this study therefore was to identify novel regulators of the DNA damage response among human DUBs, which control H2AX and PCNA ubiquitination.

The key objectives in this study were the following:

1. To perform a screen by overexpressing individual plasmids of a DUB library containing most human DUBs and some SUMO hydrolases. Examining the effect of each enzyme on the monoubiquitination of H2AX and PCNA.

2. After identifying a novel ubiquitin hydrolase for H2AX, studying the mechanism of action: the effect of DUB overexpression on the recruitment of other proteins in the pathway, interaction of DUB with H2AX and the *in vitro* activity of the DUB towards H2AX. Finally, examining the influence of this DUB for genomic stability.

3. Testing the candidate enzyme(s) for PCNA for interaction with PCNA and its effect on PCNA-regulated translesion synthesis.

4. Identify possible novel chromatin regulators involved in the DNA damage response by an additional screening using a small library consisting of several acetyl-transferases and de-methylases with recruitment of mediator proteins to sites of DNA lesions as readout.

# VI. MATERIALS AND METHODS

## 1. CELL CULTURE

### 1.1. Cell lines

The following human tumor cell lines were used in our experiments:

- HEK 293T: cell line derived from human embryonic kidney cells, with epithelial morphology.
- U2OS: human osteosarcoma cell line expressing wild type p53 and Rb, but lacking p16.
- HCT116: human colorectal carcinoma cell line. This cell line has a mutation of the Ras proto-oncogene.
- 639V: cell line from human urinary tract, from bladder carcinoma.
- KATO III: human gastric signet ring carcinoma cell line.
- HL-60: human promyelocytic leukemia cell line that proliferates continuously in suspension culture.
- T24: cell line from human bladder carcinoma.
- BJ: Epstein-Barr Virus transformed lymphoblastoid cellline which is part of the Human Leukocyte Antigen (HLA) Typed Collection and was growth in suspension culture.
- HT1080: human fibrosarcoma cell line.
- Mcf7: breast cancer cell line.
- NIH-3T3: standard fibroblast cell line.
- LOVO: human colon adenocarcinoma cell line.
- Mrc5: cell line derived from normal lung tissue.
- A549: adenocarcinomic human alveolar basal epithelial cell line.
- LS174: human colorectal adenocarcinoma.

### 1.2. Medium and culture conditions

Cell lines were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Lonza/Gibco), supplemented with 10% fetal bovine serum (FBS, Biowest/Gibco), 1,5 mM L-glutamine (Lonza/SIGMA), 150 U/ml penicillin and 150 μg/ml streptomycin (Lonza/SIGMA). Cells were grown at 37ºC in a 5% of $CO_2$ atmosphere in 6 and 10 cm nonpyrogenic polystyrene dishes (Nunc, Falcon).

## 2. CELL HANDLING

### 2.1. Plasmid transfection

Transfection of plasmids was carried out with the calcium phosphate transfection method (amounts below for a 10 cm TC dish). First, 10 μg of DNA plasmid was mixed with 450 µl milliQ water and 50 µl of 2,5 M $CaCl_2$. Then 500 µl of HBS 2X buffer was slowly added to the mix, after which this was added to cells cultured at 30% of confluence in 10 ml of medium. After 16 hours of incubation, the cells were washed with PBS and incubated in fresh medium for 24 hours, after which the cells were harvested.

| **HBS 2X buffer pH 7,02** |
|---|
| 280 mM NaCl<br>1,5 mM $Na_2HPO_4$<br>12 mM Glucose<br>10 mM KCl<br>50 mM Hepes |

### 2.2. siRNA oligo transfection

Transfection of siRNA oligos was carried out following the next steps (amounts for a 6 cm TC dish): 5 µl of Lipofectamine RNAiMAX (Invitrogen) was added to 250 µl of Opti-MEM (Invitrogen) – Solution A. In another tube, 5 µl of oligonucleotide (20 µM) was added to 250 µl of Opti-MEM – Solution B. Then solution A and B were mixed and incubated for 20 minutes at room temperature. During this incubation, the medium of the cells (20-40% confluency) was refreshed. After incubation, the mix was added drop by drop into the plate and incubated at 37ºC. Cells were collected 48-72 hours later. For proteins with long half-lives, a second round of transfection was performed 24 hours after the first round and cells were collected 48-72 hours thereafter.

| siRNA oligo | Sequence 5' → 3' |
|---|---|
| **Luciferase** | CGUACGCGGAAUACUUCGAdTdT |
| **DUB3 #2** | CCUCCGUGAUGUUGCUUGAdTdT |
| **SENP5 #1** | AAGUCCACUGGUCUCUCAUUAdTdT |
| **SENP5 #2** | UAAUGAGAGACCAGUGGACUUdTdT |
| **KDM3A** | AAGAUCGGAAUAUGGAACAAdTdT |

### 2.3. DNA damage induction

Various DNA damaging agents were used to induce different types of DNA lesions:

- Etoposide (ETP) is an inhibitor of Topoisomerase II that cuts both strands of the DNA duplex and thereby induces double strand breaks. Cells were treated with 20 µM (A.G. Scientific, Inc.) and collected 1 hour after treatment.

- Hydroxyurea (HU) interferes with DNA replication by decreasing the production of deoxyribonucleotides via inhibition of ribonucleotide reductase. Cells were treated with 2 mM HU (Alfa Aesar) for 16 hours.
- Ionizing radiation (IR) induces predominantly DNA double strand breaks. Cells were irradiated with 2 Gy (Radiotherapy equipment in Hospital Universitario de Canarias, LINAC) and collected 1 hour after treatment.
- Phleomycin (Phleo) induces DNA double strand breaks by binding and intercalating DNA, thus destroying the integrity of the double helix. Cells were treated with 10 μg/ml (IBIAN Technologies) and collected 1 hour later.
- Ultraviolet (UV) light causes thymine base pairs next to each other to bind together into pyrimidine dimers and 6-4 photoproducts. Cells were treated with 40 J/m$^2$ (Philips UV lamp) and were collected 1 hour later.

## 3. PLASMIDS AND CLONING

The library of expression vectors for most human ubiquitin hydrolases and some human SUMO hydrolases. The plasmids have different vector backbones but all with a CMV promoter

| Enzyme | Tag | Resistance | MW(kDa) (incl. tag) |
|---|---|---|---|
| **ATXN3** | FLAG | Amp | 43,4 |
| **BAP1** | FLAG-HA | Amp | 80,4 |
| **CEZANNE** | HA | Amp | 92,5 |
| **CEZANNE** | GFP | Kan | 119,5 |
| **COPS5** | FLAG-HA | Amp | 37,6 |
| **CXORF53** | FLAG-HA | Amp | 36,0 |
| **CYLD** | FLAG-HA | Amp | 107,3 |
| **DUB3** | FLAG-HA | Amp | 59,6 |
| **DUBA1** | FLAG-HA | Amp | 60,6 |
| **DUBA3** | FLAG-HA | Amp | 134,3 |
| **EIF3S3** | FLAG-HA | Amp | 39,9 |
| **EIF3S5** | FLAG-HA | Amp | 37,5 |
| **HDAC6** | FLAG | Amp | 131,4 |
| **JOSD1** | FLAG-HA | Amp | 23,3 |
| **JOSD2** | FLAG-HA | Amp | 20,8 |
| **JOSD3** | FLAG-HA | Amp | 32,1 |
| **MYSM1** | FLAG | Amp | 95,0 |
| **OTUB1** | FLAG-HA | Amp | 31,3 |
| **OTUB2** | FLAG-HA | Amp | 27,2 |
| **OTUD1** | FLAG-HA | Amp | 51,1 |
| **OTUD4** | FLAG-HA | Amp | 124,0 |

| | | | |
|---|---|---|---|
| **OTUD6B** | FLAG-HA | Amp | 33,8 |
| **PARP11** | FLAG-HA | Amp | 38,7 |
| **PSMD14** | FLAG-HA | Amp | 34,5 |
| **SENP1** | FLAG-HA | Amp | 73,5 |
| **SENP2** | FLAG | Amp | 67,9 |
| **SENP2** | GFP | Kan | 94,9 |
| **SENP5** | 3xFLAG | Amp | 86,7 |
| **SENP6** | GFP | Kan | 153,1 |
| **SENP7** | GFP | Kan | 139,2 |
| **SENP8** | FLAG-HA | Amp | 24,1 |
| **STAMBP** | FLAG | Amp | 48,1 |
| **STAMBPL1** | FLAG | Amp | 49,8 |
| **TNFAIP3** | FLAG-HA | Amp | 89,6 |
| **TRABID** | HA | Amp | 81,0 |
| **UCHL1** | FLAG | Amp | 24,8 |
| **UCHL3** | FLAG | Amp | 26,2 |
| **UCHL5** | FLAG | Amp | 37,6 |
| **USP1** | FLAG | Kan | 88,2 |
| **USP10** | FLAG | Kan | 87,1 |
| **USP11** | FLAG | Kan | 109.8 |
| **USP12** | FLAG-HA | Amp | 42,9 |
| **USP13** | FLAG-HA | Amp | 97,3 |
| **USP14** | FLAG | Amp | 56,2 |
| **USP15** | FLAG-HA | Amp | 112,4 |
| **USP16** | FLAG-HA | Amp | 93,6 |
| **USP18** | FLAG | Amp | 43,0 |
| **USP19** | FLAG | Amp | 145,7 |
| **USP2** | FLAG-HA | Amp | 68,1 |
| **USP20** | FLAG-HA | Amp | 102,0 |
| **USP21** | FLAG-HA | Amp | 62,7 |
| **USP22** | FLAG | Amp | 59,9 |
| **USP25** | MYC | Kan | 125,8 |
| **USP26** | FLAG-HA | Amp | 104,0 |
| **USP28** | FLAG | Kan | 122,5 |
| **USP29** | FLAG-HA | Amp | 104,2 |
| **USP3** | FLAG-HA | Amp | 58,9 |
| **USP30** | FLAG | Amp | 58,5 |
| **USP31** | FLAG-HA | Amp | 146,7 |
| **USP33** | FLAG-HA | Amp | 106,7 |
| **USP36** | FLAG-HA | Amp | 122,7 |
| **USP37** | FLAG-HA | Amp | 110.1 |
| **USP38** | FLAG-HA | Amp | 116,6 |
| **USP39** | FLAG-HA | Amp | 65,4 |

| | | | |
|---|---|---|---|
| **USP42** | FLAG-HA | Amp | 145,6 |
| **USP43** | FLAG-HA | Amp | 123,0 |
| **USP44** | FLAG-HA | Amp | 81,2 |
| **USP45** | FLAG-HA | Amp | 91,7 |
| **USP46** | FLAG-HA | Amp | 42,4 |
| **USP47** | FLAG | Amp | 157,3 |
| **USP48** | FLAG-HA | Amp | 119,0 |
| **USP49** | FLAG-HA | Amp | 79,2 |
| **USP5** | FLAG-HA | Amp | 95,8 |
| **USP50** | FLAG-HA | Amp | 39,0 |
| **USP52** | FLAG-HA | Amp | 135,4 |
| **USP53** | FLAG-HA | Amp | 120,8 |
| **USP6** | HA | Amp | 158,7 |
| **USP7** | FLAG | Kan | 128,3 |
| **USP8** | FLAG | Amp | 127,5 |
| **USP9X** | HA | Amp | 289,5 |
| **USPL1** | FLAG-HA | Amp | 120,4 |
| **YOD1** | FLAG | Amp | 38,3 |

Library of chromatin modifiers:

| **Enzyme** | **Tag** | **Resistance** | **MW(kDa)** (incl. tag) |
|---|---|---|---|
| **CLOCK** | FLAG-HIS | Amp | 95 |
| **CUL1** | FLAG | Amp | 90 |
| **CUL2** | FLAG | Amp | 87 |
| **CUL3** | FLAG | Amp | 89 |
| **CUL4A** | FLAG | Amp | 80,8 |
| **CUL4B** | FLAG | Amp | 103 |
| **CUL5** | FLAG | Amp | 90,9 |
| **DDB1** | FLAG | Amp | 127 |
| **DDB2** | MYC | Amp | 48 |
| **HAT1** | FLAG | Amp | 49,5 |
| **KAT2A** | FLAG-HA | Amp | 94 |
| **KAT2B** | FLAG-HA | Amp | 93 |
| **KDM1A** | FLAG | Amp | 110 |
| **KDM1B** | FLAG-HA | Amp | 109 |
| **KDM2A** | FLAG | Amp | 132,8 |
| **KDM2B** | FLAG-2xSTREP2 | Amp | 255,9 |
| **KDM3A** | FLAG-HIS-MYC | Amp | 147,3 |
| **KDM3B** | HA | Amp | 192 |
| **KDM4A** | HA | Amp | 121 |
| **KDM4B** | HA | Amp | 121,9 |

| | | | |
|---|---|---|---|
| **KDM4C** | FLAG-HA | Amp | 121 |
| **KDM5A** | FLAG-HA | Amp | 192 |
| **KDM5B** | MYC | Amp | 190 |
| **KDM5C** | FLAG | Amp | 180 |
| **KDM6A** | HA | Amp | 158,8 |
| **KDM6B** | HA | Amp | 176 |
| **PHF2** | FLAG | Amp | 121 |
| **PHF8** | FLAG | Amp | 135 |
| **SKP2** | MYC | Amp | 49 |

Other plasmids used:

| **Plasmid** | **Tag** | **Resistance** | **MW (kDa)** (incl. tag) |
|---|---|---|---|
| **BRCC36** | FLAG-HA | Amp | 36 |
| **DUB3 WT** | FLAG | Amp | 59,6 |
| **DUB3 WT** | GFP | Kan | 86,6 |
| **DUB3 CS/HQ/DN** | FLAG | Amp | 59,6 |
| **DUB3 CS/HQ/DN** | GFP | Kan | 86,6 |
| **H2AX** | FLAG | Amp | 15,2 |
| **KDM3A WT** | FLAG-HIS-MYC | Amp | 147,3 |
| **KDM3A H1122A** | FLAG-HIS-MYC | Amp | 147,3 |
| **OTUB1** | FLAG-HA | Amp | 31,3 |
| **PCNA WT** | GFP | Kan | 56 |
| **PCNA WT** | MYC | Amp | 29,8 |
| **PCNA WT** | GST | Amp-Chl | 57 |
| **PCNA K164R** | GFP | Kan | 56 |
| **PCNA K164R** | MYC | Amp | 29,8 |
| **PCNA M40A** | GST | Amp-Chl | 57 |
| **Rad18** | YFP | Kan | 82,6 |
| **RNF168** | GFP | Kan | 92 |
| **RNF8** | HA | Amp | 55,5 |
| **SENP5 WT** | 3XFLAG | Amp | 86,7 |
| **SENP5 C713S** | 3XFLAG | Amp | 86,7 |
| **SupF (pSP189)** | --- | Amp | --- |
| **Ubiquitin** | HIS | Amp | 8,5 |
| **USP16** | FLAG-HA | Amp | 93,6 |
| **USP28** | FLAG | Kan | 122 |
| **USP3** | FLAG-HA | Amp | 58,9 |
| **USP44** | FLAG-HA | Amp | 81,2 |

pMEF Flag-DUB3 wild type (WT) was kindly provided by J.F. Burrows (Queen's University, Belfast, Northern Ireland).

pMEF Flag-DUB3 C89S/H334Q/D350N, a catalytic inactive version (CI), was generated using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). DUB3 WT and CI were cloned into peGFP-C1 to generate GFP-fusion vectors.

pcDNA3.1 Flag-H2AX was kindly shared by L. Penengo (University of Piemonte Orientale A. Avogadro, Novara, Italy), HA-RNF8 by T.M. Thomson (IBMB, Barcelona, Spain), RNF168-GFP by G.S. Stewart (University of Birmingham, United Kingdom) and Flag-HA-USP3 by R.A. Greenberg (University of Pennsylvania, USA). pcDNA4 Flag-His-Myc KDM3A wild type and H1122A were obtained from Addgene (#38136 and 38149). An expression plasmid for His-Ubiquitin (pMT107) was a gift from D. Bohmann (Rochester, New York, USA). YFP-RAD18 was kindly provided by A. Inagaki (Erasmus MC, Rotterdam, The Netherlands).

USP28 cDNA was a gift from G. Marfany (Barcelona University, Barcelona, Spain) and was cloned in pCMVTag2B (Agilent Technologies) to generate Flag-tagged protein.

Flag-SENP5 wild type plasmid was provided by Grace B. Gill (University of California, Berkeley, USA) and a catalytic inactive mutant (C713S) was obtained using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies).

## 4. PROTEIN DETECTION

### 4.1. Whole cell extracts

Whole cell extracts were made using Laemmli buffer and removing the lysate with a cell scraper (Greiner bio-one). After that, samples were heated at 96ºC during 5 minutes and sonicated for 15 seconds at 80% frequency (UP1000H, Hielsher Ultrasonic). Samples were stored at -20ºC. Before loading on SDS-PAGE gel, loading buffer was added.

| Laemmli buffer (2x) | Loading buffer (10x) |
|---|---|
| 4% SDS<br>20% Glycerol<br>120 mM TRIS pH 6,8 | 0,1% Bromophenol Blue<br>5% β-Mercaptoethanol |

### 4.2. Histone acid extraction

For acid extraction of histones, cells were collected and washed twice in cold PBS. After that, cells were resuspended in Triton Extraction Buffer (TEB) at $10^7$ cells/ml density and incubated on ice for 10 minutes. Subsequently, nuclei were centrifuged at 4ºC during 10 minutes at 2000 rpm after which the supernatant was discarded. The pellet was washed in half volume of TEB and centrifugation was repeated. Then the pellet was resuspended on 0,2 N HCl at 4x$10^7$ cells/ml density and incubated overnight at 4ºC. The acid was responsible for the extraction of histones. The next day, after centrifugation at 4ºC during 10 minutes at 2000 rpm, the supernatant was collected and protein content was determined. Samples were stored at -20ºC.

| **TEB** |
|---|
| PBS containing<br>0,5% Triton X-100<br>2mM PMSF<br>0,02% $NaN_3$ |

### 4.3. Protein quantification

The protein concentration of samples was quantified using the Bicinchoninic Acid (BCA) method, in a 96 well plate. A calibration curve using increasing known amounts of bovine serum albumin protein (BSA) was used as reference. BCA Protein Assay Kit (Novagen, Sigma) was used to obtain a 50:1 bicinchoninic acid:copper sulphate solution. 150 µl of this solution was added to each well. The plate was incubated at 37ºC for 30 minutes, after which the absorbance was measured at 540 nm wavelength with a spectrophotometer (Labsystems). The absorbance values obtained for the samples and the calibration curve were processed by linear regression of Pearsons, thereby obtaining the protein concentration of each sample.

Calibration curve:

| *µg BSA* | *0* | *2* | *5* | *10* | *15* | *20* | *30* | *40* |
|---|---|---|---|---|---|---|---|---|
| *BSA(2mg/ml)* | 0 µl | 1 µl | 2,5 µl | 5 µl | 7,5 µl | 10 µl | 15 µl | 20 µl |
| *$H_2O$* | 20 µl | 19 µl | 17,5 µl | 15 µl | 12,5 µl | 10 µl | 5 µl | 0 µl |
| *Lysis Buffer* | 5 µl | 5 µl | 5 µl | 5 µl | 5 µl | 5 µl | 5 µl | 5 µl |

Samples:

| *Sample* | 5 µl |
|---|---|
| *$H_2O$* | 20 µl |

## 4.4. Western blot

### 4.4.1. Electrophoresis

Protein analysis was performed in SDS polyacrylamide (PAA) gels of different percentages (between 6% and 12%) depending on the size of analysed proteins. Between 20 and 40 µg of total protein was loaded per sample, which previously were heated at 96ºC for 5 minutes. Electrophoresis was carried out using a mini-protean 3 system (BioRad), at 130-160 V in SDS-PAGE buffer, until the front left the gel.

| Stacking gel | Running gel |
|---|---|
| 125 mM TRIS pH 6,8<br>0,1% SDS<br>5% acrylamide:bisacrylamide (29:1) | 0,75 M TRIS pH 8,8<br>0,2% SDS<br>6-12% acrylamide:bisacrylamide (29:1) |

| Electrophoresis buffer |
|---|
| 25 mM TRIS<br>192 mM Glycine<br>0,1% SDS |

### 4.4.2. Gel transference and blocking

After electrophoresis, proteins were transferred to a nitrocellulose membrane (Protran BA 85, Whatman), using the mini-protean system 3 (BioRad), in transfer buffer at 265-295 mA for one hour. Blocking of the membranes was performed in a 5% skimmed milk (Sveltesse, Nestlé) in TBS + 0,1% Tween 20 (Sigma Aldrich, TBS-T) for one hour while gently shaking at room temperature.

| Transfer buffer | TBS pH 7,4 |
|---|---|
| 25 mM TRIS<br>192 mM Glycine<br>20% Ethanol | 25 mM TRIS<br>10 mM KCl<br>270 mM NaCl |

### 4.4.3 Immunodetection and antibodies

Immunodetection of transferred proteins was performed by incubating the membrane with antibodies or (purified) immune serum diluted in 5% skimmed milk in TBS-T overnight at 4°C under gentle shaking. Then, membranes were washed in TBS-T five times for 5 minutes each and subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch) diluted 1:10,000 in TBS-T, for 1 hour at room temperature. After this incubation, 5 washes of 5 minutes in

TBS-T were performed and finally the blots were incubated in a 1:1 mix of LumiSensor Chemiluminescent HRP solutions (GenScript) for 5 minutes at room temperature.

Chemiluminescence detection was carried out using X-ray films (Kodak) or the ImageQuant LAS 4000 mini equipment (GE Healthcare).

Primary antibodies used:

| **Antibody** | **Origin** | **Dilution** | **Source** |
|---|---|---|---|
| **FK2**<br>(conjugated ubiquitin) | Mouse | 1:1000 | Millipore |
| **FLAG** | Mouse | 1:1000 | GenScript |
| **HA** | Mouse | 1:2000 | Roche |
| **H2A** | Rabbit | 1:3000 | Millipore |
| **H2A** | Rabbit | 1:3000 | GenScript |
| **H2AX** | Rabbit | 1:5000 | Bethyl |
| **H2B** | Rabbit | 1:4000 | GenScript |
| **Ku86** | Goat | 1:4000 | Santa Cruz Biotechnology Inc. |
| **MYC** | Mouse | 1:2000 | Santa Cruz Biotechnology Inc. |
| **PCNA** | Mouse | 1:500 | Santa Cruz Biotechnology Inc. |
| **PCNA** | Mouse | 1:500 | Abcam |
| **Ub-H2A** | Mouse | 1:1000 | Millipore |
| **Ub-H2AX** | Mouse | 1:1000 | Millipore |
| **Ub-Lys63** | Mouse | 1:1000 | Millipore |
| **β-actin** | Mouse | 1:5000 | GenScript |
| **γ-H2AX** | Mouse | 1:1000 | Millipore |
| **γ-H2AX** | Rabbit | 1:1000 | GenScript |
| **BRCA1** | Rabbit | 1:2000 | Homemade* |
| **DUB3** | Rabbit | 1:500 | Homemade* |
| **GFP** | Rabbit | 1:2000 | Homemade* |
| **KDM3A** | Rabbit | 1:1000 | Homemade* |
| **MDC1** | Rabbit | 1:1000 | Homemade* |
| **Rad18** | Rabbit | 1:2000 | Homemade* |
| **RNF168** | Rabbit | 1:1000 | Homemade* |
| **RNF8** | Rabbit | 1:1000 | Homemade* |
| **SENP5** | Rabbit | 1:5000 | Homemade* |
| **USP28** | Rabbit | 1:2000 | Homemade* |
| **53BP1** | Rabbit | 1:2000 | Homemade* |

* Homemade antibodies were generated by injecting rabbits with His-tagged antigens that were obtained by expression in bacteria followed by purification with a Ni-NTA resin (Qiagen) following manufacturers recommendations.

## 4.5. Immunoprecipitation

For immunoprecipitations cells were washed with cold PBS by centrifugation at 1500 rpm for 5 minutes. Then the cells were lysed in 1 ml EB150 buffer (for a 10 cm TC dish) and incubated for 20 minutes on ice.

| **EB150 buffer** |
|---|
| 50 mM Hepes<br>150 mM NaCl<br>2 mM $MgCl_2$<br>5 mM EGTA<br>1 mM DTT<br>0,5% NP-40<br>10% Glycerol<br>1 mM $Na_3VO_4$<br>5 mM NaF<br>Protease inhibitor cocktail (Calbiochem) |

Lysates were cleared by centrifugation at maximum speed at 4ºC for 20 minutes. 50 µl of supernatant was taken for input, to which sample buffer was added. The rest of the lysate was incubated with beads for 3 hours under agitation at 4°C. This step was performed in three different ways depending on the experiment: For immunoprecipitation against Flag-tag, 10 µl of anti-Flag-sepharose (M2, Sigma) was used. For immunoprecipitation against HA-tag, 20 µl anti-HA affinity matrix (Roche) was used. For other immunoprecipitations, 3 µl of antibody against the target protein was added together with 20 µl of Protein A resin (GenScript). The resins were collected by gentle centrifugation, 4000 rpm for 1 minute, and washed 4 times with EB150 buffer. Proteins bound to the resin were eluted using 50 µl of sample buffer and heated at 96°C for 5 minutes.

| **Sample buffer** |
|---|
| 250 mM TRIS pH 6,8<br>40% Glycerol<br>8% SDS<br>0,1% Bromophenol Blue<br>2% β-Mercaptoethanol |

## 4.6. GST - pulldown

### 4.6.1. Protein expression

GST constructs (GST, GST-PCNA and GST-PCNA M40A) were transformed into bacterial cells optimized for protein expression (BL21). The transformation reaction was inoculated in 1 ml SOC medium (Sigma Aldrich) and incubated for 1 hour at 37ºC for recovery. Transformed bacteria were inoculated in 50 ml LB (25 g/l, Fluka Analytical) with Ampicillin 50 µg/ml and Chloramphenicol 25 µg/ml culture (LB-Amp-Chl).

The next morning, the 50 ml culture was transferred into a big flask with 300 ml fresh LB-Amp-Chl medium and grown at 37ºC until OD600=0.6 (about 1 hour) when protein expression was induced by adding IPTG (Sigma Aldrich) at 1 mM. The culture was incubated for 5 hours at room temperature before centrifugation at 6000 g for 15 minutes. Samples were taken before and after induction as controls, which were analysed on a Coomassie gel.

| **Coomassie Staining Solution** | **Destaining solution** |
|---|---|
| 0,1% Coomassie Brilliant Blue<br>50% Methanol<br>10% Glacial Acetic Acid | 40% Methanol<br>10% Glacial Acetic Acid |

### 4.6.2. GST protein purification

For lysis, the bacterial pellet was resuspended and vortexed in 20 ml cold lysis.

| **Lysis buffer (50 ml)** |
|---|
| PBS supplemented with<br>50 mg lysozyme<br>1mM PMSF<br>1mM EDTA<br>1 protease inhibitor cocktail pill (Roche) |

The lysate was incubated at 4ºC for 30 minutes with rotation, then sonicated 6 times 30 seconds (output level 3). 2 ml of Triton 10% (Calbiochem) was added and incubated at 4ºC for 30 minutes with rotation. Susequently, the lysate was cleared by centrifugation for 30 minutes at 18000 g at 4ºC. An “extract” sample was taken, to which sample buffer was added. 300 µl of prewashed Glutathione Sepharose beads (GenScript) was added, incubated for 3 hours at 4ºC with rotation followed by centrifugation for 1 minute at 2000 rpm, 4ºC. The supernatant was transferred to new tubes, and a “supernatant” sample was taken. The beads were washed once with Buffer

1, twice with Buffer 2 and finally once with PBS. The washes were performed as follows: 5 minutes incubation at 4ºC with rotation, followed by centrifugation for 1 minute at 2000 rpm at 4ºC, aspiration of supernatant and adding the next buffer.

| GST Buffer 1 | GST Buffer 2 |
|---|---|
| PBS supplemented with<br>300 mM NaCl<br>1% Triton | PBS supplemented with<br>0,1% Triton |

After washing, beads were pelleted, 150 µl cold PBS was added and the slurry was stored at 4ºC. A "beads" sample was taken. The induction and expression of the proteins was checked on a Coomassie gel with all control samples.

### 4.6.3. GST-binding

For studying *in vitro* interactions, 293T cells were transfected with the protein of interest. Two days later, cells were washed in PBS and lysed for 30 min in 1 ml IP lysis buffer + inhibitors (PMSF, NaF, NEM and complete protease inhibitors) at 4ºC.

| IP lysis buffer |
|---|
| 150 mM NaCl<br>1% NP40<br>50 mM TRIS pH 7,8<br>2 mM EDTA |

The lysate was cleared by centrifugation at maximum speed for 10 minutes at 4ºC. The supernatant was transferred in new tube, 30 µl of precleaning beads was added and incubated for 30 minutes at 4ºC with rotation. The beads were centrifuged at 2000 rpm for 1 minute and a small sample of the supernatant was taken for input. The lysate was then incubated with 60 µl GST-protein beads, previously purified as above, and incubated at 4ºC for 3 hours with rotation. Samples were centrifuged at 2000 rpm for 1 minute and washed 6-8 times with 750 µl of IP lysis buffer. Proteins were eluted from the beads with 30 µl of sample buffer.

## 4.7. Immunofluorescence

For immunostaining, cells grown on coverslips, were washed twice with PBS, fixed for 20 minutes at room temperature and then permeabilized for 5 minutes. After washing with PBS, samples were blocked in PBS+1% FBS and incubated with primary antibodies diluted in PBS+1% FBS at 4ºC overnight.

| Fixing buffer | Permeabilizing buffer |
|---|---|
| PBS supplemented with<br>2% Paraformaldehyde<br>0,2% Triton | PBS supplemented with<br>0,1% Triton |

Primary antibodies used for immunofluorescence:

| Antibody | Origin | Dilution | Source |
|---|---|---|---|
| **FK2**<br>(conjugated ubiquitin) | Mouse | 1:1000 | Millipore |
| **FLAG** | Mouse | 1:1000 | GenScript |
| **γ-H2AX** | Mouse | 1:800 | Millipore |
| **BRCA1** | Rabbit | 1:500 | Homemade |
| **MDC1** | Rabbit | 1:800 | Homemade |
| **53BP1** | Rabbit | 1:500 | Homemade |

The next day, the coverslips were washed in PBS and incubated with Alexa-conjugated secondary antibodies (Invitrogen) diluted 1:600 in PBS for 1 hour, protected from the light. After washing, the coverslip was assembled on a slide, at the same time staining the DNA with DAPI (SlowFade, Invitrogen) and sealed with nail polish.

Images were taken using a Cell Observer fluorescent microscope equipped with Axiovision software (Zeiss). For quantification of focus formation, more than 100 cells were analysed for each sample and error bars present the standard error. Cells with more than 5 foci were scored as positive.

## 5. PROTEIN PURIFICATION AND ENZYMATIC ASSAYS

### 5.1. Protein purification

For protein purification of ubiquitinated Flag-H2AX, Flag-DUB3 WT/CI and Flag-USP28, 293T cells were transfected with the corresponding expression vectors. Cells overexpressing Flag-H2AX were treated with UV (40 J/m2, 1 hour) to increase ubiquitination of H2AX. Cells were washed in PBS and lysed in EB150 lysis buffer (described before) for 20 minutes on ice. For the DUBs, no inhibitors were added. In case of Flag-H2AX, the buffer was supplemented with protease inhibitors and 2 mM N-ethylmaleimide (NEM, Sigma) and extracts were sonicated 8 times 15 seconds. After centrifugation at 13000 rpm for 20 minutes, extracts were incubated with anti-Flag M2 agarose (Sigma) for 2 hours at 4°C, followed by 4 washes with lysis buffer and 1 wash in elution buffer. Proteins were eluted in elution buffer supplemented with 330 μg/ml Flag peptide (DYKDDDDK, Genscript) for 1,5 hours at 4°C.

| Elution buffer |
|---|
| 50 mM TRIS pH 7,5 |

The supernatant was collected, 10% glycerol was added and aliquots were stored at -20°C. Samples to check for expression and purification were taken before and after purification and loaded on a PAA gel for western blotting with the corresponding antibodies.

### 5.2. *In vitro* deubiquitin assay

To investigate deubiquitination *in vitro,* ubiquitinated H2AX and different ubiquitin hydrolases (DUB3 WT, DUB3 CI and USP28) were purified as described above. Then H2AX and DUB were mixed in deubiquitin assay buffer and incubated for 2 hours at 37°C. When indicated, 2 mM N-ethylmaleimide (NEM), a DUB inhibitor, was added as negative control. Sample buffer was added to stop the reaction. H2AX ubiquitination was subsequently analysed by western blotting with anti-H2AX antibodies.

| Deubiquitin assay buffer |
|---|
| 50 mM TRIS pH 7,5<br>4 mM DTT |

### 5.3. DUB activity assay

For the DUB activity assay, cells were transfected with different DUB expression vectors. Then the cells were collected, washed in PBS and lysed in lysis buffer for 20 minutes on ice.

| DUB activity lysis buffer |
|---|
| 50 mM TRIS pH 7,4<br>5 mM $MgCl_2$<br>250 mM sucrose<br>0,1% NP40<br>1 mM DTT<br>2 mM ATP |

The extracts were cleared by centrifugation at 13000 rpm for 20 minutes and incubated with 50 μM HA-Ubiquitin-Vinyl Sulfone (Boston Biochemicals) for 1 hour at 37°C, when indicated in the presence of 2 mM NEM. Subsequently, the samples were incubated with washed anti-HA affinity matrix (Roche Diagnostics) for 2 hours at 4°C, followed by 4 washes with lysis buffer, after which sample buffer was added to stop the reaction. Samples were analysed by western blotting for the DUB of interest. An active DUB irreversibly binds HA-Ub-VS and consequently runs with lower mobility on an SDS-PAGE gel.

## 6. TRANSLESION SYNTHESIS ASSAYS BY MUTAGENESIS OF PLASMID pSP189

Translesion synthesis assay was carried out in 293T cells. During the protocol, cell confluency was monitored carefully. When cells became confluent before the day of lysis, the cells were split. Cells (6 cm TC dish), at 30-40% confluency, were transfected with siRNA oligos as described above. Cells were washed the next day. 48 hours after transfection, the SupF (psp189) plasmid was transfected using GeneJuice reagent (Novagen). To induce mutagenesis, the plasmid was UV irradiated just before transfection: drops of plasmid were put on parafilm and exposed to 1000 J/m$^2$ in a UV Stratalinker. 24 hours later, the medium was replaced and cells were harvested two days after by scraping and centrifuging. Plasmid DNA was extracted from the pellet using Promega Wizard Miniprep SV Kit. For elution 4x 100 μl of pre-warmed nuclease free water was used. The plasmid DNA was digested with Dpnl (NEB) for 2-3 hours at 37ºC and subsequently precipitated at 4ºC overnight using 3 M NaAcetate pH 5,2 and chilled 100% EtOH. Finally, plasmid DNA was centrifuged, washed and the dried pellet was resuspended in 10 μl nuclease-free water. 1 μl DNA was used to transform MBM7070 indicator bacteria by electroporation (Biorad GenePulser; 2,5 kV, 25 μF, 200 ohms).

LB-Amp plates were coated with 40 μl of 50mg/ml X-Gal (Promega) and 100 μl of 50 mg/ml IPTG and dried at 37ºC. After electroporation and recovery in SOC media, 10 and 100 μl transformed bacteria were plated in these plates and incubated at 37ºC for 2 days in the dark. The rest was stored in the fridge for the next day, when bacteria were plated again based on the plating efficiency obtained. The aim was to obtain 2,000 colonies/plate. Whereas most colonies were blue, around 1% was white due to expression of the mutated plasmid. Mutation efficiency was calculated by counting at least 10,000 colonies in total. The ratio of white (mutant) to total (blue + white) colonies was scored as mutation frequency.

# III. RESULTS

## 1. DUB SCREENING FOR H2AX AND PCNA

The DNA damage response (DDR) is a tightly regulated process in which post-translational modifications play a crucial role. Whereas it was initially thought that the DNA damage checkpoints predominantly function as a kinase cascade, recent research demonstrated that ubiquitination or SUMOylation of DDR proteins also significantly contribute to this response. Proteins can be modified by monoubiquitination or polyubiquitination. Monoubiquitination is the conjugation of a single ubiquitin molecule to a lysine residue in the target protein. Numerous cellular proteins are described to be monoubiquitinated and this monoubiquitination often acts as a regulator of the localization and activity of the target proteins (193).

Moreover, ubiquitin itself carries lysines that act as sites of self-conjugation by which poly-ubiquitin chains can be formed. In these poly-ubiquitin chains the C-terminal glycine of ubiquitin is attached to a lysine residue of the other ubiquitin molecule (194). Polyubiquitination via Lys48 linkage generally directs a protein for proteasome-dependent degradation. Other functions for polyubiquitination controlled by different ways of ubiquitin conjugation, for example signalling, are known. As such, Lys63 polyubiquitination of H2AX acts to recruit other proteins to sites of DNA lesions (29; 30).

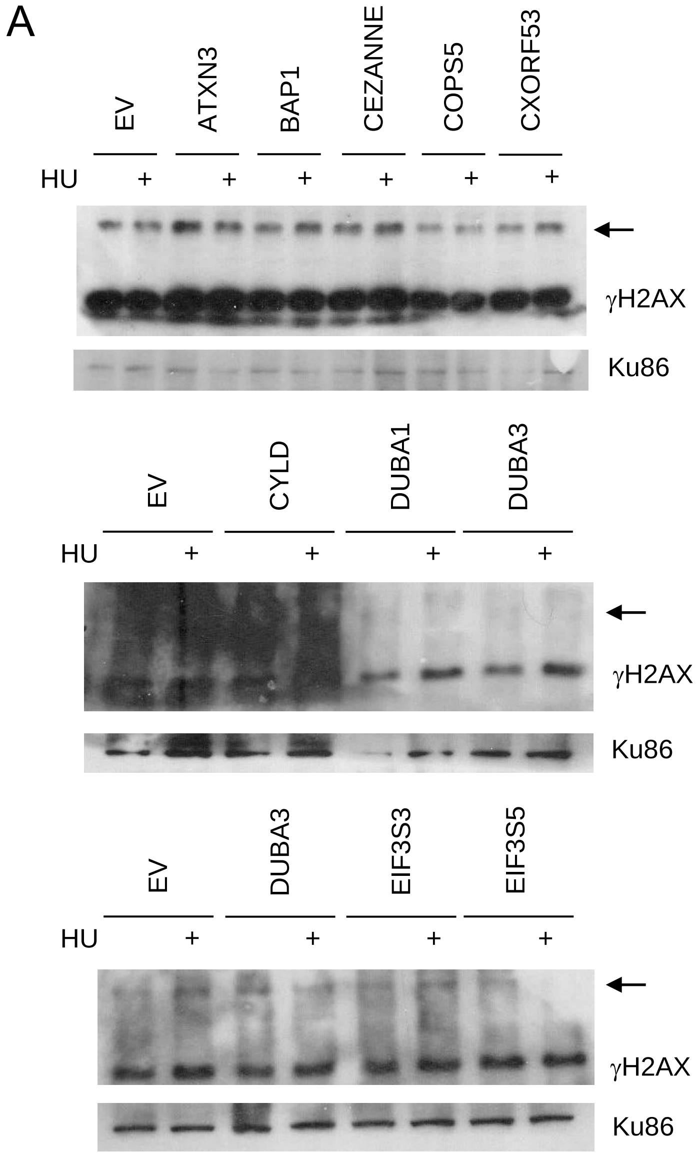
Many DDR proteins are regulated by ubiquitination: protein levels are regulated by poly-ubiquitin-mediated proteasomal degradation but also mono- and polyubiquitination is used as ´signalling´. The human genome encodes over 600 putative E3 ligases, the ubiquitin enzyme responsible for substrate recognition, providing tremendous substrate diversity. Ubiquitination is a reversible process and deubiquitinating enzymes (DUBs) are proteases that cleave ubiquitin from substrate proteins. Various ubiquitin E3 ligases have been identified in the DDR, for instance, RNF8 and RNF168 have been described as E3 ligases for H2AX and Rad18 as the E3 ligase for PCNA (26; 27; 122; 146). However, less is known about the reversible process by ubiquitin hydrolases in this response.

We set out to identify novel regulators in the DDR among the nearly 100 DUB genes existing in humans, by performing a screening overexpressing ubiquitin hydrolases. We considered this type of screening a good complementation to the existing siRNA screenings as redundancy might mask some potential candidates in conditions of downregulation. We chose to search for novel enzymes regulating histone H2AX and PCNA, both monoubiquitinated in response to genotoxic stress and crucial for different aspects of the DDR.

### 1.1. H2AX screening

To perform the DUB screening for novel enzymes responsible for deubiquitination of H2AX, the optimal conditions for the screening were characterized. We chose 293T cells because of the high transfection efficiency achieved in these cells. 293T cells were subjected to different treatments inducing DNA damage and changes in monoubiquitination levels were detected by western blot using different H2AX antibodies. The treatments used were: 2mM hydroxyurea (HU) for 16 hours, 10mM HU for 2 hours, 20 μM etoposide (ETP) for 1 hour and 0,5 μM doxorubicin (DOX) for 1 hour. Treatment of cells with 2mM HU for 16 hours followed by detection of H2AX monoubiquitination by mobility shift using a γH2AX (phosphorylated H2AX) antibody gave the best results for DNA damage-induced H2AX monoubiquitination (data not shown).

For the screening, 293T cells were transfected with an empty vector as negative control in addition to 6-8 different DUB expression vectors in every experiment. Cells were left untreated and/or treated with 2mM HU for 16 hours. As the amount of DNA damage-induced H2AX monoubiquitination varied somewhat between experiments, the effect of overexpression of each DUB was compared to the empty vector control in each experiment (Figure 1A-G). In some cases, when the possible effect on H2AX monoubiquitination was not clear, transfection of a DUB expression vector was repeated.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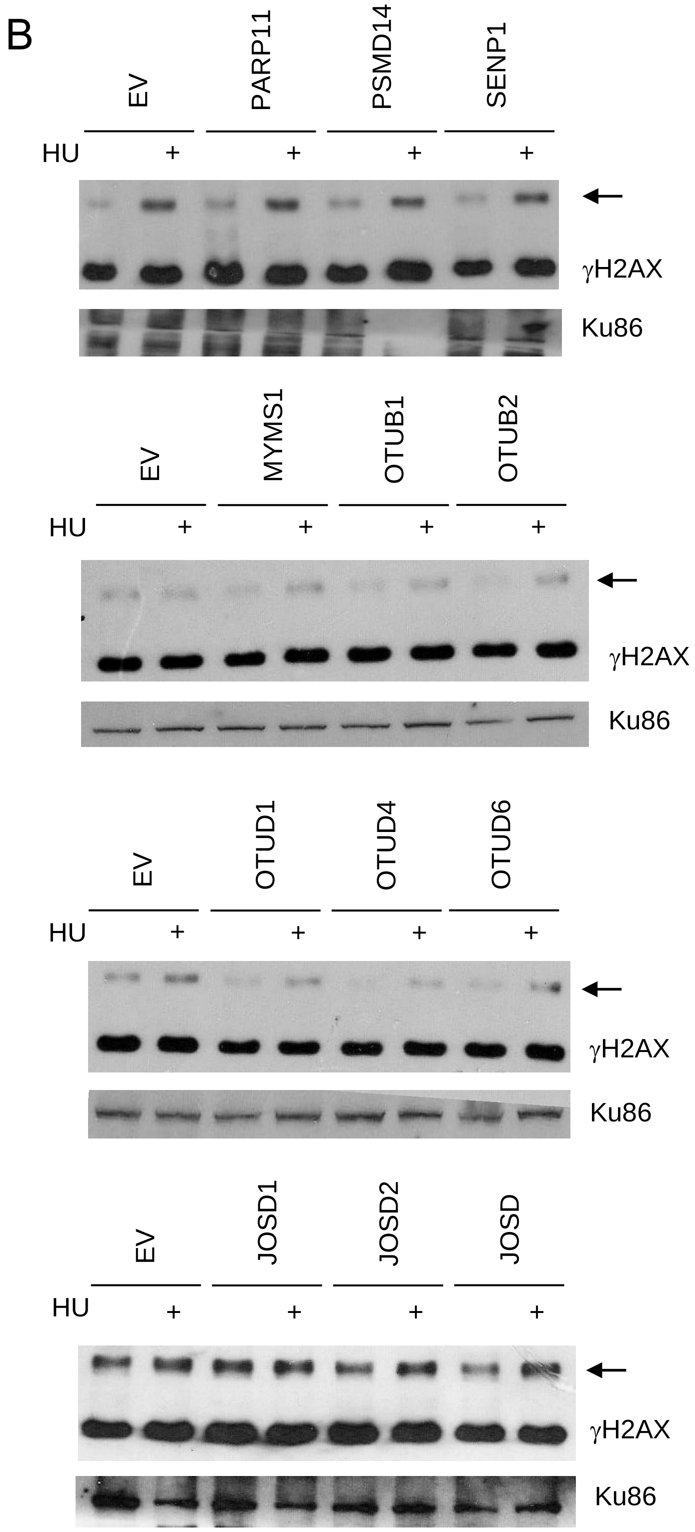
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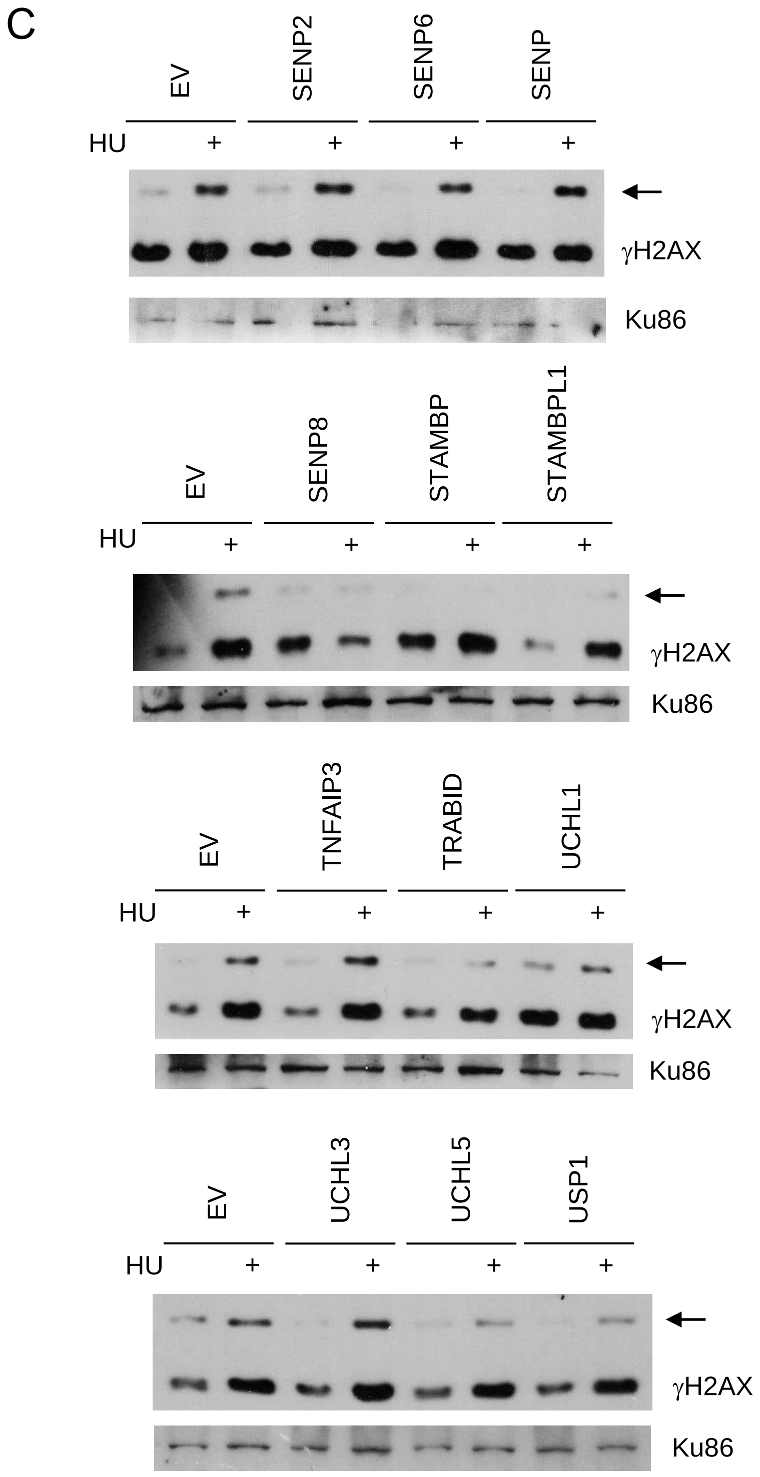
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+
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TNFAIP3
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HU
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+
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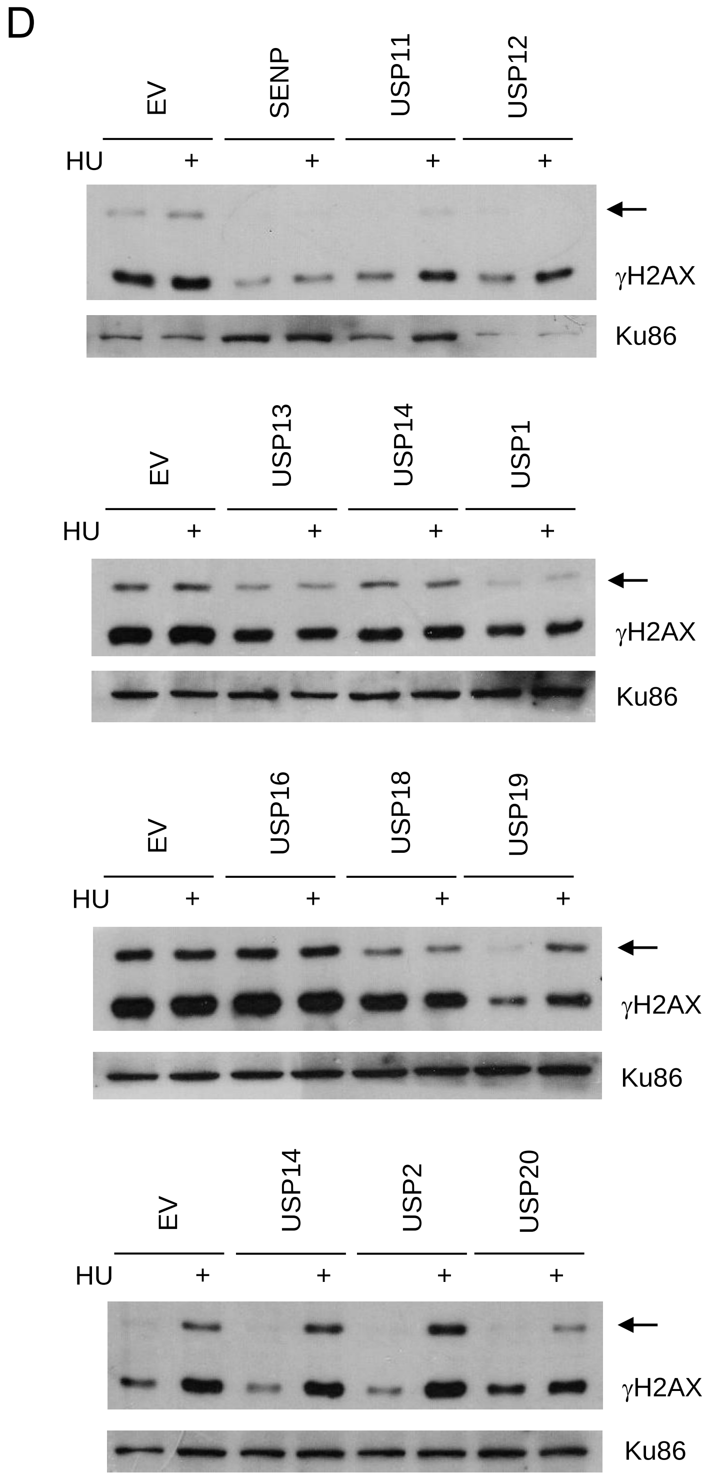
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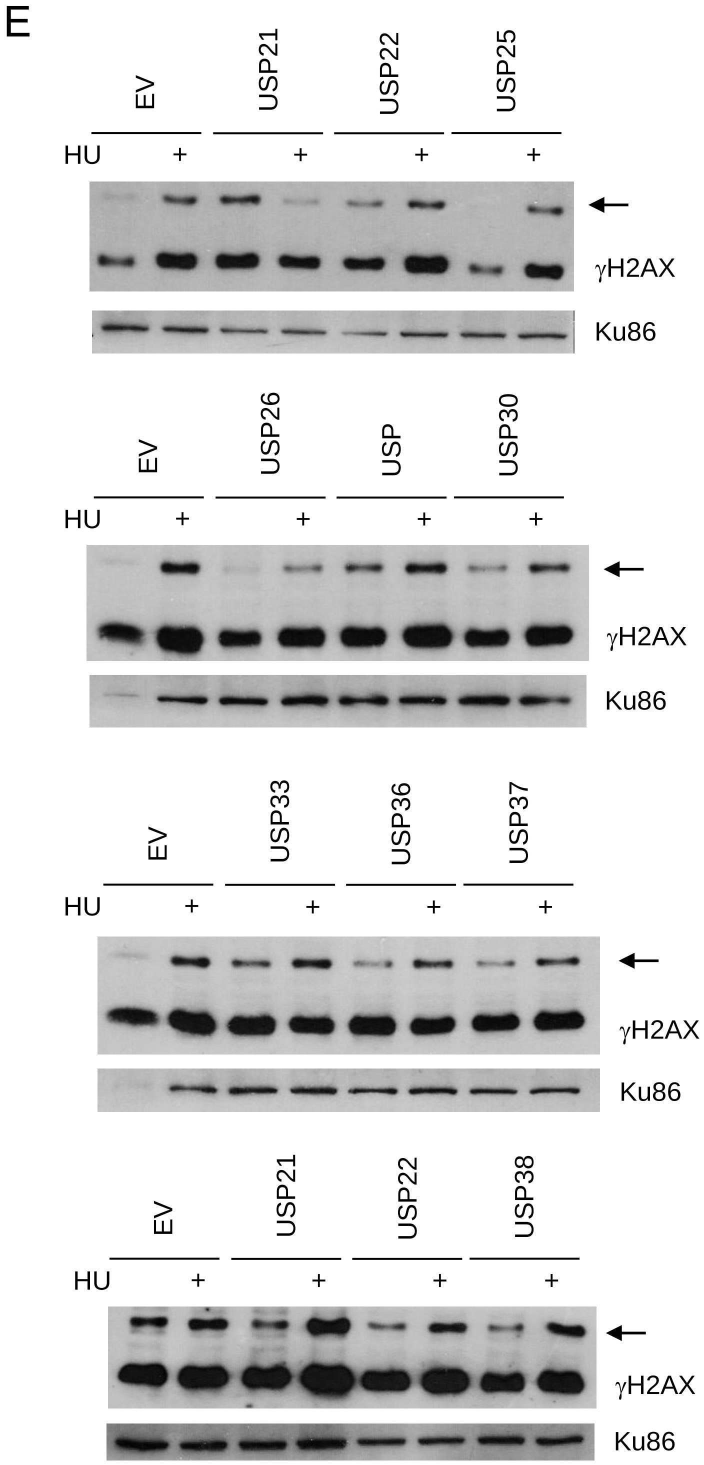
Ku86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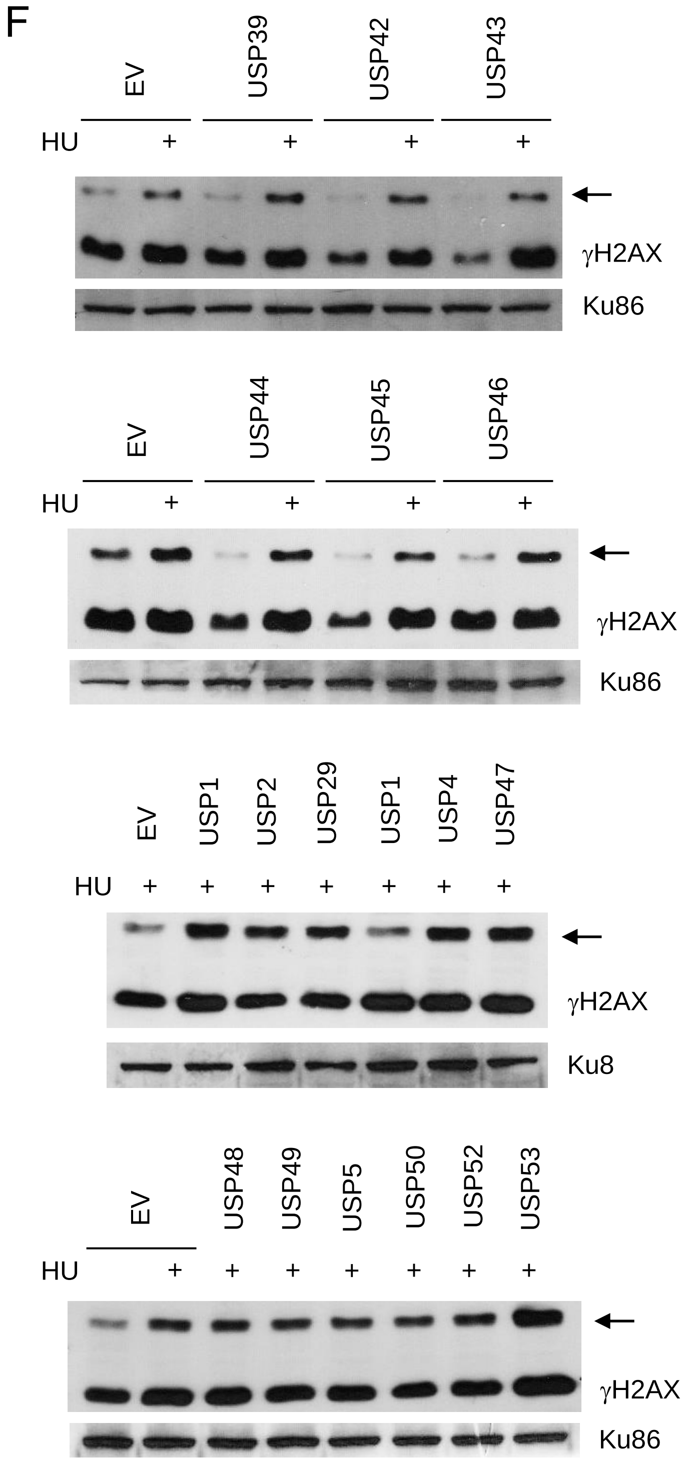
Ku86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USP42
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γH2AX
Ku86
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Ku86
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USP4
USP47
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Ku86

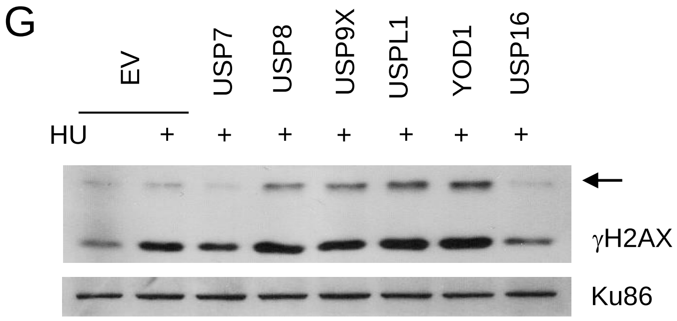


**Figure 1: DUB overexpression screening for H2AX ubiquitination in 293T cells.** A-G) Cells transfected with the indicated expression vectors were left untreated or treated with 2 mM HU for 16 h. Whole cell extracts were analysed by western blot with the indicated antibodies. Arrow indicates monoubiquitination of H2AX. Ku86 serves as loading control.

Expression of most DUBs did not result in changes in levels of HU-induced H2AX monoubiquitination (Figure 1A-G). However, H2AX monoubiquitination decreased, both in untreated conditions and after treatment with HU, after overexpression of ubiquitin hydrolase DUB3 (USP17L2) (Figure 2). DUB3 therefore was a potential candidate enzyme responsible for the deubiquitination of histone H2AX.

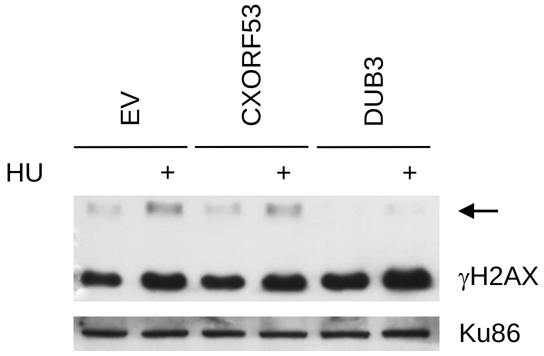
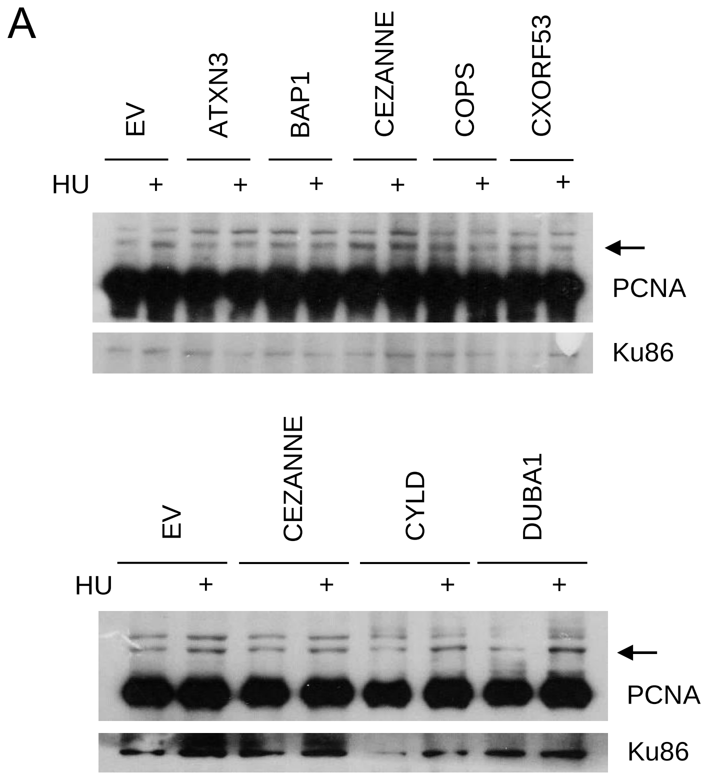


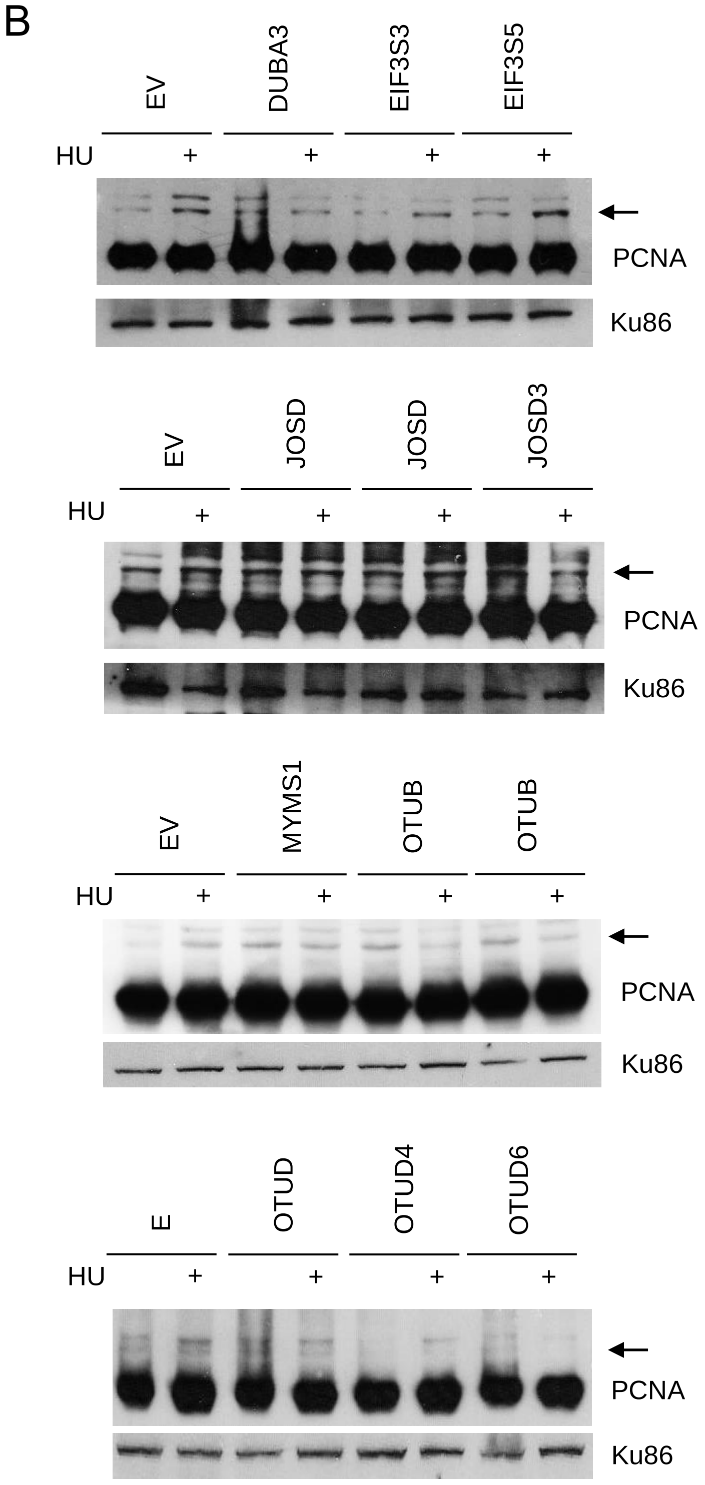
**Figure 2: DUB3 as candidate for regulating H2AX monoubiquitination.** 293T cells were transfected with the indicated vectors and treated with 2 mM HU for 16 h. H2AX monoubiquitination was analysed by western blot. Arrow indicates monoubiquitination of H2AX.

### 1.2. PCNA screening

To search for possible candidates involved in the regulation of PCNA monoubiquitination, the optimal conditions for visualizing PCNA monoubiquitination by western blot were determined. 293T cells were subjected to different DNA damaging treatments to induce PCNA monoubiquitination. The treatments used were: 2 mM HU for 16 hours, 10 mM HU for 2 hours, UV light (20 and 40 J/m$^2$) for 1 hour and 0,02% methyl methanesulfonate (MMS) for 1 hour. Treating cells with HU (2 mM, 16 hours) or UV light (40 J/m$^2$, 1 hour) both resulted in efficient PCNA monoubiquitination (data not shown). For practical reasons overnight treatment with HU was chosen.

All DUBs present in the library were individually transfected in 293T cells. As for the H2AX screening, an empty vector was transfected in every experiment as a negative control. Cells were left untreated or treated with 2 mM HU for 16 hours to stimulate monoubiquitination of PCNA. The effect of overexpression of all DUBs on HU-induced PCNA monoubiquitination was shown in Figure 3A-G. In some cases, when the result was not clear, transfection of a DUB expression vector was repeated.

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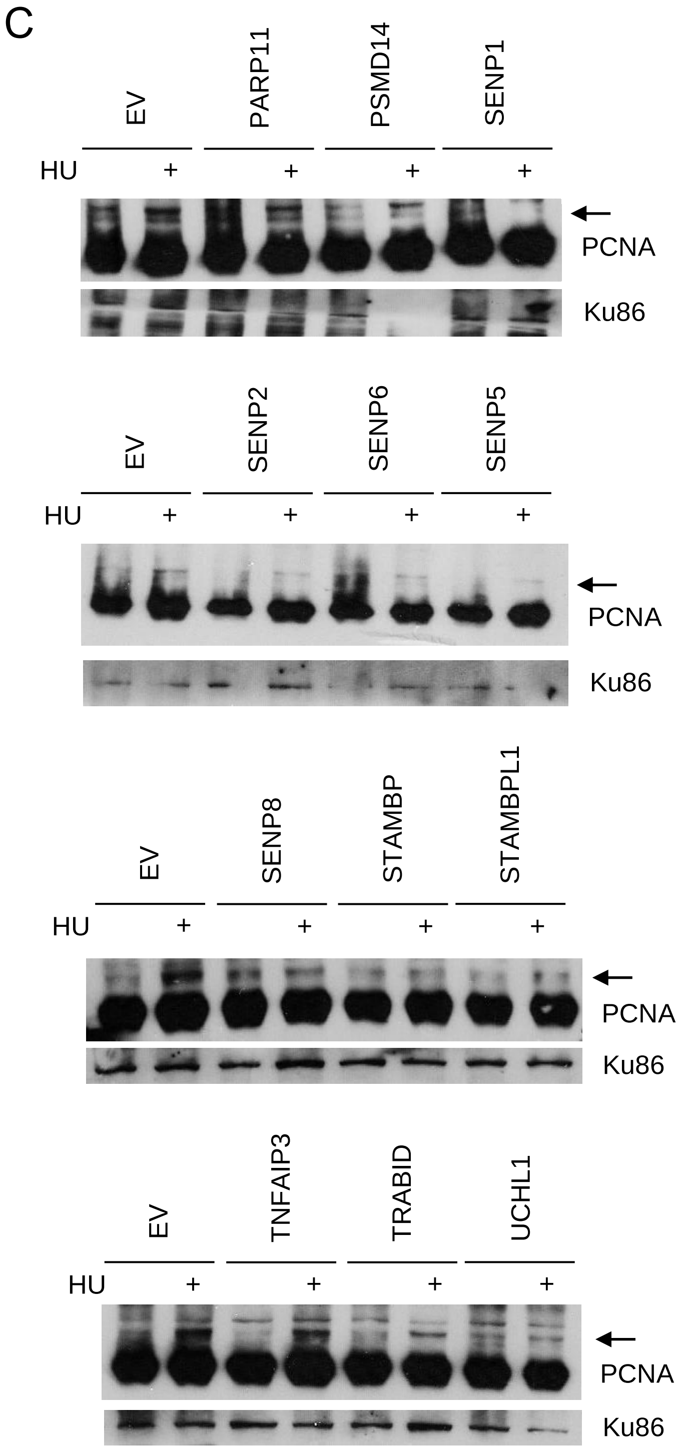
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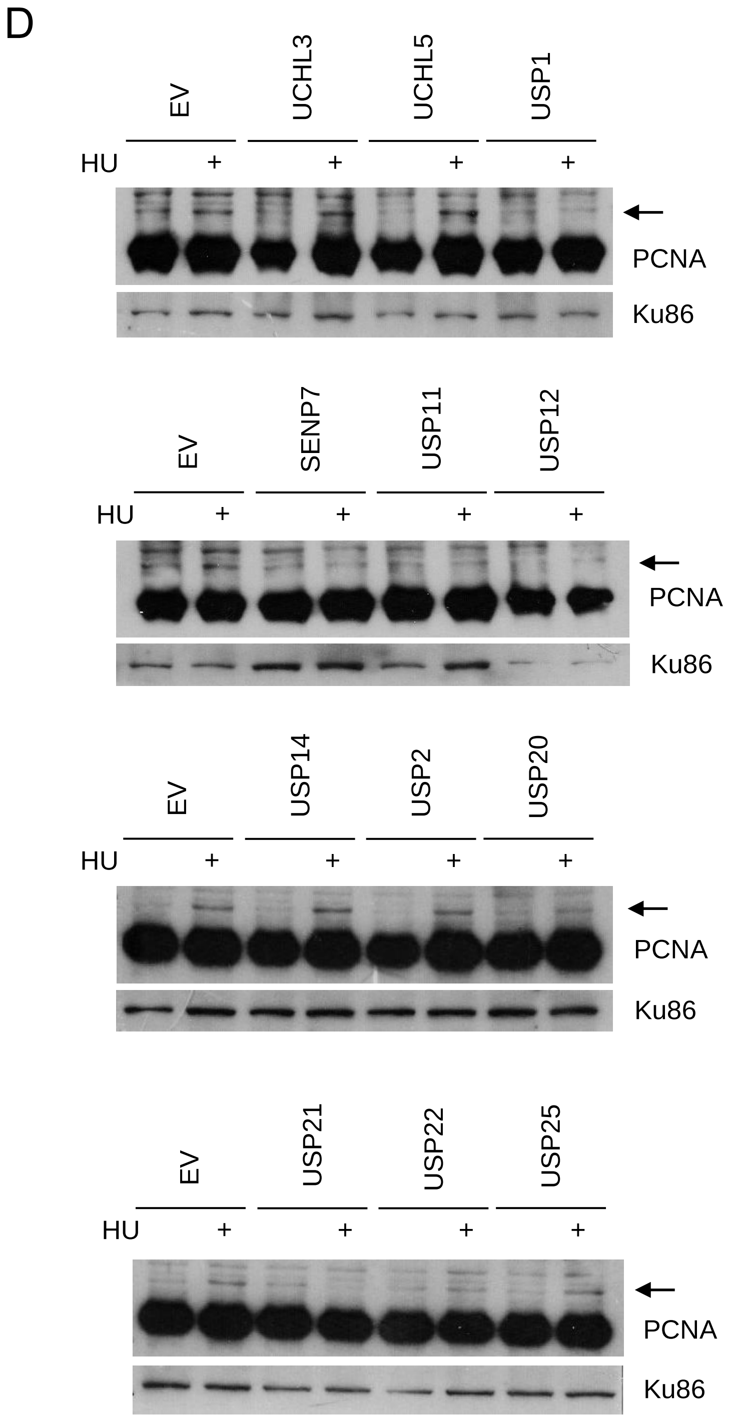
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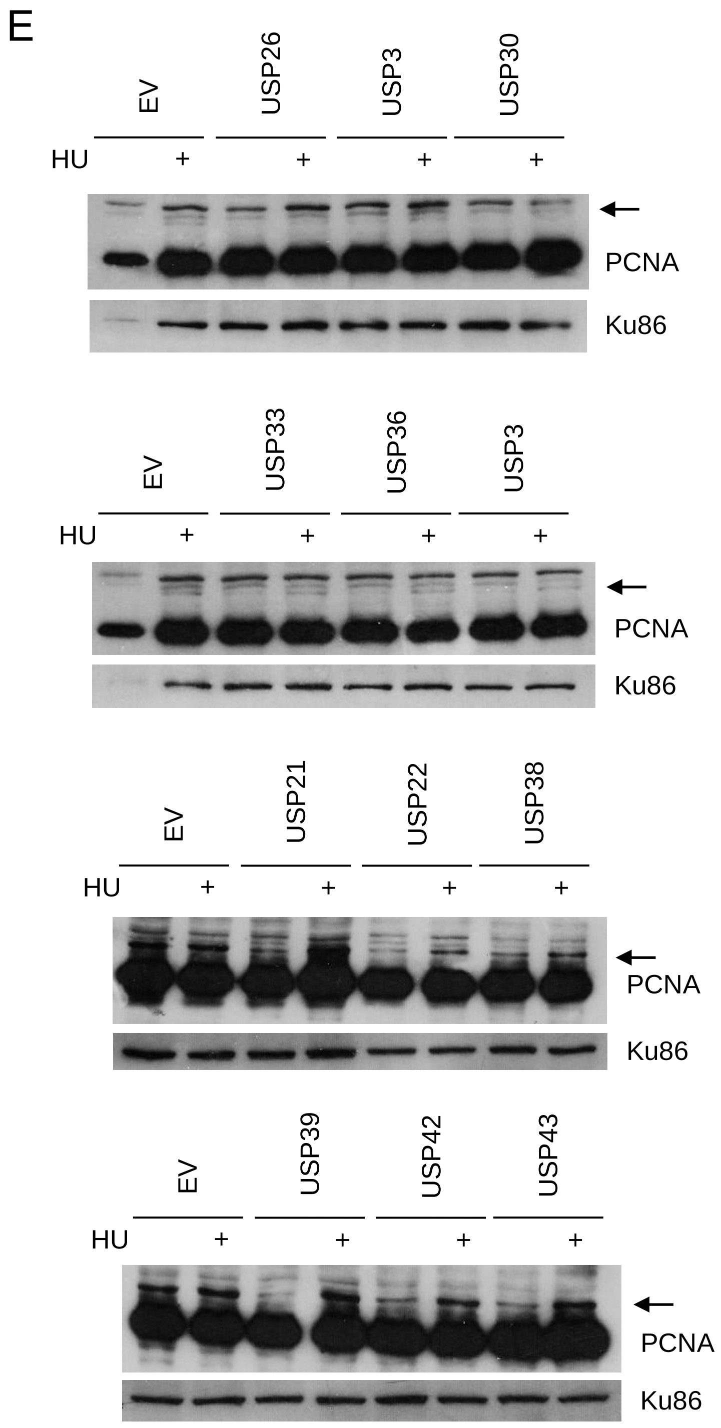
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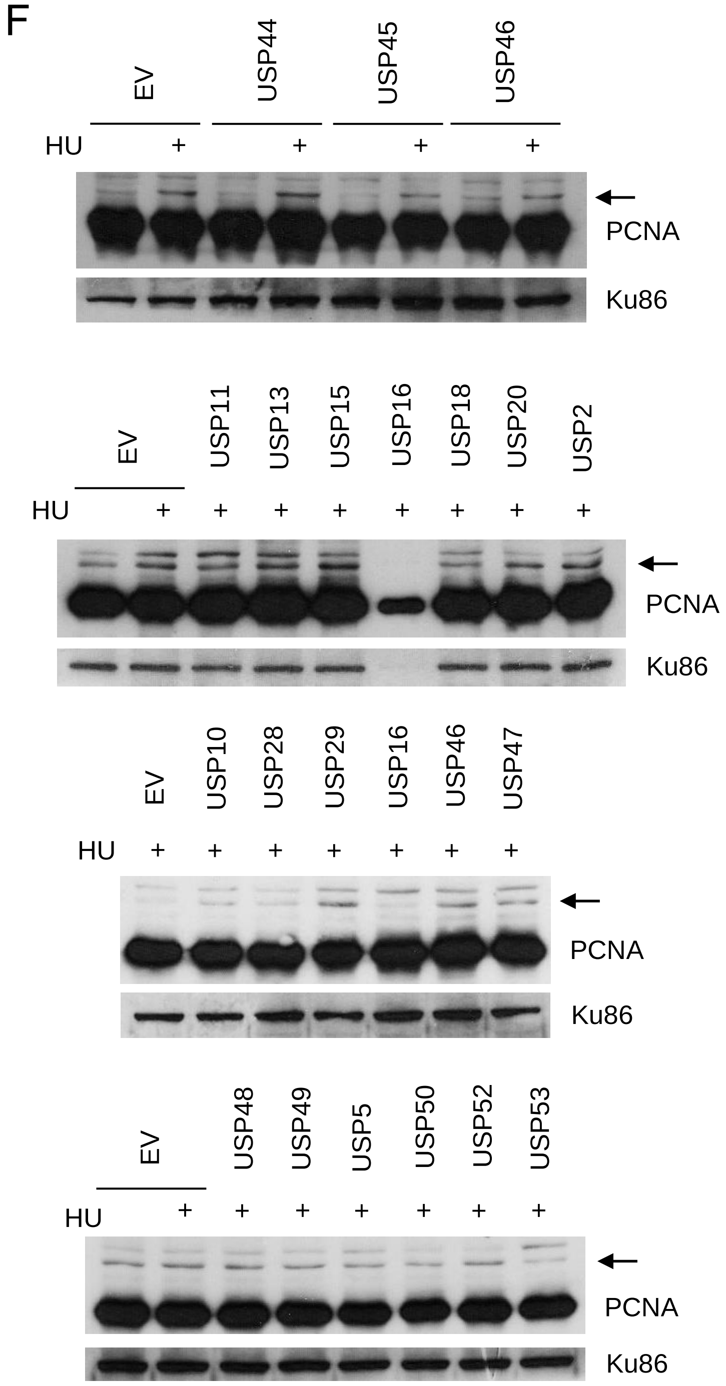
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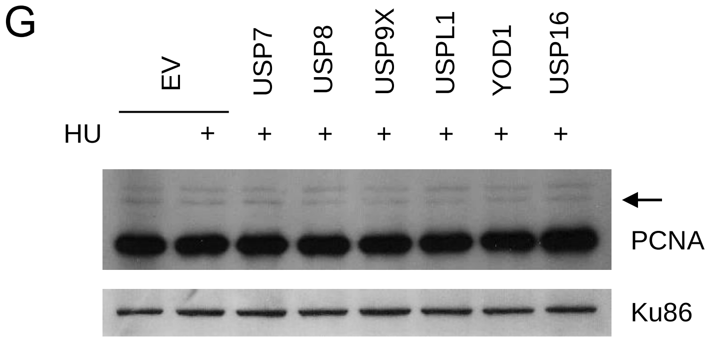


**Figure 3: Screening for PCNA ubiquitination by DUB overexpression.** A-F) 293T cells were transfected with the indicated expression vectors and thereafter left untreated or treated with 2 mM HU for 16 h. Whole cell extracts were analysed by western blot with the indicated antibodies. Arrow indicates PCNA monoubiquitination and Ku86 serves as loading control.

USP1 was previously described to regulate PCNA monoubiquitination (195). Overexpression of this ubiquitin hydrolase served as a positive control and resulted, as expected, in decreased monoubiquitination of PCNA (Figure 4). In the same experiment, SENP5, a SUMO hydrolase, was identified as a possible candidate as overexpression of SENP5 resulted in lower levels of HU-induced monoubiquitinated PCNA (Figure 4).

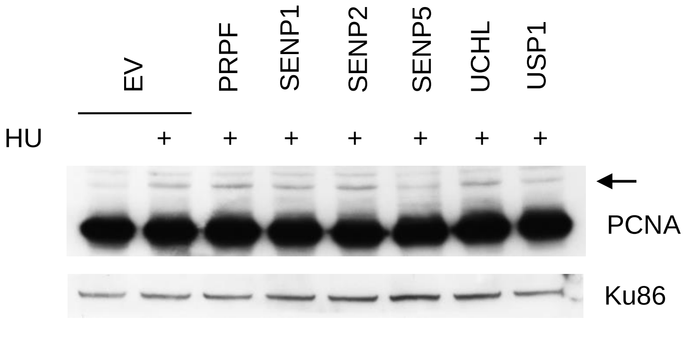


**Figure 4: Overexpression of USP1 and SENP5 results in decreased PCNA monoubiquitination.** 293T cells were transfected with the indicated expression vectors and treated with 2 mM HU for 16 h, followed by analysis by western blot with the indicated antibodies. Arrow indicates monoubiquitination of PCNA.

In addition, overexpression of ubiquitin hydrolase DUB3 caused a reduction in PCNA monoubiquitination, both in untreated conditions and after HU treatment (Figure 5).

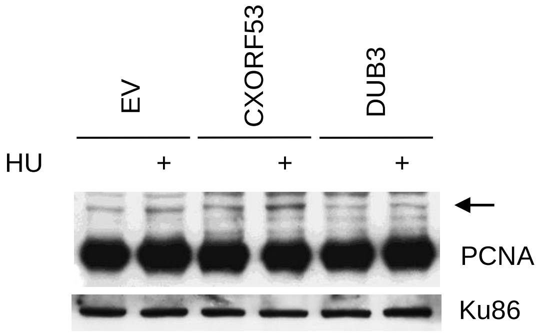


**Figure 5: Lower levels of PCNA monoubiquitination after overexpression of DUB3.** 293T cells were transfected with the indicated expression vectors and thereafter treated with 2 mM HU for 16 h, followed by analysis by western blot with the indicated antibodies. Arrow indicates monoubiquitination of PCNA.

In conclusion, our DUB screening identified two novel candidate enzymes regulating the DDR: ubiquitin hydrolase DUB3 and SUMO hydrolase SENP5. Whereas elevating the levels of SENP5 decreased PCNA monoubiquitination, overexpression of DUB3 affected both H2AX and PCNA monoubiquitination, suggesting that DUB3 might play a more general role in the DDR.

## 2. DUB3 CONTROLS DNA DAMAGE SIGNALLING BY DIRECT DEUBIQUITINATION OF H2AX

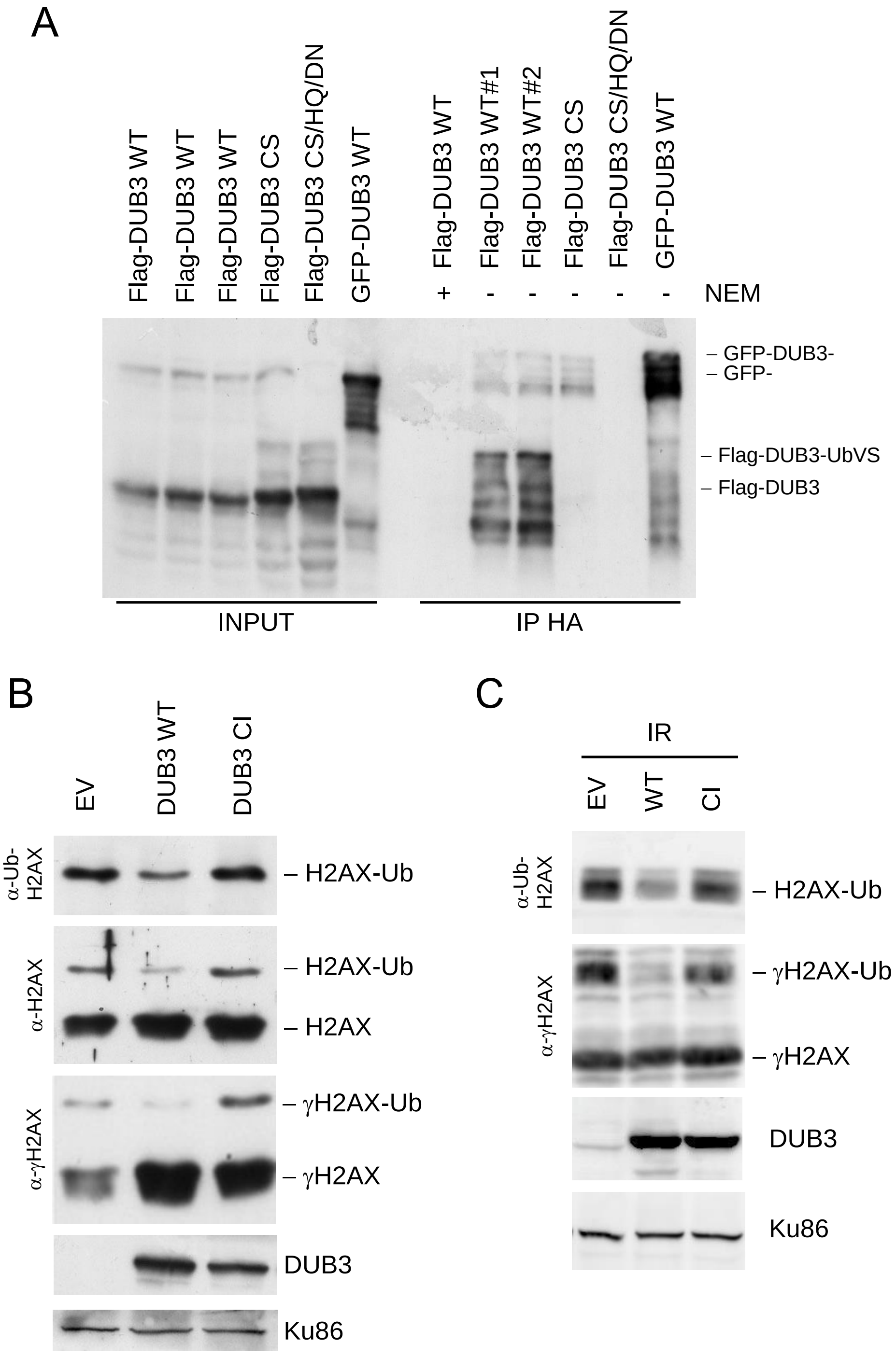
### 2.1. DUB3 catalytic activity reduces monoubiquitination of H2A(X)

DUB3 (USP17L2) was the most obvious hit of our screen for regulators of H2AX monoubiquitination. Overexpression of DUB3 reduced the levels of genotoxic stress-induced H2AX monoubiquitination (Figure 2).

DUB3 is an ubiquitin-specific protease (USP) family member. In this family the active site that contributes to protease activity contains a cysteine (196). To generate a catalytic inactive mutant, this active cysteine was mutated to serine (CI, C89S). An additional histidine and aspartic acid (H334 and D350, respectively) were mutated in a second catalytic inactive version of DUB3 (C89S/H334Q/D350N) as these three amino acids form a ´catalytic triad´. To test the activity of the wild type (WT) and catalytic inactive versions of DUB3, an *in vitro* activity assay, with HA-Ub-VS as artificial substrate, was performed. 293T cells expressing the different versions of DUB3 were lysed and incubated with HA-Ub-VS, followed by an anti-HA immunoprecipitation and western blot for DUB3. An active DUB irreversibly binds HA-Ub-VS and therefore runs with a lower mobility. As shown in Figure 6A, this assay demonstrated that both catalytic inactive versions of DUB3 had lost its catalytic capacity.

As described above, the screening was performed using an antibody for phosphorylated H2AX (γH2AX). To confirm the effect of DUB3 on H2AX, antibodies against total H2AX and ubiquitinated H2AX (Ub-H2AX) were used. The levels of HU-induced H2AX monoubiquitination decreased after DUB3 overexpression and this was appreciated using the three different antibodies (Figure 6B). The same effect of DUB3 overexpression was obtained when inducing H2AX monoubiquitination by ionizing radiation (IR), followed by western blot analysis with antibodies against γH2AX and Ub-H2AX (Figure 6C). The C89S catalytic mutant of DUB3 was subsequently used to determine if the effect of DUB3 on H2AX monoubiquitination was due to its catalytic activity. Overexpression of the C89S catalytic inactive mutant of DUB3 did not diminish the monoubiquitination on H2AX both after HU and IR-treatment (Figure 6B and 6C), indicating that the catalytic domain is critical for this effect of DUB3.

To examine if overexpression of DUB3 also affected the monoubiquitination of histone H2A, a histone acid extraction was performed, to separate histones from chromatin. Analysing such extracts demonstrated that overexpression of DUB3 wild type, but not catalytic inactive, also reduced the monoubiquitination of H2A (Figure 7).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Flag-DUB3 WT#1
Flag-DUB3 WT#2
Flag-DUB3 CS
Flag-DUB3 CS/HQ/DN
GFP-DUB3 WT
+ - - - - - NEM
– GFP-DUB3-
– GFP-
– Flag-DUB3-UbVS
– Flag-DUB3
INPUT
IP HA
B
EV
DUB3 WT
DUB3 CI
α-Ub-H2AX
– H2AX-Ub
α-H2AX
– H2AX-Ub
– H2AX
α-γH2AX
– γH2AX-Ub
– γH2AX
DUB3
Ku86
C
IR
EV
WT
CI
α-Ub-H2AX
– H2AX-Ub
α-γH2AX
– γH2AX-Ub
– γH2AX
DUB3
Ku86

**Figure 6: DUB3 overexpression decreases H2AX monoubiquitination.** A) 293T cells were transfected with the different DUB3 expression vectors. After 36 h, cells were lysed, and a DUB activity assay was performed using HA-Ub-VS as described in materials and methods. Active DUB3 binds HA-Ub-VS and consequently runs with lower mobility. DUB-inhibitor NEM was used as a negative control. B) 293T cells were transfected with EV, DUB3 WT or DUB3 C89S (CI) expression vector. Cells were treated o/n with 2 mM HU. Western blotting was performed with different H2AX antibodies. C) 293T cells were transfected with the indicated expression vectors. Cells were treated with IR (10 Gy) and harvested 1 h later. Western blotting was performed with the indicated antibodies.

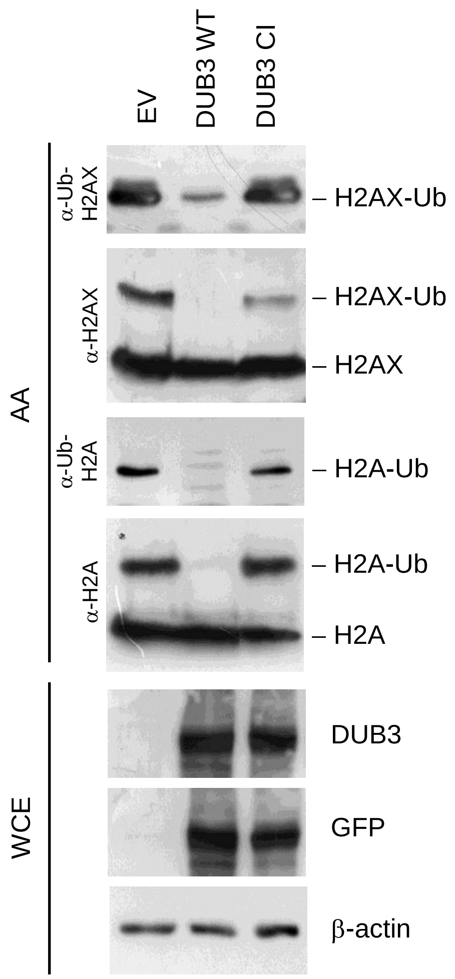


**Figure 7: DUB3 overexpression diminishes histone H2A monoubiquitination levels after acid extraction**. 293T cells were transfected with EV, GFP-DUB3 WT or GFP-DUB3 CI, treated with HU after which acid extraction (AA) was performed and whole cell extracts (WCE) were prepared. Western blotting was performed with the indicated antibodies.

To exclude that overexpression of DUB3 had a pan-cellular effect, for example by decreasing cellular ubiquitin pools, the FK2 antibody, which detects both mono- and polyubiquitinated proteins (conjugated ubiquitin), was used (197). 293T cells were transfected with DUB3 wild type and catalytic mutant and an anti-FK2 western blot was performed. The effect of DUB3 wild type seemed specific for the histone, only decreasing the levels of (a) protein(s) at the molecular weight of monoubiquitinated H2A(X) in the FK2 western, which, as expected, was also recognized by the Ub-H2AX antibody (Figure 8A). Importantly, no major changes in conjugated ubiquitin in general were detected after expression of DUB3 wild type, strongly suggesting that the effect was not general.

As GFP-Ub was used as a marker for DNA damage–induced H2A ubiquitination in fluorescence studies, and the FK2 antibody strongly recognised monoubiquitinated H2A(X) in our hands (Figure 8A), we used the FK2 antibody to demonstrate H2A(X) ubiquitination in an immunofluorescence experiment (198). Indeed, whereas etoposide (ETP)-treated U2OS cells expressing catalytic inactive GFP-DUB3 stained positive for FK2 by immunofluorescence, after expression of wild type GFP-DUB3 this signal was strongly diminished (Figure 8B). These results confirm our earlier experiments showing that expression of DUB3 specifically affects the ubiquitination of H2A(X).

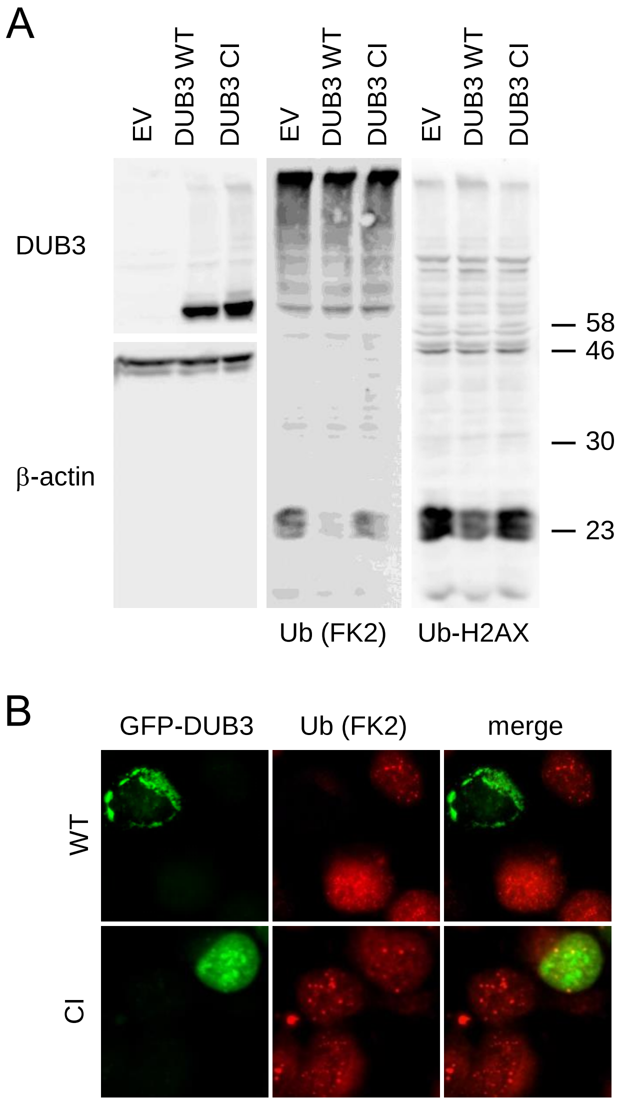
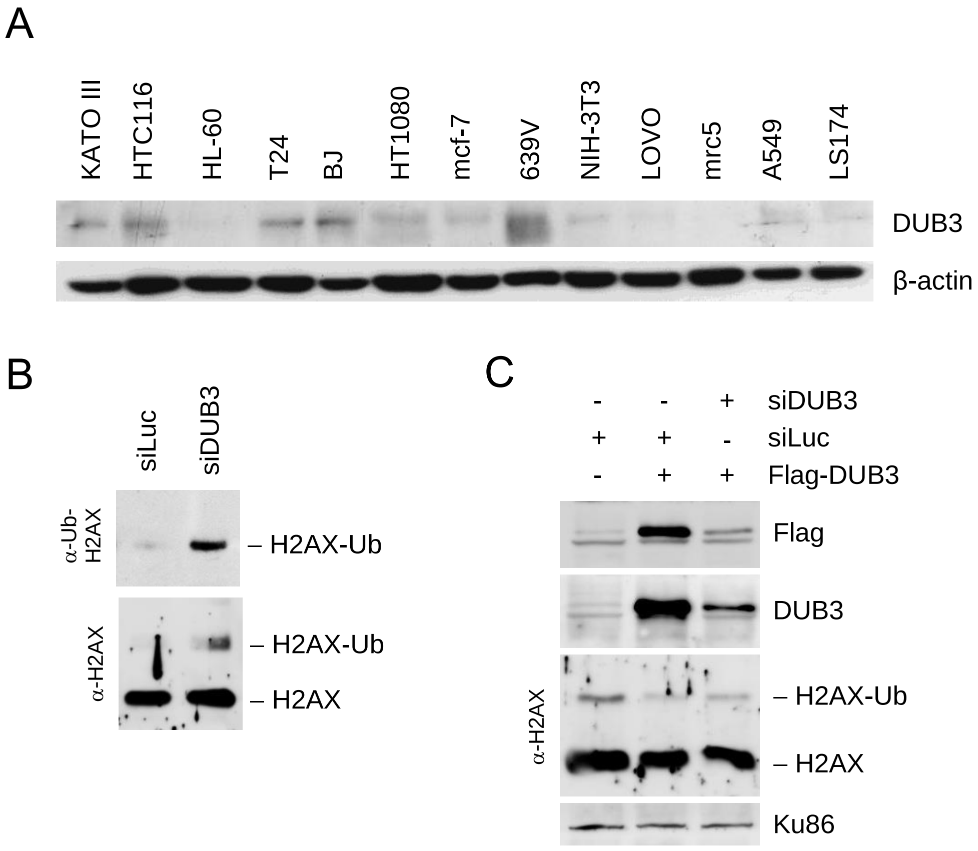


**Figure 8: The effect of DUB3 WT is specific for H2A(X).** A) 293T cells were transfected with EV, DUB3 WT or DUB3 CI. Western blotting with indicated antibodies. Ubiquitinated H2AX (23kD) is detected in the FK2 western. B) U2OS cells were transfected with GFP-DUB3 WT or GFP-DUB3 CI, treated with ETP (20 μM) and fixed after 1 h. Immunofluorescence analysis was carried out with FK2 antibody. Transfected cells were detected by GFP fluorescence.

## 2.2. DUB3 downregulation increases H2AX monoubiquitination

A major drawback of working with DUB3 was the low endogenous level of this protein, practically undetectable by western blot in the cell lines standardly used in our laboratory. As we wanted to study the effect of DUB3 depletion on H2AX ubiquitination, detecting the endogenous protein was critical for verifying the efficiency of the downregulation by western blot. To find a cell line that expressed higher levels of endogenous DUB3 for such depletion experiments, endogenous levels of DUB3 were compared in different cell lines of human origin. As shown in Figure 9A, the highest levels of endogenous DUB3 were expressed in 639V cells, a cell line from bladder carcinoma.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LS174
DUB3
β-actin
B
siLuc
siDUB3
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– H2AX-Ub
α-H2AX
– H2AX-Ub
– H2AX
C
- - + siDUB3
+ + - siLuc
- + + Flag-DUB3
Flag
DUB3
α-H2AX
– H2AX-Ub
– H2AX
Ku86

**Figure 9: DUB3 downregulation increases H2AX monoubiquitination.** A) Whole cell extracts of indicated cell lines were analysed by western blot for DUB3 to detect endogenous protein. B) 639V cells were transfected with Luc or DUB3 siRNA oligos. Cells were left untreated, and whole cell extracts were analysed by western blot with the indicated antibodies. C) Simultaneous transfection of Flag-DUB3 plasmid and DUB3 siRNA oligos in U2OS cells, as described in materials and methods. Western blot analysis with the indicated antibodies.

When depleting DUB3 by siRNA in these cells, the monoubiquitination of H2AX increased in the absence of exogenous DNA damage (Figure 9B). However, despite numerous efforts, we could not efficiently demonstrate DUB3 knockdown by western blot in these extracts. To show the efficiency of our DUB3 siRNA oligo, Flag-DUB3 was overexpressed and DUB3 was simultaneously downregulated in U2OS cells. Levels of Flag-DUB3 decreased by co-transfection of DUB3 siRNA oligos (Figure 9C). In addition, the diminished levels of H2AX monoubiquitination after Flag-DUB3 expression were somewhat rescued upon simultaneous depletion of DUB3 (Figure 9C), together suggesting that with this siRNA oligo, DUB3 can indeed be depleted. These results confirm that DUB3 regulates levels of H2A(X) monoubiquitination.

### 2.3. DUB3 is more effective in inhibiting H2AX ubiquitination than other DUBs previously described for H2A(X)

The first ubiquitin hydrolase described for H2A(X) was USP3 (31). To compare the activity of DUB3 on H2AX monoubiquitination to that of USP3, expression vectors of both DUBs were transfected in 293T cells. Overexpression of DUB3 resulted in equal or more efficient decrease in H2AX monoubiquitination as USP3 overexpression in conditions of similar expression levels of the ubiquitin hydrolases (Figure 10A).

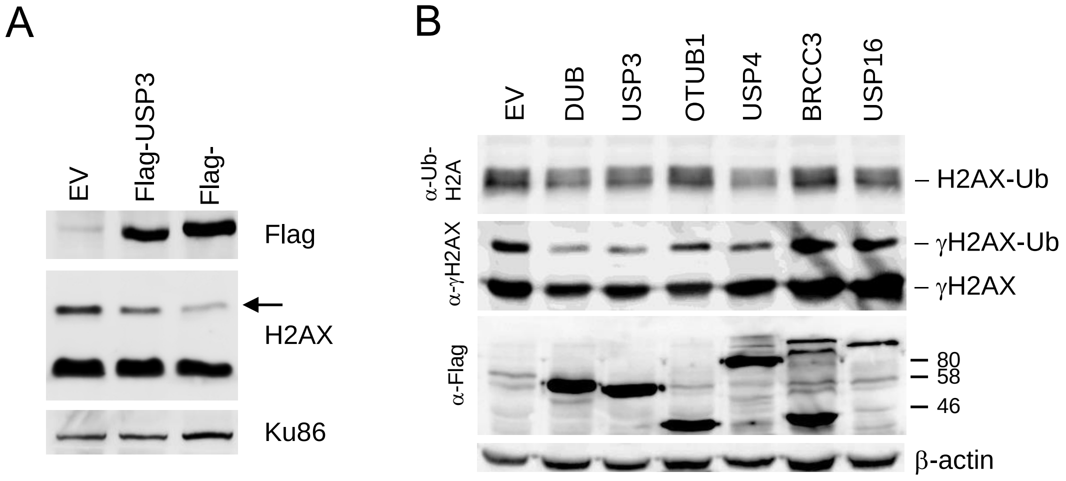

**Figure 10: DUB3 is highly efficient in inhibiting H2AX monoubiquitination levels as compared to other described ubiquitin hydrolases.** A) 293T cells overexpressing empty vector (EV), Flag-USP3 or Flag-DUB3 were treated with HU (2 mM, 16 h). Western blotting was performed with the indicated antibodies. B) 293T cells were transfected with empty vector (EV), Flag-DUB3 (60kD), Flag-USP3 (59kD), Flag-OTUB1 (31kD), Flag-USP44 (81kD), Flag-BRCC36 (36kD) or Flag-USP16 (94kD). Treatment with HU was followed by western blot analysis with the indicated antibodies.

Subsequent DUBs that were described to affect H2A(X) monoubiquitination, directly or more indirectly, were OTUB1, USP44, BRCC36 and USP16 (32; 33; 34; 35). The efficiency of these DUBs was compared to DUB3 by expression of all these ubiquitin hydrolases in 293T cells. Also in this experiment, DUB3 was among the more effective DUBs in decreasing H2AX monoubiquitination (Figure 10B), again suggesting a firm role for DUB3 in the DDR.

### 2.4. Overexpression of DUB3 abrogates recruitment of 53BP1 and BRCA1 to sites of DNA lesions, while γH2AX and MDC1 focus formation is not affected

Monoubiquitination of H2AX is a crucial event in the recruitment of mediator proteins 53BP1 and BRCA1 to sites of DNA lesions (26; 27). As DUB3 affects H2AX monoubiquitination, the consequences of DUB3 overexpression on DNA damage-induced focus formation of these proteins were studied. U2OS cells were transfected with GFP-tagged versions of DUB3, wild type and catalytic inactive mutant, after which IR-induced focus formation was analysed by immunofluorescence. GFP-negative cells were used as non-transfected controls in the same samples, while GFP-positive cells were scored as cells expressing DUB3. As shown in Figure 11A and B, the IR-induced 53BP1 focus formation was completely abrogated by overexpression of DUB3 wild type, but not affected in cells expressing DUB3 catalytic inactive. Next, Flag-tagged DUB3 wild type and DUB3 catalytic inactive were expressed and transfected cells were identified by staining with an anti-Flag antibody. As for GFP-DUB3 wild type expressing cells, expression of Flag-DUB3 wild type completely prevented 53BP1 foci induced by IR (Figure 11C), whereas Flag- DUB3 catalytic inactive had no effect.

As described above, also BRCA1, a protein important for homologous recombination, is also recruited to damaged sites in a manner dependent on H2AX monoubiquitination (26; 27). To analyse the effect of DUB3 on BRCA1 localization at these sites, GFP-DUB3 wild type or catalytic inactive were overexpressed in U2OS cells and IR-induced focus formation of BRCA1 was analysed by immunofluorescence. DUB3 wild type, but not catalytic inactive, completely inhibited BRCA1 focus formation upon IR treatment (Figure 12A).

If DUB3 directly controls H2AX monoubiquitination, earlier events in the DDR should not to be affected by DUB3 overexpression. Phosphorylation of H2AX (18; 19) and accumulation of MDC1 into IR-induced foci are two events that occur before the ubiquitination of H2AX (22, 26; 27; 146). To confirm that these events were not affected by DUB3, U2OS cells were transfected with GFP-DUB3 wild type and catalytic inactive versions and irradiated to induce damage. H2AX phosphorylation and MDC1 focus formation were analysed by immunofluorescence in GFP-positive cells. As shown in Figure 12 B-D, both events were left unchanged by DUB3 overexpression. Together these data strongly suggest that DUB3 directly impacts on the monoubiquitination of H2AX.

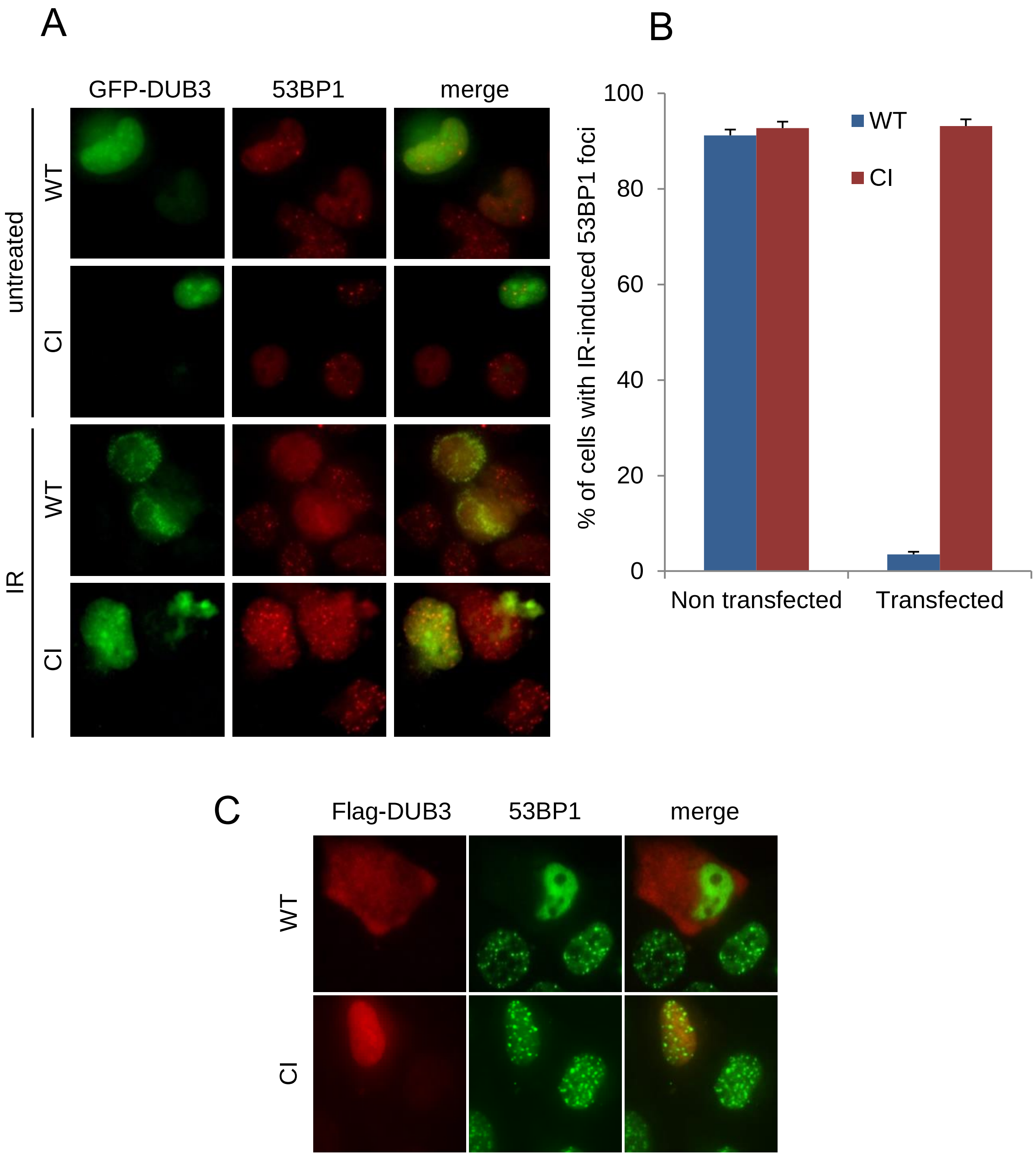
A
GFP-DUB3
53BP1
merge
untreated
WT
CI
IR
WT
CI
B
100
80
60
40
20
0
% of cells with IR-induced 53BP1 foci
■ WT
■ CI
Non transfected
Transfected
C
Flag-DUB3
53BP1
merge
WT
CI

**Figure 11: Expression of DUB3 WT abrogates 53BP1 focus formation.** A) U2OS cells were transfected with GFP-tagged DUB3 WT and DUB3 CI. Cells were left untreated or treated with IR (2 Gy). One hour later cells were fixed and analysed by immunofluorescence using 53BP1 antibody. B Quantification of 53BP1 focus formation upon DUB3 WT expression in three different experiments. GFP-positive (transfected) and GFP-negative (non transfected) cells were scored. Error bars represent standard error. C) Flag-tagged DUB3 WT or DUB3 CI was expressed in U2OS cells. Cells were treated with IR (2 Gy) and fixed one hour later. Immunofluorescence analysis was carried out using 53BP1 and Flag antibodies.

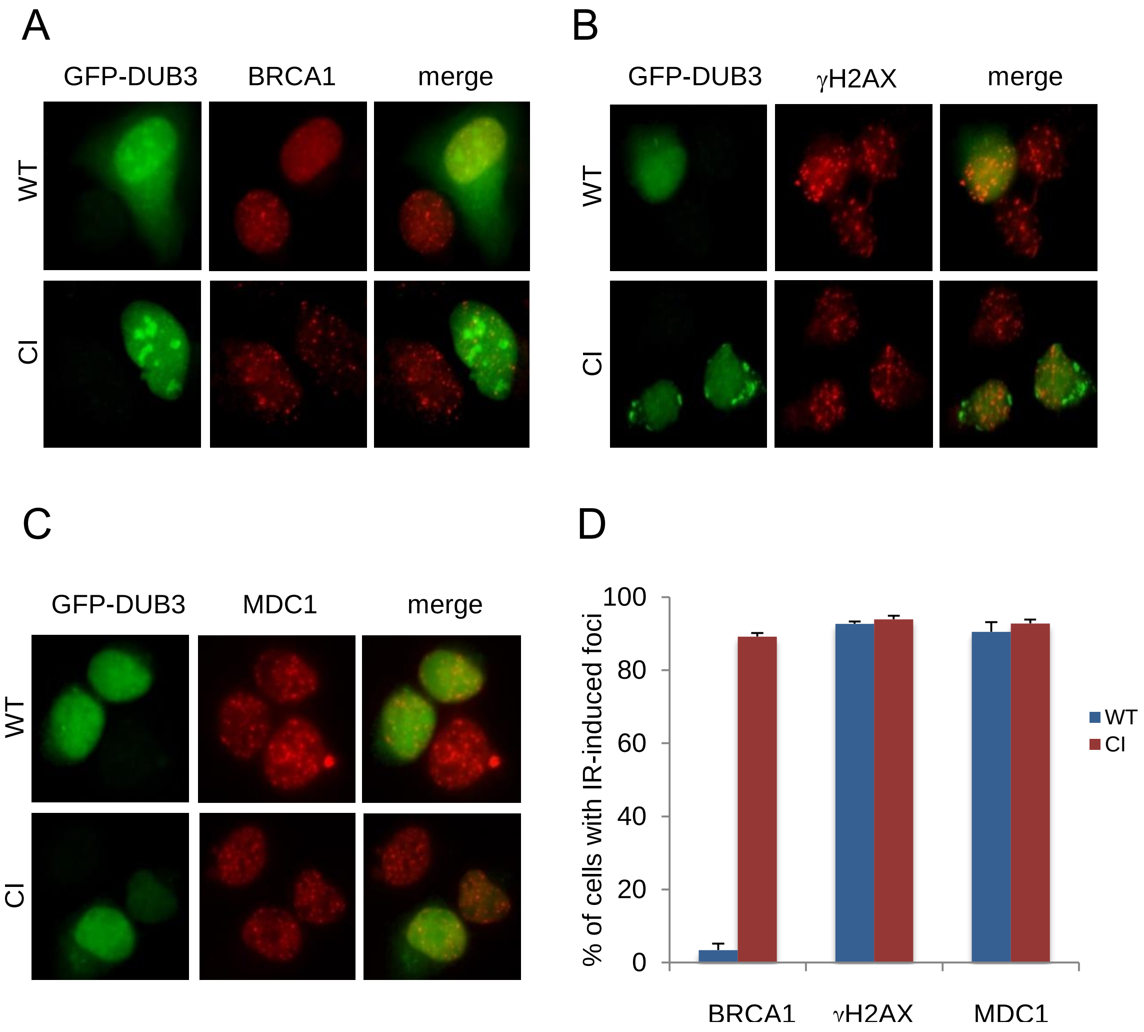
A
GFP-DUB3
BRCA1
merge
WT
CI
B
GFP-DUB3
γH2AX
merge
WT
CI
C
GFP-DUB3
MDC1
merge
WT
CI
D
% of cells with IR-induced foci
100
80
60
40
20
0
BRCA1
γH2AX
MDC1
WT
CI

**Figure 12: DUB3 abrogates BRCA1 focus formation whereas it does not affect H2AX phophorylation or MDC1 recruitment to sites of DNA damage.** A) GFP-tagged DUB3 WT or CI was transfected in U2OS cells, which were treated with IR (2 Gy, 1 h). Analysis by immunofluorescence for BRCA1. B) As in A, but now for γH2AX. C) As in A, but now for MDC1. D) Quantification of IR-induced focus formation of BRCA1, γH2AX and MDC1 of A, B and C in three different experiments. Error bars represent standard error.

### 2.5. DUB3 antagonizes E3 ligases RNF8 and RNF168 and restrains recruitment of RNF168 to sites of DNA lesions

E3 ubiquitin ligase RNF8 is recruited to DNA damage sites by interaction with MDC1 and subsequently initiates monoubiquitination of H2A(X) (22; 26; 146). A second E3 ligase, RNF168, is generally thought to be recruited by recognition of RNF8 ubiquitination products and then amplifies the H2A(X) ubiquitination response, thereby triggering the recruitment of 53BP1 and BRCA1 (29; 30).

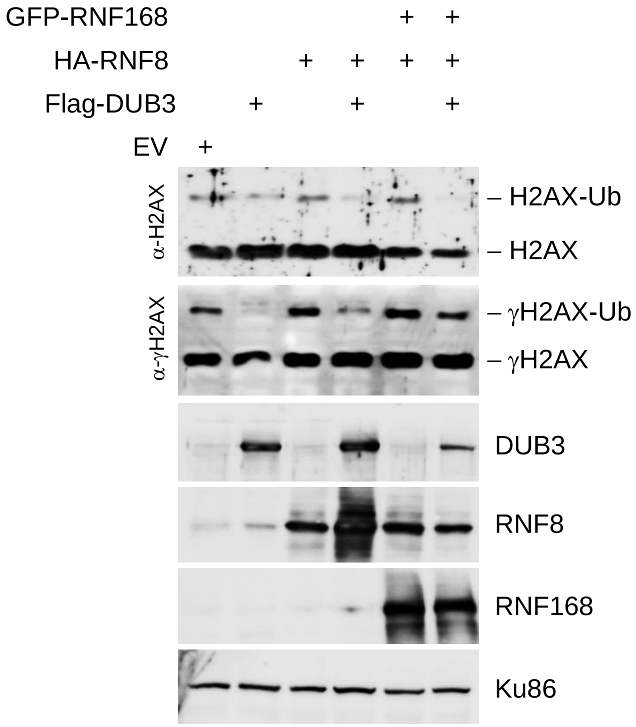

**Figure 13: DUB3 overexpression counteracts the effect of E3 ligases RNF8 and RNF168 on H2AX monoubiquitination.** 293T cells were transfected with the indicated plasmids, treated with HU (2 mM, 16 h), lysed and analysed by western blotting using the indicated antibodies.

Several ubiquitin hydrolases were shown to counterbalance the ubiquitination cascade by RNF8 and RNF168. For instance, USP16 and USP44 overexpression reverses RNF8/RNF168-mediated ubiquitination (33; 199). To study if DUB3 counteracts RNF8 and RNF168 function, these E3 ligases were overexpressed in the presence and absence of Flag-DUB3 and H2AX monoubiquitination was analysed by western blotting. Overexpression of RNF8 only, or RNF8 and RNF168 together, led to elevated H2AX monoubiquitination levels as compared to empty vector transfected cells. However, co-expression of DUB3 reverted this increase in monoubiquitination of H2AX (Figure 13), indicating that this ubiquitin hydrolase counteracts RNF8 and RNF168 ligase activities.

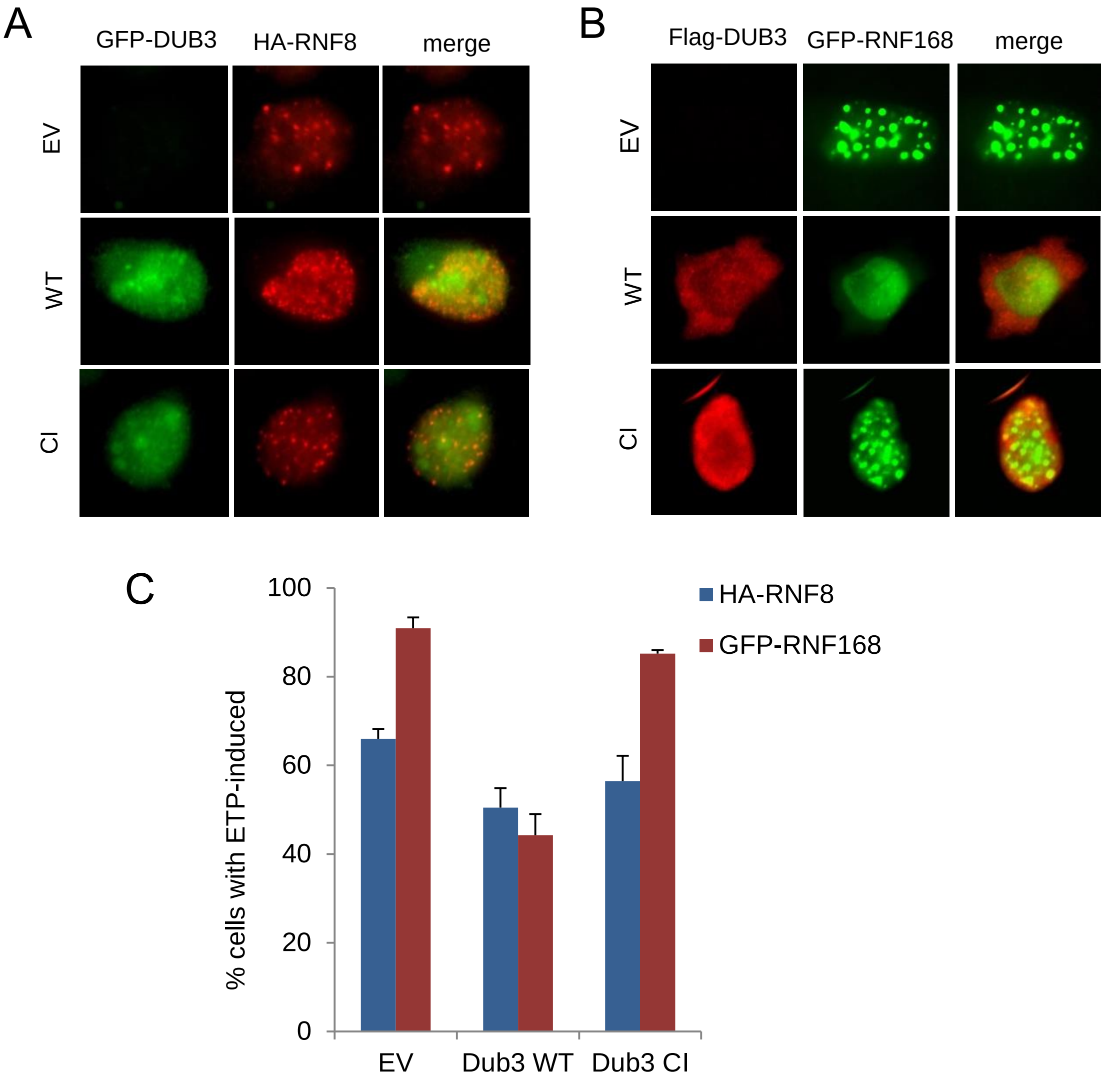
A
GFP-DUB3
HA-RNF8
merge
EV
WT
CI
B
Flag-DUB3
GFP-RNF168
merge
EV
WT
CI
C
% cells with ETP-induced
100
80
60
40
20
0
EV
Dub3 WT
Dub3 CI
■ HA-RNF8
■ GFP-RNF168

**Figure 14: DUB3 overexpression does not alter the recruitment of RNF8 in sites of DNA lesions but diminishes RNF168 focus formation.** A) U2OS cells were transfected with EV, GFP-DUB3 WT or CI in combination with HA-RNF8. Cells were treated with ETP (20 μM) and fixed 1h later. RNF8 foci were scored in GFP-positive cells by immunofluorescence with an HA antibody. B) U2OS cells were transfected with EV, Flag-DUB3 WT or Flag-DUB3 CI in combination with GFP-RNF168. Cells were treated with ETP (20 μM) and fixed 1h later. GFP-RNF168 foci were scored in Flag-positive cells. C) Quantification of A) and B). Error bars represent standard error.

To investigate if DUB3 could affect the recruitment of these E3 ligases to the sites of DNA lesions, an immunofluorescence analysis was carried out. U2OS cells were transfected with different versions of GFP-tagged DUB3 and HA-RNF8, or Flag-DUB3 and GFP-RNF168. Subsequently, RNF8 or RNF168 focus formation in response to etoposide was scored by immunofluorescence. The percentage of cells with HA-RNF8 foci did not significantly change after expression of DUB3 (Figures 14A and C), while the percentage of cells with GFP-RNF168 foci was inhibited by around 50% upon co-expression of DUB3 wild type, but not catalytic inactive (Figures 14B and C). As described above, as RNF168 recruitment to damaged sites has been reported to be dependent on RNF8 and ubiquitination of H2AX (29; 30), and the inefficient DNA damage-induced focus formation of RNF168 in DUB3-overexpressing cells could therefore be a consequence of lower levels of H2AX monoubiquitination.

### 2.6. DUB3 deubiquitinates H2AX *in vitro*

Our results demonstrate that DUB3 overexpression affects H2AX ubiquitination. To investigate if this effect is due to direct deubiquitination of H2AX, a possible interaction between purified proteins was determined. Indeed, purified exogenous H2AX co-immunoprecipitated with purified exogenous DUB3 in an anti-DUB3 immunoprecipitation (Figure 15A). Next, these purified proteins were mixed and an *in vitro* deubiquitination assay was performed. Figure 15B shows that DUB3 wild type, but not catalytic inactive, was able to deubiquitinate H2AX *in vitro*. Inhibition of DUB activity by N-ethylmaleimide (NEM) prevented deubiquitination of H2AX by DUB3 wild type. In addition, to support the specificity of the assay, USP28, a nonspecific DUB, was used, which was not able to deubiquitinate H2AX in these conditions (Figure 15B). These data demonstrate that DUB3 directly affects H2AX by deubiquitination.

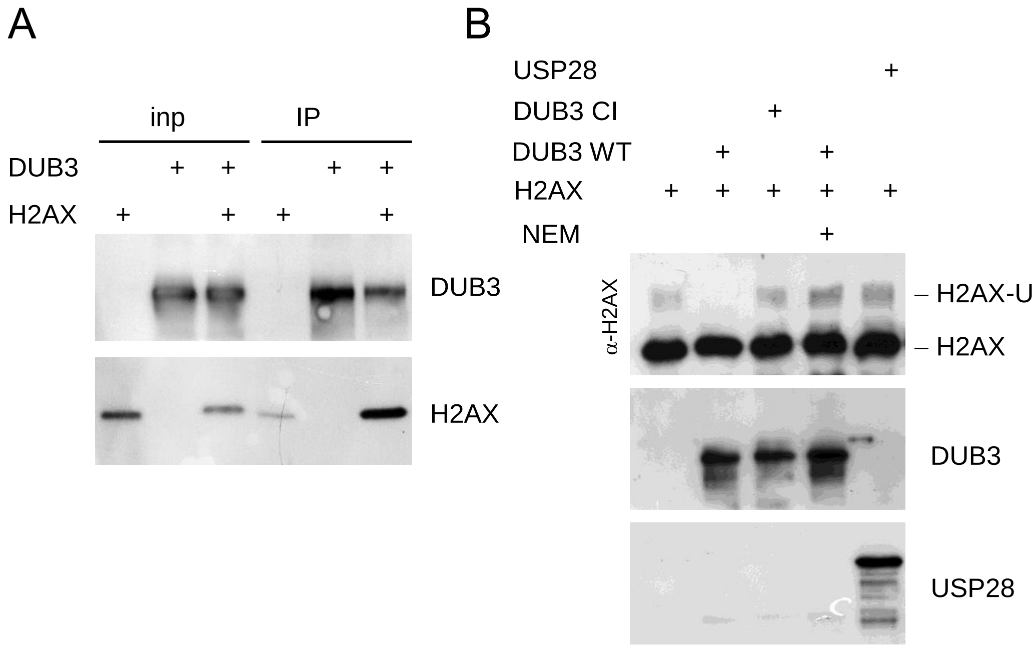

**Figure 15: DUB3 deubiquitinates H2AX *in vitro*.** A) immunoprecipitation of purified H2AX and DUB3 with an anti-DUB3 antibody, followed by western blotting using the indicated antibodies. B) *In vitro* deubiquitination assay of purified ubiquitinated H2AX and DUB3 WT, DUB3 CI or USP28 WT (as described in materials and methods), analysed by western blotting using the indicated antibodies.

## 2.7. DUB3 regulates proper DDR function

We identified DUB3 as a novel regulator of H2AX monoubiquitination, which enhanced the complexity of the response that helps maintaining genome stability. It was expected that deregulation of H2AX monoubiquitination would have consequences for the DDR, for example an impaired response or DNA repair upon overexpression of DUB3. This hypothesis was tested by studying H2AX phosphorylation (γH2AX) and MDC1 focus formation, both markers of an activated DDR, after overexpression of DUB3. 293T cells expressing DUB3 wild type or catalytic inactive were treated with ETP for 1 hour, after which ETP was removed and cells were incubated with fresh medium. At different time points, cells were harvested to see the status of the DDR. Western blot analysis demonstrated that whereas in cells overexpressing DUB3 catalytic inactive or control cells transfected with an empty vector, H2AX phosphorylation returned to basal levels 6 hours after the damage induction, H2AX phosphorylation was maintained high at late time points (6-24 hours) after overexpression of DUB3 wild type (Figure 16), suggesting that deregulation of H2AX monoubiquitination results in a problematic DDR.

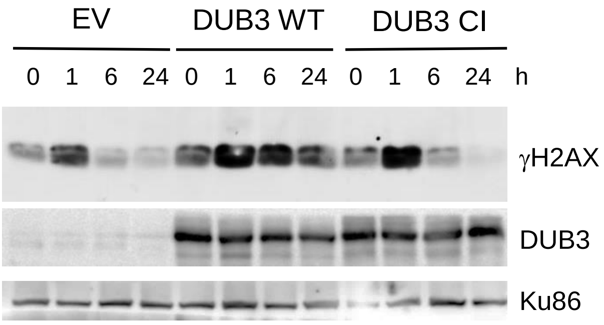

**Figure 16: DUB3 WT overexpression maintains H2AX phosphorylation high at late time points after damage.** 293T cells were transfected with EV, DUB3 WT or CI plasmids. After 36 hours, cells were incubated with ETP (1 μM) for 1 hour, then washed and incubated with fresh medium for the indicated time periods. Then cells were lysed and western blotting with the indicated antibodies was performed.

Focus formation of γH2AX and MDC1, analysed by immunofluorescence, at late time points showed the same result. Whereas the same amount of γH2AX-positive cells were counted 1 hour after damage induction, indicative of a similar damage load, significantly more cells with γH2AX foci were identified at 6 hours after ETP treatment in DUB3 wild type as compared to cells expressing the catalytic mutant or empty vector (Figures 17A and C). MDC1 foci were also sustained at later time points after damage in cells expressing wild type DUB3 but not in DUB3 catalytic inactive (Figures 17B and C). Together these results indicate that a balanced level of H2AX monoubiquitination is required for a correct DDR: activation upon the detection of DNA lesions and switch off when the lesions are repaired, to stimulate recovery.

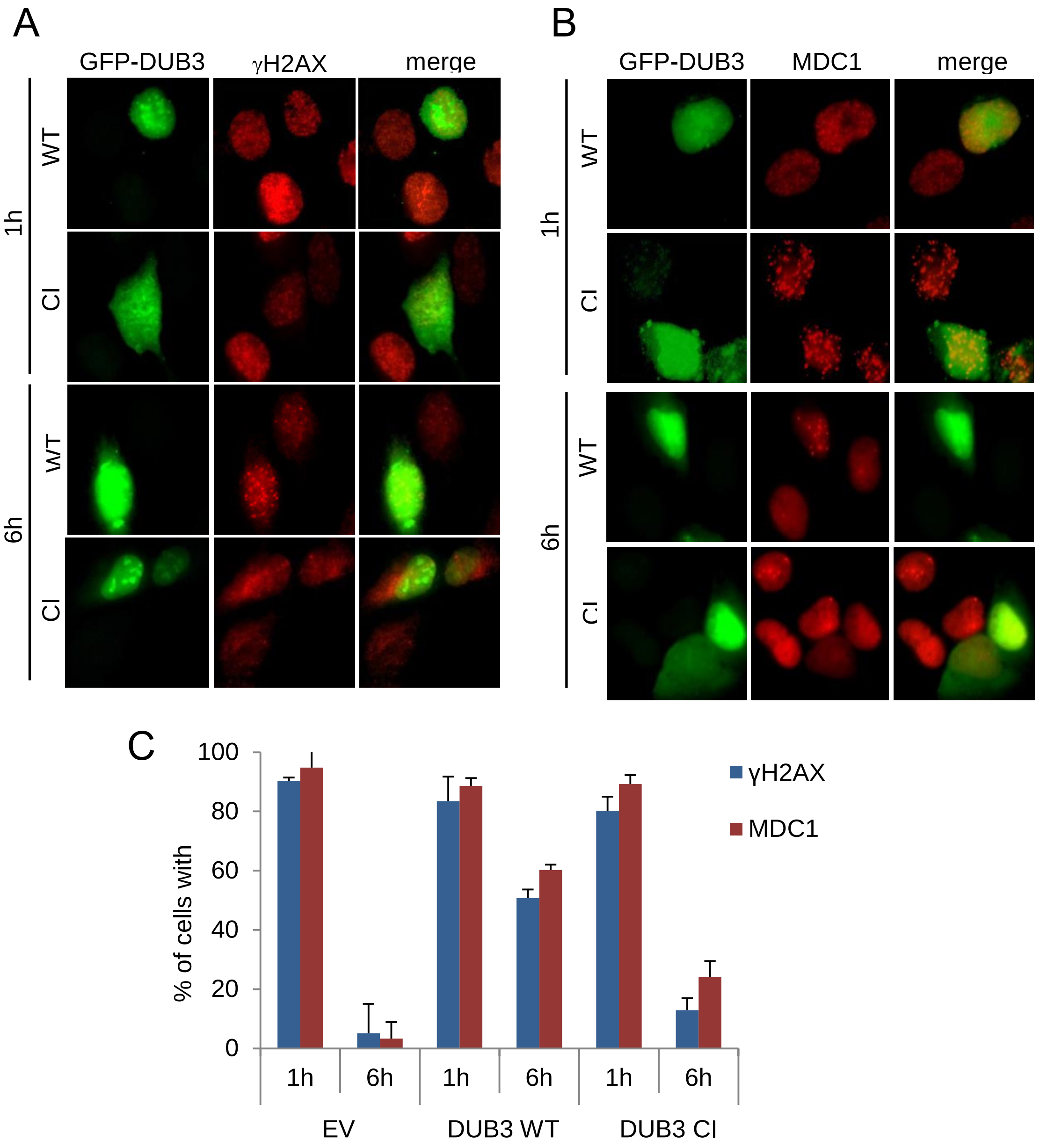
A
GFP-DUB3
γH2AX
merge
1h
WT
CI
6h
WT
CI
B
GFP-DUB3
MDC1
merge
1h
WT
CI
6h
WT
CI
C
% of cells with
100
80
60
40
20
0
1h
6h
1h
6h
1h
6h
EV
DUB3 WT
DUB3 CI
γH2AX
MDC1

**Figure 17: DUB3 overexpression impairs an efficient DDR.** A) U2OS cells were transfected with the indicated plasmids. Cells were treated with 1 μM ETP for 1 h. Cells were fixed or washed and left to recover for another 5 h. Cells were analysed by immunofluorescence for γH2AX. GFP-positive cells were scored for γH2AX foci and GFP-negative cells served as untransfected controls. B) As in A) but for MDC1. C) Quantification of A and B. Error bars represent standard error.

To study the effect of the absence of DUB3, DUB3 was downregulated from HeLa cells and ETP-induced H2AX phosphorylation was analysed by western blotting. In these conditions, phosphorylation of H2AX recovered quicker at late time points in DUB3-depleted cells as compared to control cells (Figure 18A). At these time points, γH2AX focus formation by immunofluorescence also disappeared quicker after DUB3 downregulation than in control transfected cells (Figure 18B).

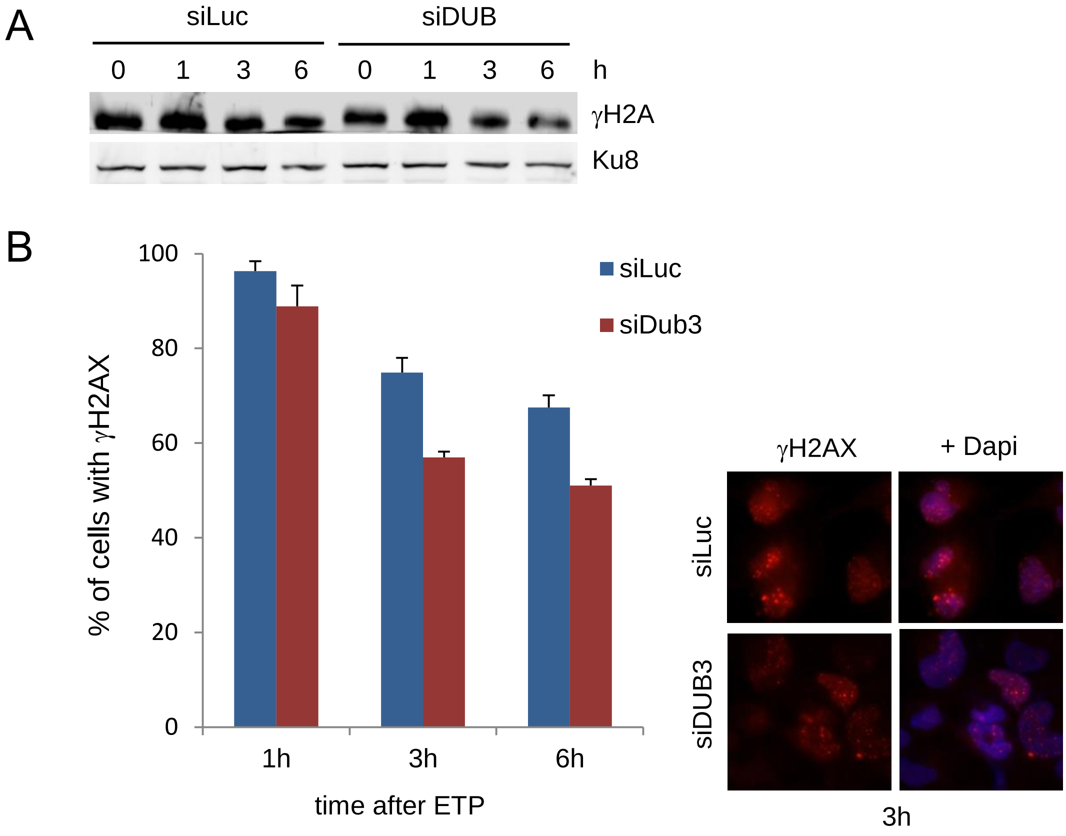

**Figure 18: DUB3 depletion produces a quicker recovery of the DDR.** A) HeLa cells were transfected twice with Luc or DUB3 siRNA oligos. 48 h later, cells were treated with 2 μM ETP for 1 h, then washed, incubated with fresh medium for the indicated time points. Western blot analysis of lysates using the indicated antibodies was carried out. B) HeLa cells were transfected and treated as in A) but now cells were analysed by immunofluorescence. γH2AX foci were scored and quantified (left panel). Right panel shows representative images of 3 h time point.

The data presented in this chapter characterize a novel role for DUB3 in the DDR by direct deubiquitination of H2AX. DUB3 regulates the localization of DNA repair factors 53BP1 and BRCA1 to the sites of DNA lesions and thereby ensures a correct DDR. These data demonstrate that a tight regulation of DNA damage checkpoint activation is crucial for the maintenance of genomic integrity.

# 3. DUB3 AND SENP5 AS NOVEL REGULATORS OF PCNA MONOUBIQUITINATION AND THE IMPLICATIONS FOR TRANSLESION SYNTHESIS

## 3.1. DUB3 and SENP5 catalytic activity regulate PCNA ubiquitination

PCNA ubiquitination was discovered over a decade ago and it subsequently emerged as a prominent marker for replication problems associated with DNA damage or replication fork stalling (122; 200). By means of its downstream effectors, ubiquitinated PCNA controls several aspects of damage tolerance, defined as a mechanism that allows the replication machinery to bypass or avoid lesions in the template DNA (123; 154). PCNA is monoubiquitinated on Lys164 in a manner dependent on E3 ligase Rad18, which can be followed by Lys63-mediated polyubiquitination by HLTF and SHPRH (156; 157). The same Lys164 residue is also modified by SUMO, as demonstrated in budding yeast, chicken DT40 cells, *Xenopus* egg extracts and more recently in human cells (122; 201; 202; 203). In contrast to ubiquitination, PCNA SUMOylation is a damage-independent modification (122). Similar to ubiquitination however, the modification is strongly enhanced by loading of PCNA onto DNA (204). Preventing PCNA SUMOylation suppresses the damage sensitivity of budding yeast mutants defective in damage bypass. This was interpreted as an antagonistic relationship between SUMO and ubiquitin (122).

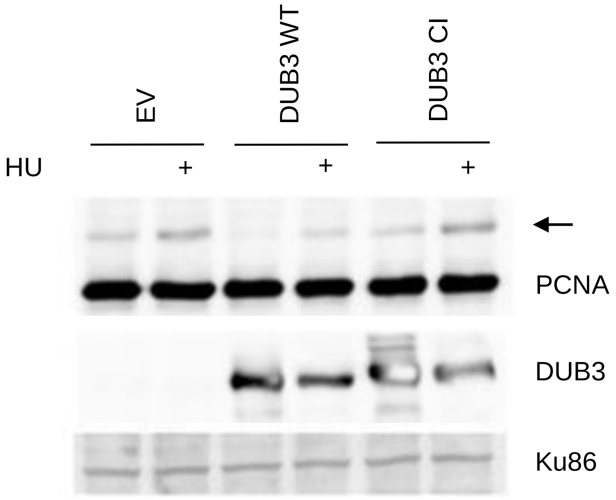

**Figure 19: Overexpression of DUB3 wild type, but not catalytic inactive, reduces PCNA monoubiquitination.** 293T cells were transfected with EV, DUB3 WT or DUB3 CI and left untreated or treated with 2 mM HU for 16 h. Whole cell extracts were analysed by western blot using indicated antibodies (arrow points to monoubiquitinated PCNA).

Deubiquitinase DUB3 and SUMO hydrolase SENP5 were the most obvious candidates of our screening for regulation of genotoxic stress-induced monoubiquitination of PCNA (Figures 4 and 5). As overexpression of DUB3 led to a decreased PCNA monoubiquitination, we wanted to investigate that this effect is due to its catalytic activity, for which our catalytic inactive version of this protein (C89S, see before) was used. In contrast to wild type, overexpression of the catalytic inactive mutant did not diminish the HU-induced monoubiquitination of PCNA (Figure 19), indicating that the catalytic domain is essential for DUB3 activity on PCNA.

The same was true for the effect of SENP5 on PCNA monoubiquitination. SENP5 contains a cysteine in the active site that contributes to its protease activity. To generate a catalytic inactive mutant, this active cysteine was mutated to a serine (C713S). Subsequently, wild type and catalytic mutant versions of SENP5 were overexpressed in 293T cells and in this experiment, PCNA monoubiquitination was triggered by irradiation with UV light. Expression of SENP5 wild type reduced UV-induced PCNA monoubiquitination, while two clones of the same catalytic inactive version did not have any effect (Figure 20).

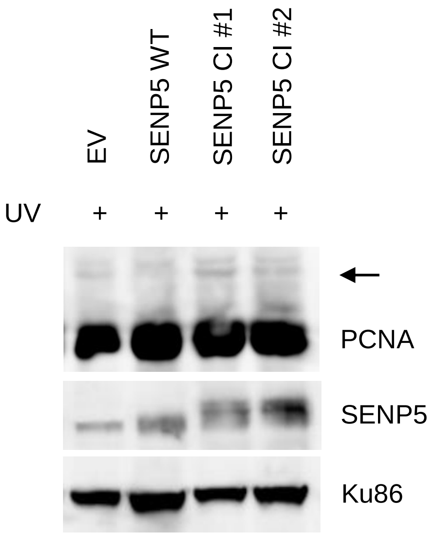

**Figure 20: SENP5 wild type overexpression diminishes UV-induced PCNA monoubiquitination.** 293T cells were transfected with indicated plasmids and treated with UV light (40 J/m$^2$, 1 h). Western blot analysis was performed with mentioned antibodies (arrow points to monoubiquitinated PCNA).

### 3.2. DUB3 and SENP5 counteract E3 ubiquitin ligase Rad18

DNA damage-induced monoubiquitination of PCNA is mediated by E3 ubiquitin ligase Rad18 (122). To confirm that DUB3 and SENP5 antagonize the effect of this E3 ligase, Rad18 was overexpressed in the presence and absence of exogenous DUB3 or SENP5, and PCNA monoubiquitination was analysed by western blot. As described by others, Rad18 overexpression increased PCNA monoubiquitination without treatment with DNA damaging agents (Figures 21 and 22). Coexpression with DUB3 wild type resulted in a decrease in PCNA monoubiquitination, whereas this did not occur when the catalytic inactive version of DUB3 was expressed with Rad18 (Figure 21). Transfection of SENP5 had the same effect. Wild type SENP5 inhibited the Rad18-induced monoubiquitination of PCNA, while expressing the catalytic mutant did not result in any change in PCNA monoubiquitination levels (Figure 22).

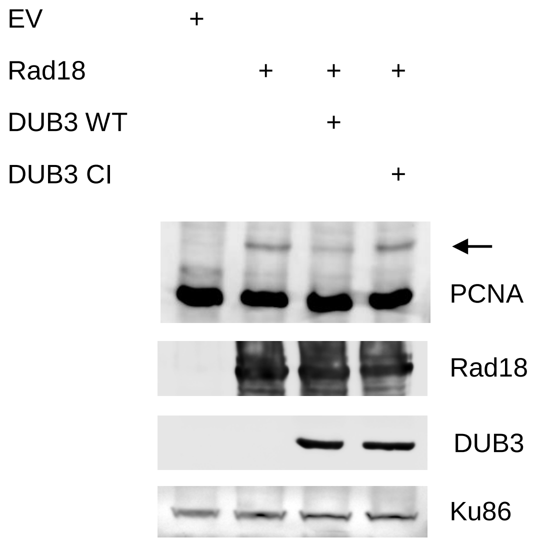

**Figure 21: DUB3 counteracts Rad18-induced monoubiquitination of PCNA.** 293T cells were transfected with indicated plasmids and analysis was carried out by western blotting. Arrow points to PCNA monoubiquitination.

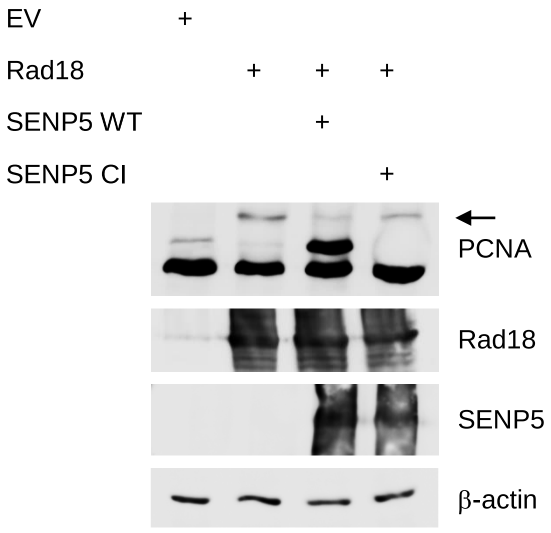

**Figure 22: Overexpression of SENP5 antagonizes Rad18 activity on PCNA.** 293T cells were transfected as indicated and analysed by western blotting. Arrow points to monoubiquitinated PCNA.

## 3.3. PCNA interacts with DUB3 and SENP5

We next wanted to study if the effect of DUB3 and SENP5 on PCNA monoubiquitination was direct. For DUB3, a deubiquitinase, this could be likely but as SENP5 is a SUMO hydrolase, we suspected the effect on PCNA monoubiquitination to be indirect. Either by regulating possible PCNA SUMOylation, which might affect monoubiquitination or by controlling SUMOylation of a protein involved in PCNA ubiquitination.

As a first approach, the possible interaction of PCNA with our candidate proteins was examined. Since ubiquitin hydrolases generally interact with their substrates, in the event of a direct effect, interaction was expected. For this experiment, wild type or catalytic mutant Flag-DUB3 was expressed with GFP-PCNA in 293T cells. Flag-tagged proteins were immunoprecipitated and PCNA was detected by western blot. PCNA was pulled down with both versions of DUB3, as shown in anti-PCNA and anti-GFP western blots (Figure 23).

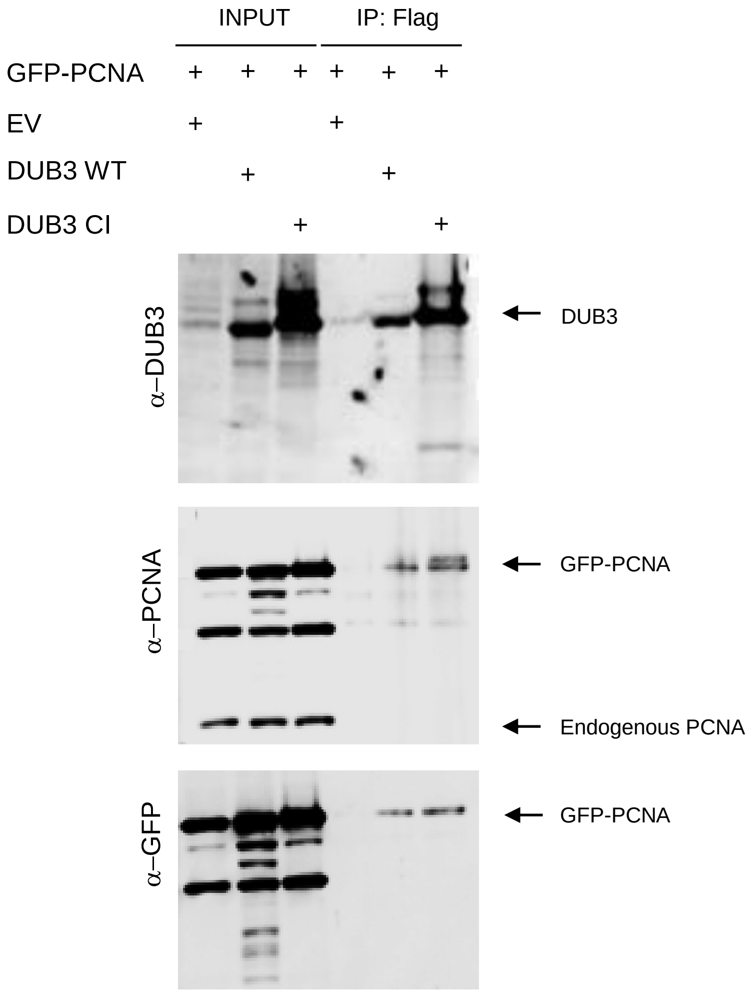

**Figure 23: GFP-PCNA co-immunoprecipitates with Flag-DUB3.** 293T cells were transfected were transfected with EV, Flag-DUB3 WT or CI in combination with GFP-PCNA. Flag-tagged proteins were immunoprecipitated and western blot was performed with DUB3, PCNA and GFP antibodies.

Next, to demonstrate interaction between DUB3 and endogenous PCNA, GFP-DUB3 was overexpressed, PCNA immunoprecipitations were carried out and DUB3 was detected using a DUB3 antibody. GFP-DUB3 co-immunoprecipitated with endogenous PCNA (Figure 24). Together these independent experiments demonstrate that DUB3 and PCNA interact *in vivo*, suggesting that DUB3 might directly deubiquitinate PCNA.

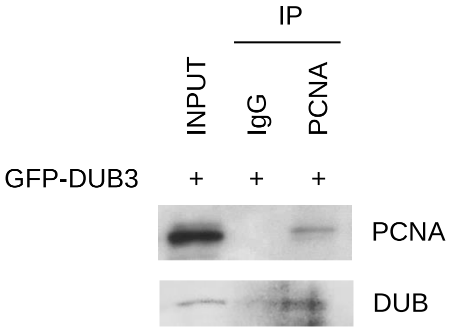

**Figure 24: Interaction between endogenous PCNA and GFP-DUB3.** 293T cells were transfected with GFP-DUB3 WT. Endogenous PCNA was immunoprecipitated using a PCNA antibody, followed by western blot with indicated antibodies.

A potential interaction between SENP5 and PCNA was tested by immunoprecipitation of endogenous PCNA from extracts of Flag-SENP5-expressing 293T cells. To our surprise, Flag-SENP5 was abundantly present in PCNA immunoprecipitations (Figure 25). In addition, a pulldown experiment was performed using purified GST-tagged PCNA and extracts from 293T cells expressing Flag-SENP5. Figure 26 shows that Flag-SENP5 was found in GST-PCNA wild type pulldowns. Proteins that interact with PCNA usually contain a consensus sequence named PCNA-interacting protein (PIP) box. PCNA Met40 (M40) and His44 (H44) are essential residues for PCNA/PIP-box interactions (205). In contrast to GST-PCNA wild type, Flag-SENP5 did not bind a binding domain mutant (M40A) of GST-PCNA (Figure 26).

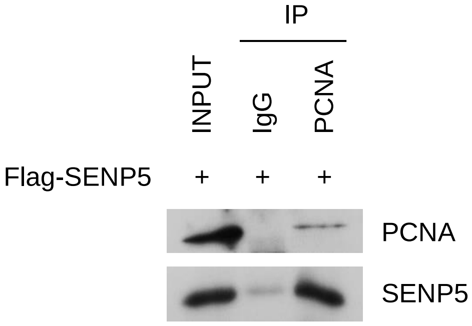

**Figure 25: Flag-SENP5 binds endogenous PCNA.** 293T cells were transfected as described. Endogenous PCNA was immunoprecipitated using PCNA antibody, followed by western blot with indicated antibodies.

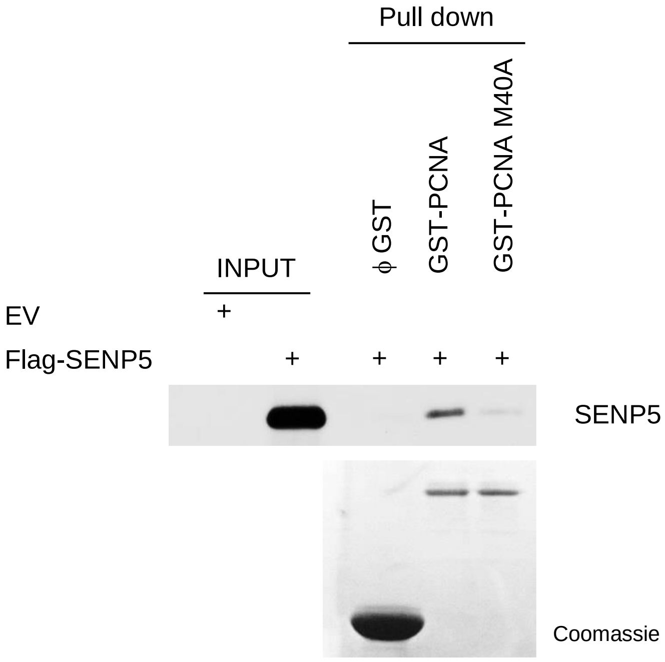

**Figure 26: GST-pull down demonstrates that PCNA and SENP5 interact through the PCNA binding domain.** Flag-SENP5 WT was transfected in 293T cells. GST-pulldown was performed with purified wild type or M40A mutant GST-PCNA. Western blot against SENP5 was performed. Coomassie staining was used to demonstrate loading of purified proteins.

From these experiments we conclude that PCNA interacts with SUMO hydrolase SENP5, likely via a possible PIP motif in SENP5. However, we should stress that does not demonstrate that the effect of SENP5 on PCNA monoubiquitination is direct.

### 3.4. DUB3 deubiquitinates PCNA in vivo, but not SENP5

As mentioned, further experiments were required to answer the question if DUB3 and especially SENP5 directly deubiquitinate PCNA. As a start, an *in vivo* assay for PCNA ubiquitination was performed in which PCNA monoubiquitination was forced by expressing His-tagged ubiquitin followed by a His-pull down. As shown in Figure 27, under these conditions, very efficient monoubiquitination of wild type PCNA was observed, both after pulling down ubiquitinated proteins as well as in the input. A K164R mutant version of PCNA, which cannot be ubiquitinated, served as a negative control (122). Co-transfection of DUB3 wild type, but not catalytic inactive, inhibited the in vivo monoubiquitination of PCNA (Figure 27), supporting the hypothesis that DUB3 might directly deubiquitinate PCNA. In contrast, co-expression of SENP5 did not change PCNA monoubiquitination levels (Figure 27). This result might not seem in accordance with our previous results showing that overexpression of SENP5 wild type without exogenous ubiquitin (Figure 20 and 22). However, we reason that by forcing

PCNA monoubiquitination by expressing exogenous ubiquitin, a potential indirect effect of SENP5 is masked. These results thereby suggest, as expected from a SUMO hydrolase, that it is unlikely that SENP5 directly deubiquitinates PCNA.

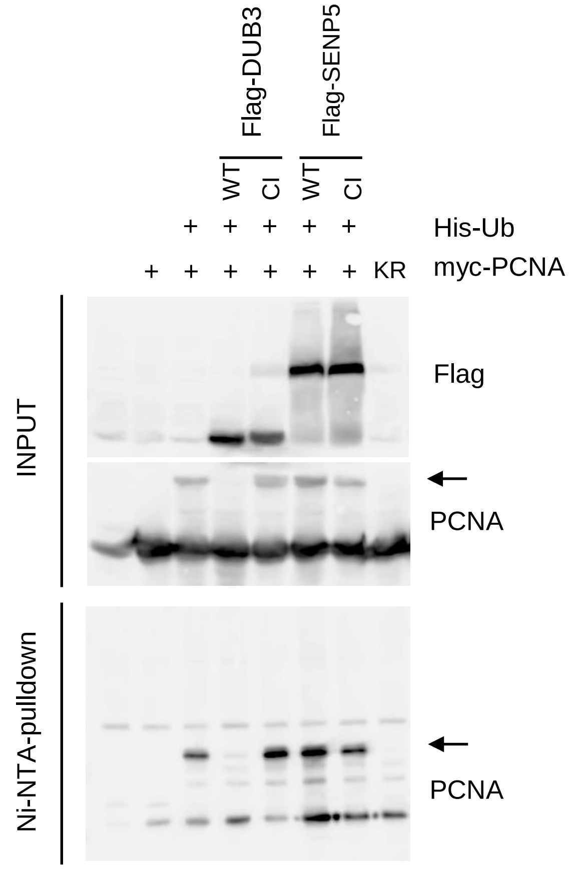

**Figure 27: Forced monoubiquitination of PCNA is inhibited by DUB3, but not SENP5.** 293T cells were transfected with His-ubiquitin (His-Ub) together with myc-PCNA and WT or CI versions of Flag-DUB3 or Flag-SENP5. PCNA K164R mutant was used as a negative control. A Ni-NTA-pull down was performed and western blot with the indicated antibodies was used as readout. Arrow indicates monoubiquitinated PCNA.

### 3.5. DUB3 and SENP5 affect PCNA function

PCNA monoubiquitination was shown to be important for the activation of the translesion synthesis pathway, an error prone repair pathway (121; 206). PCNA monoubiquitination initiates a polymerase switch from a replicative polymerase to a TLS polymerase (125; 207).

As our results suggested that DUB3 and SENP5 affect PCNA monoubiquitination, we wanted to study if modulating the levels of these enzymes affects translesion synthesis. DUB3 and SENP5 depletion was expected to increase PCNA monoubiquitination, which would promote translesion synthesis repair. The efficiency of translesion synthesis was determined by measuring the mutation frequency in a mutagenesis assay. For this assay, a SupF plasmid was damaged by UV light and was subsequently transfected in 293T cells depleted of DUB3 or SENP5. The SupF plasmid was recovered and transformed into bacteria, using colony colours to quantify the mutation frequency (blue: normal, white: mutated). Cells downregulated for DUB3 displayed a higher mutation frequency as compared to control cells (Figure 28A). SENP5 depletion had the same effect. Knock down of SENP5 by two independent siRNA oligos, resulted in an increase in mutation frequency (Figure 28B). These experiments indicate that there is a hyperactivation in the translesion synthesis repair pathway upon UV-induced DNA damage in cells depleted for DUB3 and SENP5, pointing to DUB3 and SENP5 as important players in translesion synthesis through controlling PCNA monoubiquitination.

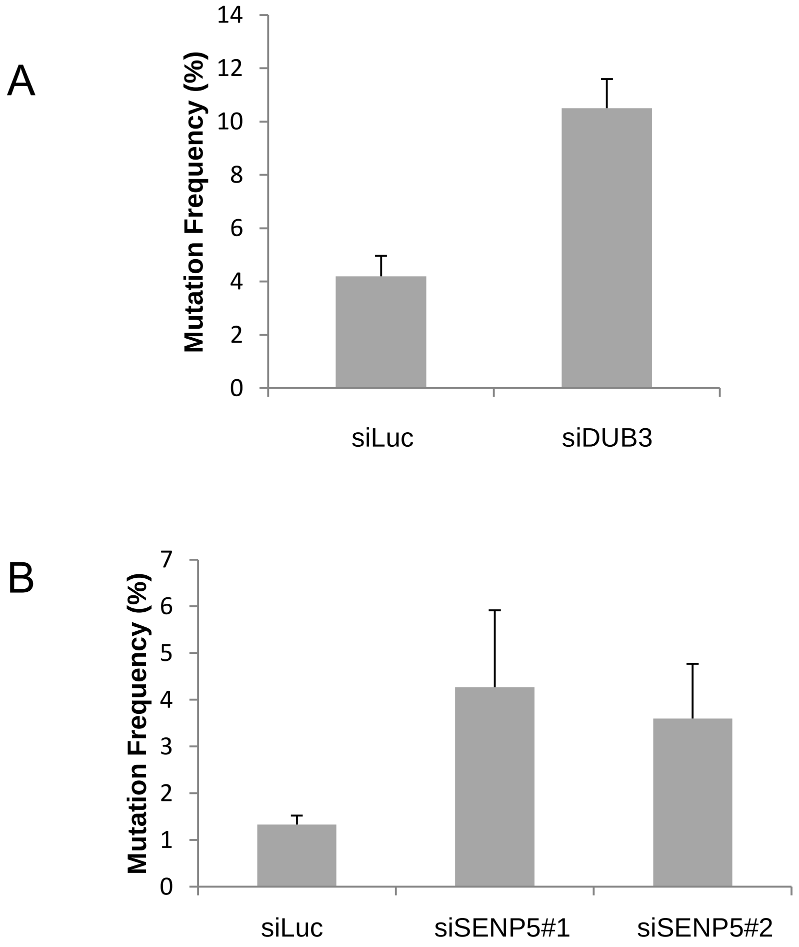

**Figure 28: DUB3 or SENP5 depletion results in increased mutation frequency**. A) DUB3 was downregulated in 293T cells by siRNA. Subsequently, the UV-irradiated SupF plasmid was transfected. Two days later, the SupF was recovered, transformed into bacteria which were plated. The ratio of mutants (white) to total (blue + white) colonies was scored as mutation frequency. B) As in A, but after SENP5 downregulation using two different siRNA oligos.

## 4. PRELIMINARY RESULTS OF SCREENING CHROMATIN REGULATORS ON 53BP1 AND BRCA1 FOCUS FORMATION

There is increasing evidence that histone modifications and chromatin remodelling play a role before and after DNA repair, among others to locally open up the structure to facilitate accessibility of the repair machinery and to restore the chromatin structure after completion of the DNA repair (208; 209). This makes the identification of novel regulators of these processes interesting. A screening similar to the DUB screening was therefore carried out to search for novel chromatin modifying enzymes involved in the DDR. A small library consisting of expression vectors for several acetyl transferases, demethylases and related enzymes was collected. Recruitment of 53BP1 and BRCA1 to sites of DNA lesions was studied because this event is crucial for two pathways of DSB repair: homologous recombination (BRCA1) and non homologous end joining (53BP1) (97). The plasmids of the library were individually overexpressed in U2OS cells and phleomycin-induced accumulation of 53BP1 and BRCA1 into foci was analysed by immunofluorescence, detecting transfected cells through different tags and using non-transfected cells as negative control.

Overexpression of the following proteins affected DNA damage-induced focus formation of 53BP1 and/or BRCA1 (to different extends) and these enzymes were therefore considered as candidates.

| Name | Activity | Synonyms | Effect on focus formation |
|---|---|---|---|
| CUL1 | E3 ligase component | Cullin1 | 53BP1 (30% foci reduction)<br>BRCA1 (no changes) |
| CUL3 | E3 ligase component | Cullin 3, PHA2E | 53BP1 (30% foci reduction)<br>BRCA1 (no changes) |
| KAT2B | Lys acetyl-transferase | PCAF | 53BP1 (30% foci reduction)<br>BRCA1 (no foci 40%) |
| KDM3A | Lys specific de-methylase | JMJD1, JMJD1A<br>JHDM2A | 53BP1 (no changes)<br>BRCA1 (80% foci reduction) |
| KDM3B | Lys specific de-methylase | C5orf7, JMJD1B | 53BP1 (20% foci reduction)<br>BRCA1 (no foci 50%) |
| KDM4A | Lys specific de-methylase | JMJD2, JMJD2A<br>JHDM3A | 53BP1 (20% foci reduction)<br>BRCA1 (no foci 45%) |
| PHF2 | PHD finger protein | | 53BP1 (30% foci reduction)<br>BRCA1 (no changes) |
| PHF8 | PHD finger protein | | 53BP1 (no changes)<br>BRCA1 (25% foci reduction) |

The most interesting candidate protein was KDM3A, a demethylase, previously described to demethylate mono- and di-methyl Lys9 of histone H3 (210). Overexpression of KDM3A resulted in a decrease in phleomycin-induced BRCA1 focus formation while it did not affect to 53BP1 recruitment to sites of DNA damage (Figure 29). We therefore decided to focus first on KDM3A.

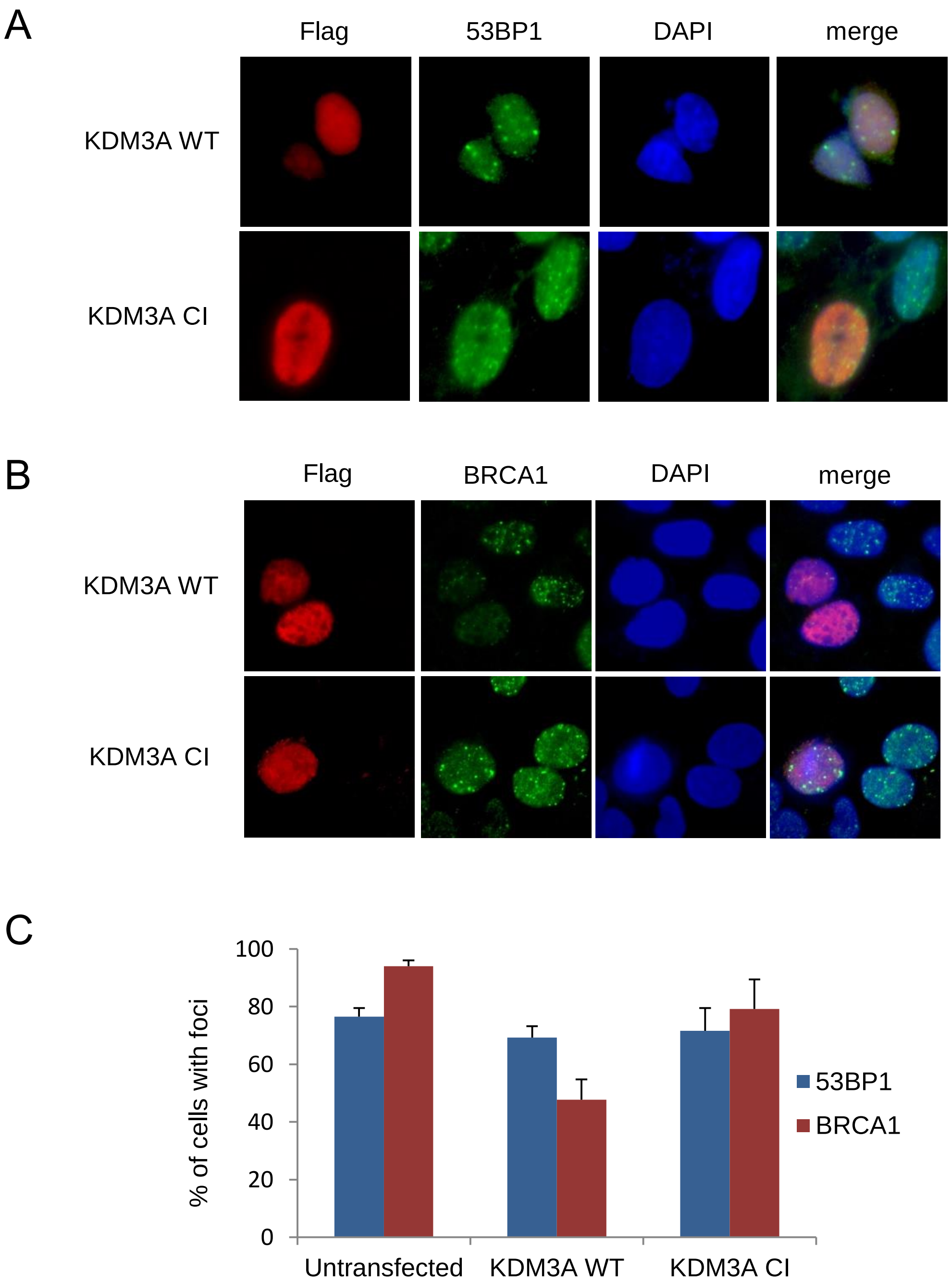
A
Flag
53BP1
DAPI
merge
KDM3A WT
KDM3A CI
B
Flag
BRCA1
DAPI
merge
KDM3A WT
KDM3A CI
C
100
80
60
40
20
0
% of cells with foci
53BP1
BRCA1
Untransfected
KDM3A WT
KDM3A CI

**Figure 29: KDM3A overexpression reduces recruitment of BRCA1 to sites of DNA lesions but does not affect DNA damage-induced focus formation of 53BP1.** A) Expression of KDM3A WT and CI in U2OS cells treated with phleomycin (10 µg/ml, 1 h). Flag staining was performed to detect transfected cells and 53BP1 antibody was used to detect focus formation. Non-transfected cells were used as control. B) As in A, but staining against BRCA1 to detect focus formation. C) Quantification of A and B of three different experiments. Error bars represent standard error.

His1120 and His1122 are important residues for KDM3A catalytic activity, involved in co-factor binding during the oxidative demethylation reaction (211). His1122 was mutated to alanine to generate a catalytic inactive version of KDM3A for use in this study. KDM3A wild type and catalytic inactive version were overexpressed in U2OS where after phleomycin-induced 53BP1 and BRCA1 focus formation were analysed by immunofluorescence. KDM3A did not affect recruitment of 53BP1 to sites of DNA lesions (Figure 29A and C). In contrast, wild type KDM3A reduced the focus formation of BRCA1 while catalytic mutant did not have any effect on BRCA1 recruitment (Figure 29B and C).

In the initial screening, BRCA1 focus formation was reduced by 80% after overexpression of KDM3A, whereas the quantification in Figure 29C table resulted in a 50% reduction. This difference could be due to different KDM3A expression levels or a difference in the efficiency of the induction of foci. Moreover, the initial screening was performed once while the quantification was the average of three different experiments. This result suggests that KDM3A might be involved in regulating homologous recombination while it is not involved in non homologous end joining. Further research is needed to investigate the role of this demethylase in DSB repair into more detail.

# IV. DISCUSSION

## 1. IMPORTANCE OF POST-TRANSLATIONAL MODIFICATIONS IN THE DNA DAMAGE RESPONSE

In mammals, DNA lesions occur naturally and this occurrence is aggravated after metabolic and oxidative stress. Consequently, DNA repair processes have evolved to maintain cellular viability and genomic stability. As the severity of DNA damage increases from single base damage to DSBs, different mechanisms have developed to carry out repair. In the last years, numerous studies in the DNA damage response field demonstrated that post-translational modifications are critical in DNA damage response for the rapid execution of checkpoint signalling, DNA repair and restoration of chromatin. Ubiquitination, SUMOylation, phosphorylation, methylation and acetylation seem to be important modes of these modifications.

### 1.1. Histone modifications

It is a fact that immediately upon induction of DNA damage, chromatin is locally destabilized to facilitate the access of the repair machinery to DNA lesions. After DNA repair is completed, chromatin is restored to its original state in the vicinity of damage sites. This demonstrates how DNA repair might occur in chromatin environment (212; 213; 214). Histone modifications can be integrated into this damage-access-repair-restore model.

One well characterized change in chromatin organization is the rapid formation of open chromatin structures at DSBs. It is demonstrated that this process is associated with increased acetylation of histones H2A and H4 on nucleosomes near DSBs (166; 215; 216; 217). The Tip60 acetyltransferase is rapidly recruited to DSBs where it can acetylate multiple proteins, such as histones H2A and H4 (147; 215; 218). The increase in acetylation of histones H2A and H4 at DSBs subsequently promotes chromatin unpacking and direct the formation of open, relaxed chromatin structures (219). Histone H3 acetylation also contributes to opening up the chromatin structure. In response to DNA damage, histone H3 is first acetylated at Lys14 and Lys23, which promotes chromatin relaxation and in this way facilitates ubiquitination of phosphorylated H2AX (γH2AX), checkpoint activation, and DNA repair (147). In contrast, acetylation of histone H3 at Lys56 at later time points, after repair has been completed, leads to restoration of chromatin by recruiting the histone chaperone Asf1 and termination of checkpoint activation (220). These data suggest that the timing and characteristics of each different post-translational modification are critical factors in determining the physiological effect of a specific modification.

The same concept seems to be true for histone ubiquitination. Ubiquitination of H2AX and H2A contributes to checkpoint signalling by initiating a cascade that assembles DNA damage response components at the breakage site (21; 221). Deubiquitination of H2A(X) at later time points has been proposed to function to switch off checkpoint signalling (36). These scenarios raise the question whether these modifications are an extension of histone code in the field of DNA damage response.

Answer to this question is important for our understanding of the detailed mechanisms of this response.

It was found that USP3 is required for deubiquitination of ubiquitin-conjugates of H2A and H2B and for dephosphorylation of H2AX at later time points after damage. This way, USP3 depletion resulted in a delay of S phase progression after DNA damage and an accumulation of DNA breaks. These findings suggest that ubiquitin needs to be removed from H2A and H2B for checkpoint recovery (31). As USP3, deubiquitinating enzymes POH1, OTUB1, BRCC36, USP16 and USP44 were shown to counteract RNF8/RNF168-mediated H2A(X) ubiquitination (33; 34; 35; 199; 222). The Rap80/BRCC36 complex contains BRCC36, which functions to prevent Ubc13/RNF8 activity to provide a balanced level of ubiquitination around the DNA lesions (34). OTUB1 was shown to suppress RNF168-dependent ubiquitination in a non-catalytic manner, by interacting with and inhibiting Ubc13 and 19S proteasome ubiquitin protease POH1 was shown to restrict 53BP1 recruitment by counteracting RNF8/RNF168-mediated polyubiquitination and retention of JMJD2A on the chromatin (35; 222). In this thesis we demonstrated similar things for DUB3 and our experiments show that the efficiency of H2AX deubiquitination by DUB3 is comparable or even stronger to the other known DUBs for H2AX (36). Moreover, depletion of only two of these known DUBs, USP3 and BRCC36, led to an increase in ubiquitinated H2A(X), as we have seen for DUB3 (31; 34; 36). Among them, knockdown of BRCC36 increases the DNA damage-induced H2AX ubiquitination. Depletion of DUB3 and USP3 however, already led to elevated H2AX ubiquitination levels in undamaged conditions. The involvement of multiple enzymes in the same process indicate that strict regulation of an active turnover of histone H2A(X)/H2B ubiquitination is critical in the response to genotoxic stress.

As shown in chapter 2.4, DUB3 overexpression has important effects on 53BP1 and BRCA1 focus formation and we show that DUB3 and H2AX interact directly and finally, DUB3 deubiquitinates H2AX *in vitro* (36). Our results confirm that a balanced level of H2AX ubiquitination is required for a correct DNA damage response: activation upon the detection of DNA lesions and switch of when the lesions are repaired, to stimulate switching off this pathway. This thesis therby described a novel role for DUB3 in this process in the DNA damage response by regulating H2AX ubiquitination.

The molecular mechanisms involved in protein recruitment and activation during the DNA damage response are palpably complex. The mechanisms described here are only a small part of this complexity, and crosstalk mechanisms that involve other modifications exist. A good example is the recruitment of 53BP1 and BRCA1, required for regulating DSB repair, to sites of DNA lesions. Recent work suggests that DNA damage-induced histone H4 neddylation (another ubiquitin-like modification) is important to induce ubiquitination events at sites of damage, since the polyneddylation chain can be recognized by the MIU (Motif Interacting with Ubiquitin) domain of RNF168 (223). Loss of DNA damage-induced neddylation negatively regulates focus formation of RNF168 and its downstream functional partners, such as 53BP1 and BRCA1, thereby affecting the normal DNA repair process (223). The recruitment of

53BP1 to DSBs is additionally dependent on binding of 53BP1 tandem Tudor domains to histone H4K20me2, which requires RNF8 and RNF168-induced VCP-mediated displacement of polycomb protein L3MBTL1 to unmask 53BP1 chromatin binding sites and RNF8-dependent proteasome degradation of competing H4K20me2 readers, such as the KMD4A/JMJD2A demethylase (177; 178; 224; 225). Also H4K16 deacetylation and RNF168-mediated histone H2AK15 ubiquitination influence 53BP1 recruitment to DNA lesions (226; 227; 228).

In contrast, demethylase JMJD1C contributes to the BRCA1 recruitment, but not 53BP1. JMJD1C binds to RNF8 and MDC1, and demethylates MDC1 at Lys45. JMJD1C thereby promotes the MDC1-RNF8 interaction, the RNF8-dependent ubiquitination of MDC1 and the recruitment of BRCA1 to polyubiquitinated MDC1 (229). Our screening of chromatin modifiers, with DNA damage-induced focus formation of BRCA1 and 53BP1 as readout, could serve to identify novel regulators of these pathways. The strongest candidate was KDM3A/JMJD1A, a histone H3K9 demethylase, as a possible regulator of BRCA1 recruitment to sites of DNA lesions. Overexpression of the wild type, but not catalytic active version of this demethylase inhibited phleomycin-induced focus formation of BRCA1 but not 53BP1. It should be stressed however, that these data are preliminary and need to be reproduced and followed up, for example by investigating if KDM3A can also affect MDC1 methylation or another protein/histone in the pathway.

It will be interesting to determine if combinations of specific modifications regulate the choice between different repair mechanisms, such as HR versus NHEJ. Some studies suggest that histone H3K4 methylation plays a role in promoting HR, showing that KDM1A can demethylate histone H3K4 at sites of DNA damage and loss of this demethylase increases HR (230; 231). Interestingly, another study demonstrates that the histone H3K9 methyltransferase PRDM2 is required for the recruitment of BRCA1, but not 53BP1, to DSBs (232). Would KDM3A, which appeared in our screening, and whose overexpression resulted in a decrease in damage-induced BRCA1 focus formation, counteract PRDM2 and thereby balance the level of H3K9 methylation? The combination of Lys63 polyubiquitination and histone H3K9 methylation may potentially serve as a signal for BRCA1 recruitment thereby promoting HR. Future work will clarify the molecular mechanisms responsible for recruiting repair factors and displaying distinct pathways of DSB repair.

### 1.2. PCNA post-translational modifications

Good progress has been made in understanding the regulation of PCNA post-translational modifications and its role in DNA damage bypass and the maintenance of genome stability. We now know that monoubiquitination of PCNA at Lys164 by Rad6/Rad18 functions to recruit the Y-family DNA polymerases and activate translesion synthesis, whereas further extension of this modification to polyubiquitination by UBC13/MMS2/Rad5 initiates an error-free template switching mechanism (122; 155). PCNA SUMOylation occurs at the same lysine residue as ubiquitination and serves to suppress HR at the site of the stalled replication fork, mainly during unperturbed S phase. In yeast this functions through the recruitment of Srs2, a helicase that blocks Rad51 nucleofilament formation (122; 125; 162). Recently, a low level of SUMOylation of PCNA has also been reported in human cells (203; 233).

As important as PCNA ubiquitination is PCNA deubiquitination, which is in yeast carried out by the USP1-UAF1 complex in conjunction with ELG1, a protein that forms an alternative PCNA-interacting RFC complex (234). Cleavage of PCNA monoubiquitination in mammals is also carried out by USP1 (195). Human ELG1 specifically directs USP1-UAF1 to PCNA at the damage site, and initiates the switch from the error prone and poorly processive translesion synthesis polymerases to the faithful replicative polymerases Polδ and Polε (195). Cleavage of PCNA monoubiquitination in mammals is also carried out by USP1 (235). Recently, USP7 was identified as additional regulator of PCNA monoubiquitination, specifically induced by $H_2O_2$ (236).

Here we identified DUB3 and SENP5 as novel regulators for PCNA monoubiquitination. We have demonstrated that overexpression of a wild type version of either DUB3 or SENP5 diminishes the DNA damage-induced monoubiquitination of PCNA and thereby counteracts the E3 ubiquitin ligase Rad18. We have also shown *in vivo* interaction of PCNA with DUB3 and SENP5. Nevertheless, we could only demonstrate *in vivo* deubiquitination of PCNA by DUB3 as expression of SENP5 in conditions of forced monoubiquitination of PCNA by expression of His-Ub did not decrease PCNA monoubiquitination. These results suggests, as expected from a SUMO hydrolase, that it is unlikely that SENP5 directly deubiquitinates PCNA. Despite this and in accordance to lower levels of PCNA monoubiquitination upon overexpression of DUB3 or SENP5, we detected increased mutation frequency, indicative of more efficient translesion synthesis, in cells depleted for DUB3 or SENP5. This indicates that DUB3 and SENP5 play a role in regulation of translesion synthesis through controlling PCNA monoubiquitination.

Switching back to replicative polymerases after DNA damage bypass is necessary to reduce the mutagenic effects induced by the translesion synthesis polymerases. Many questions about this process remain. For example, exactly when does DNA damage bypass occur? And what is the mechanism to return to the replicative polymerases? By controlling PCNA monoubiquitination, DUB3 and/or SENP5 could regulate switching back to the replicative polymerases. If this were the case, these hydrolases should be strictly controlled as well. As no changes in DUB3 or SENP5 protein levels after DNA damage were observed (data not shown), DUB3 and SENP5 activity should be regulated in a different manner. Many ubiquitin hydrolases are known to auto-deubiquitinate and this might be a way to regulate enzymatic activity. It will be interesting to investigate possible regulation of DUB3 and SENP5 during the cell cycle and in response to DNA damage in more detail.

How SENP5, a SUMO hydrolase, controls PCNA monoubiquitination, remains a question. As mentioned, PCNA is also SUMOylated, following replication stress or during unperturbed S phase. In yeast, PCNA is SUMOylated at Lys164 and Lys127 by the E2 Ubc9 and the E3 Siz1 (43; 44). PCNA SUMOylation is a reversible modification that can be removed by the SUMO protease Ubl-specific protease 1 (ULP1) in yeast (125; 237; 238). Modification of PCNA by SUMO has not been very well reported in human cells, but PCNA interacting partner PARI contains a PIP domain and a SUMO-binding motif and was described as a possible analogue of yeast Srs2 in higher eukaryotes (233). If SENP5 would affect PCNA SUMOylation, SENP5 could act as an ULP1 analogue in human cells. *In vivo* PCNA SUMOylation assays in cells overexpressing SENP5 should be performed to verify this hypothesis.

Alternatively and quite likely, SENP5 could regulate PCNA monoubiquitination via an indirect mechanism. It was described that *S.cerevisiae* Rad18, responsible for monoubiquitination of PCNA in response to DNA damage, can recognize SUMOylated proteins via its SIM as substrates for ubiquitination. As such, Rad18's activity towards PCNA is strongly enhanced by the presence of SUMO on PCNA. Rad18 itself is also SUMOylated (239). If we extrapolate this information to humans, our results with SENP5 could be explained since SENP5 overexpression results in less SUMOylated PCNA by which the activity of Rad18 towards PCNA monoubiquitination is not optimal. However, it should be mentioned that human Rad18 does not contain an obvious SIM and fusing SUMO-2 to human PCNA did not stimulate Rad18 activity in mammalian cells (239). An alternative explanation for our results is that SENP5 regulates Rad18 activity by directly regulating Rad18 SUMOylation. More experiments are planned to test these possibilities. Unfortunately, identification of SENP5 interactors by mass spectrometry did not result in any possible binding partners that were functionally related to PCNA (data not shown).

Although it is clear that ubiquitin and SUMO hydrolases are critical regulators of the DNA damage response, we are only beginning to understand their molecular mechanisms of action, the consequences of their possible deregulation on genomic stability and their impact on physiology and human disease. Gaining more insight into such enzymes could open possibilities as targeting these enzymes in the treatment of cancer.

## 2. RELATION OF DUB3 AND SENP5 WITH GENOMIC STABILITY MAINTENANCE

Very little is known about DUB3. One of the first reports about this ubiquitin hydrolase described that DUB3 expression was induced in response to cytokines IL-4 and IL-6 stimulation and constitutive expression of DUB3 slowed proliferation and led to an increase in the rate of apoptosis in certain cell types, suggesting that DUB3 is involved in both cell proliferation and survival (240).

After that, the DUB3 gene was shown to be a direct target of the estrogen-related receptor β, a key transcription factor of the self-renewal machinery (241). DUB3 expression was strongly downregulated during neural conversion and preceded Cdc25A destabilization, while forced DUB3 expression in mouse embryonic stem cells became lethal upon differentiation, concomitant to cell cycle remodelling and lineage commitment. Moreover, knockdown of either DUB3 or Cdc25A induced spontaneous differentiation of embryonic stem cells. These findings link the self-renewal machinery to cell cycle control through DUB3 deubiquitinase in embryonic stem cells (241).

DUB3 was linked first to the DNA damage response by a paper describing regulation of Cdc25A stability by DUB3, the phosphatase that activates cyclin/Cdk complexes and a downstream target of Chk1 (242). Pereg and coworkers additionally demonstrated that DUB3 overexpression produced accumulation in S and G2 phases due to replication stress and activated the DNA damage response. DUB3 overexpression was also shown to be responsible of an abnormally high level of Cdc25A in a subset of human breast cancer (242). Interestingly, control of Cdc25A levels by DUB3 was also demonstrated in mouse embryonic stem cells. In such cells, high Cdc25A levels persist after DNA damage, which is thought to lead to bypass of the G1/S checkpoint. In cells with high Cdc25A levels, also DUB3 levels were high (241). As regulator of Cdc25A, DUB3 is an example of a transforming ubiquitin hydrolase that forms a key component of the cell cycle machinery.

The effect of DUB3 on H2AX and PCNA, as described in this thesis, is independent of its role in controlling proteasome-dependent degradation of Cdc25A. Nevertheless, by controlling the DNA damage response at different levels, DUB3 emerges as a putative important regulator in maintaining genome integrity. Future studies should focus on regulation of endogenous DUB3, during the cell cycle and in response to DNA damage, as this could be a mechanism to counterbalance DNA damage checkpoint activation, and/or to contribute in resuming of cell cycle progression after completion of DNA repair.

DUB3 has an established role in tumor proliferation (242; 243). Last year, a relation between DUB3 and human ovarian cancer was described. High levels of DUB3 expression were found in cystadenomas, borderline tumors, and in ovarian carcinomas and DUB3 expression was significantly associated with lymph node metastasis and clinical staging (243). Multivariate survival analysis indicated that DUB3 expression can be an independent prognostic indicator of the survival of patients with ovarian cancer. Furthermore, the expression of Cdc25A closely correlates with that of DUB3 in ovarian cancer cells and DUB3 depletion can inhibit the proliferation of such cells and increase cell apoptosis (243). These results indicate that ovarian cancer cells could be a good model system to study regulation of endogenous DUB3.

In addition to the obvious role of ubiquitination and DUBs in the DNA damage response, direct involvement of SUMOylation in this pathway was recently shown by the demonstration of enrichment of all three SUMO isoforms (SUMO1 and SUMO2/3) at sites of DNA damage in human cells (96; 244). SENP5, the SUMO-specific protease that we identified as regulator of PCNA, has specificity for SUMO2 and SUMO3 but less for SUMO1 (244).

Depletion of SENP5 revealed a role for this enzyme during mitosis and/or cytokinesis as demonstrated by defects in nuclear morphology and inhibition of cell proliferation (245). This finding of a nonredundant function for SENP5 in cell proliferation provides further support for the model that, analogous to phosphorylation, cycles of SUMOylation and deSUMOylation regulate orderly progression through cell division. More studies of substrates of SENP5 and other SUMO hydrolases in the DNA damage response will give more insight in the biological activities of these enzymes.

SENP5 was shown to primarily localize in the nucleus and nucleoli in COS-7 cells, but there was also a substantial amount of the protein found in the cytosol (246). Interestingly, increased subcellular localization of SENP5 in the cytosol was found in oral squamous cell carcinoma. In addition, there was a correlation between SENP5 and the differentiation of this type of carcinoma, showing higher SENP5 expression in differentiated oral squamous cell carcinoma as compared to paracarcinoma epithelium (247). These findings suggest that cellular localization of SENP5 could be a way of regulation for this SUMO protease and changes in subcellular localization might be an important clinicopathological parameter for diagnosis of oral squamous cell carcinoma. Overexpression of SENP5 was observed in oral cancer tissue specimens and oxidative stress induced higher stability of SENP5 in such oral cancer cells. The combination of SENP5 silencing and $H_2O_2$ treatment led to mitochondria fragmentation and a significant increase in cell apoptosis (248). Consequently, SENP5 protected oral squamous cell carcinoma cells from oxidative stress-induced apoptosis. SENP5 is also overexpressed in osteosarcoma cells, such U2OS cells that were used in our experiments, and its depletion suppresses cell growth and promotes apoptosis in these cells (249). A correlation between SENP5 and breast cancer invasion through a TGFβRI SUMOylation cascade was also demonstrated and low expression of SENP5 was associated with good prognosis among breast cancer patients (250). Together, these studies demonstrate that SUMO hydrolases like SENP5, involved in oral squamous cell carcinoma, osteosarcoma and breast cancer, could play important roles in development of cancer.

In conclusion, DUB3 and SENP5 have been previously linked to the development of different tumors and my studies show that their regulation may be of importance for the proper functioning of the DNA damage response. Therefore this thesis contributes to the knowledge of the role of these enzymes in/during tumorigenesis.

## 3. DUB3 AND SENP5 AS POTENTIAL ANTI-CARCINOGENIC THERAPEUTIC TARGETS

During the first stages of carcinogenesis, early precursor lesions express markers of an activated DNA damage response, such as phosphorylated ATM, Chk2, histone H2AX and p53. The DNA damage response is therefore thought to act as an anti-cancer barrier in early human tumorigenesis. Importantly, DNA damage damaging agents are also used to treat cancer. Tumor cells often have lost one of the tumor suppressor, checkpoint or repair pathways, making them dependent on fewer pathways to respond to DNA damaging agents and therefore more sensitive to treatment with chemoterapetic agents. Modulating the remaining pathways is a way to specifically target tumor cells. An example is PARP inhibitor in BRCA1/2 mutant cells. PARP inhibition leads to DSBs in replication cells, which generally are repaired by HR. BRCA1/2 mutant tumor cells are defective for HR and are consequently specifically killed by PARP inhibitors, whereas normal replicating cells are less sensitive to this treatment (251).

It is clear that ubiquitin and SUMO modifications are now considered as critical in the DNA damage response. The acceptance of Bortezomib (Velcade®), a proteasome inhibitor, for the treatment of multiple myeloma validates the targeting of the proteasome for the treatment of cancer (252). Unfortunately, extended treatment with Bortezomib is associated with toxicity and drug resistance, limiting its efficacy (253). Instead, therapeutic strategies that target specific aspects of the ubiquitin–proteasome pathway upstream of the proteasome, including DUBs, may be better tolerated. As enzymatic activity generally can be targeted by inhibitors, inhibiting ubiquitin/SUMO hydrolases might be a way to modulate the DDR in tumor cells. Indeed, several ubiquitin hydrolases have been described as possible therapeutic targets and the level of biological target validation is becoming clear (254; 255; 256). The large amount of ubiquitin modifications involved in response to DNA damage suggests that at least one DUB might be responsible to revert each modification. In addition, some DUBs appear highly expressed in tumor cells, making them potential therapeutic targets.

Examples of ubiquitin hydrolases as suitable therapeutic agents: USP7, which plays a key role regulating the ubiquitination of the RING-finger E3 ligase Mdm2/Hdm2 (257). Hdm2 binds the tumor suppressor p53 and facilitates its degradation in an ubiquitin- and proteasome-dependent manner (258). Unlike most human tumors, mutations in or deletions of p53 in common hematological malignancies are relatively rare at initial diagnosis, and the activation of p53 may offer a therapeutic benefit (259). Interestingly, some Hdm2 inhibitors have been developed, including Nutlin-3 (Roche), and these have been demonstrated to induce both p53 and apoptosis in a number of p53 wild-type tumors (260). Inducing Hdm2 degradation by inhibiting USP7 and, thus, activating the tumor suppressor p53 could offer a therapeutic benefit.

Studies demonstrated that knockdown of DUB3 significantly retarded the growth of breast tumor xenografts in nude mice, inhibited proliferation of ovarian cancer cells and increased cell apoptosis (242; 243). As discussed before, SENP5 is also involved in the development of several types of cancer (247; 249; 250). These data indicate that inhibition of DUB3 and SENP5 might be a potential strategy for treatment of specific cancers.

# V. CONCLUSIONS

1. A screening with expression plasmids of human ubiquitin hydrolases is an efficient way to identify novel regulators of the DNA damage response.

2. Ubiquitin hydrolase DUB3 regulates the monoubiquitination of H2A and H2AX by means of its catalytic activity.

3. DUB3 is equally or more effective in inhibiting monoubiquitination of H2A(X) than the other ubiquitin hydrolases previously described for H2A(X).

4. By affecting H2AX monoubiquitination, DUB3 antagonizes E3 ubiquitin ligases RNF8 and RNF168 and thereby regulates the localization of 53BP1, BRCA1 and RNF168 to sites of DNA lesions.

5. DUB3 acts on H2AX by direct interaction and deubiquitination.

6. Regulated activity of DUB3 is required for the correct functioning of the DNA damage response.

7. The catalytic activity of deubiquitinase DUB3 and SUMO hydrolase SENP5 regulate PCNA monoubiquitination.

8. By regulating PCNA monoubiquitination, DUB3 and SENP5 antagonize E3 ubiquitin ligase Rad18.

9. DUB3 binds to and deubiquitinates PCNA *in vivo*.

10. SENP5 interacts with PCNA, but cannot directly deubiquitinate PCNA *in vivo*.

11. By controlling PCNA monoubiquitination, DUB3 and SENP5 affect translesion synthesis.

12. De-methylase KDM3A might regulate DNA damage-induced recruitment of BRCA1 to DNA lesions while it leaves 53BP1 focus formation unaffected.

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