From the Department of Cell and Molecular Biology
Karolinska Institutet, Stockholm, Sweden

UBIQUITIN, SUMO AND PAR: DECIPHERING RECRUITMENT SIGNALS AT DNA DAMAGE SITES

Annika Pfeiffer

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Ubiquitin, SUMO and PAR:
Deciphering recruitment signals at DNA damage sites

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Annika Pfeiffer

Principal Supervisor:
Prof. Nico Dantuma
Karolinska Institutet
Department of Cell and Molecular Biology

Co-supervisors:
Assist. Prof. Martijn Luijsterburg
Leiden University Medical Center
Department of Human Genetics

Prof. Camilla Björkegren
Karolinska Institutet
Department of Biosciences and Nutrition

Opponent:
Prof. Joanna Morris
University of Birmingham
Institute of Cancer and Genomic Sciences

Examination Board:
Prof. Ann-Kristin Östlund Farrants
Stockholm University
Department of Molecular Biosciences
The Wenner-Gren Institute

Prof. Oscar Fernandez-Capetillo
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Assoc. Prof. Johan Holmberg
Karolinska Institutet
Department of Cell and Molecular Biology

NEC SCIRE FAS EST OMNIA.

Es ist unmöglich, alles zu wissen.

It is impossible to know everything.

ABSTRACT

Protecting genome integrity is a vital task that is constantly challenged by various genotoxic stresses. Among different types of DNA damage, double-strand breaks (DSBs) constitute a particular threat since they result in the loss of integrity of both complementary DNA strands. To cope with DNA lesions, cells have evolved efficient mechanisms to sense and repair damaged DNA, generally referred to as the DNA damage response (DDR). The DDR is particularly dependent on a multitude of post-translational modifications (PTMs) to facilitate the correct spatial and temporal recruitment of signaling and repair proteins. Among prominent PTMs that have been shown to be involved in the DDR are ubiquitylation, modification with small ubiquitin-like modifier (SUMOylation) and poly(ADP-ribos)(PAR)ylation, which are the topic of this thesis.

Paper I shows that the deubiquitylating enzyme ataxin-3 is recruited to DSBs in a SUMOylation-dependent manner. Ataxin-3 interacts with SUMO1 and its catalytic activity was stimulated *in vitro* by SUMO1. MDC1, a mediator of the DSB response, was identified as a substrate on which ataxin-3 is counteracting the RNF4 E3 ubiquitin ligase. By preventing ubiquitin-dependent removal, ataxin-3 is prolonging the chromatin retention time of MDC1, which, we propose, may ensure that the response cascade is accurately activated. Indeed, DNA damage-induced ubiquitylation downstream of MDC1 is impaired in the absence of ataxin-3 leading to inefficient recruitment of 53BP1 and BRCA1 and DSB repair.

In addition to SUMOylation, the early and transient recruitment of ataxin-3 to DSBs requires DNA damage-induced PARylation, which is presented in **paper II**. While we did not observe binding of ataxin-3 to PAR chains, ataxin-3 was found to be an interactor and substrate of PARP1. The recruitment of RNF4 to DNA damage was independent of PAR conjugation. The exact recruitment mechanism of ataxin-3 to DSBs by PARylation has not been elucidated at this stage, but the dual recruitment mode requiring SUMO and PAR likely allows spatiotemporal regulation and separates the retention of ataxin-3 from the opposed enzyme RNF4.

The recruitment of the cohesin loader NIPBL to sites of DNA damage was investigated in **paper III**. Two independent recruitment mechanisms of NIPBL were identified that depend on the type of DNA damage. The N-terminus of NIPBL is recruited by interacting with HP1, while ATM/ATR activity mediates the retention of the C-terminus. Both pathways require additional DNA damage-induced ubiquitin signaling. While NIPBL accrual at nuclease-

induced DSBs is strictly dependent on its HP1 binding motif, both recruitment mechanisms can compensate each other at laser-inflicted DNA damage.

In summary, work in this thesis presents two proteins, ataxin-3 and NIPBL, which are recruited to sites of DNA damage by combinatorial PTMs. Dual recruitment mechanisms likely enable the retention of DDR components with high spatiotemporal resolution.

LIST OF SCIENTIFIC PAPERS

I. **Pfeiffer A***, Luijsterburg MS*, Acs K, Wiegant WW, Helfricht A, Herzog LK, Minoia M, Böttcher C, Salomons FA, van Attikum H and Dantuma NP. Ataxin-3 consolidates the MDC1-dependent DNA double-strand break response by counteracting the SUMO-targeted ubiquitin ligase RNF4. *The EMBO Journal*, 2017, 36, 1066-1083.

II. **Pfeiffer A**, Luijsterburg MS, Shah R, Stoy H, Kühbacher U, van Attikum H, Shah G and Dantuma NP. Poly(ADP-ribos)ylation limits SUMO-dependent ataxin-3 recruitment to DNA double-strand breaks to the early phase of the DNA damage response. *Manuscript*

III. Bot C, **Pfeiffer A**, Giordano F, Manjeera DE, Dantuma NP and Ström L. Independent mechanisms recruit the cohesin loader protein NIPBL to sites of DNA damage. *Journal of Cell Science*, 2017, 130, 1134-1146.

* These authors contributed equally.

Publications not included in this thesis:

Dantuma NP and **Pfeiffer A**. Real estate in the DNA damage response: Ubiquitin and SUMO ligases home in on DNA double-strand breaks. *Frontiers in Genetics*, 2016, 7: 58.

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATM	Ataxia telangiectasia mutated kinase
ATR	Ataxia telangiectasia and Rad3-related protein
BARD1	BRCA1-associated RING domain 1
BRCA1	Breast cancer-associated protein 1
BRCT	BRCA1 C-terminal
BrdU	Bromodeoxyuridine
CdLS	Cornelia de Lange syndrome
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DSB	DNA double-strand break
DUB	Deubiquitylating enzyme
HEAT	Huntingtin, elongation factor 3, protein phosphatase 2A, Tor1
HERC2	HECT domain and RCC1-like domain-containing 2
HP1	Heterochromatin protein 1
HR	Homologous recombination
H2A	Histone 2A
IR	Ionizing radiation
IRIF	Ionizing radiation-induced foci
L3MBTL1	Lethal(3)malignant brain tumor-like protein 1
L3MBTL2	Lethal(3)malignant brain tumor-like protein 2
MDC1	Mediator of DNA damage checkpoint 1
MRN	MRE11-RAD50-NBS1 complex
NHEJ	Non-homologous end joining
NIPBL	Nipped-B-like protein
PAR	Poly(ADP-ribose)
PARG	Poly(ADP-ribose) glycohydrolase

PARP1	Poly(ADP-ribose) polymerase 1
PIAS	Protein inhibitor of activated STAT
PIKK	Phosphoinositide-3-kinase-related protein kinase
PTM	Post-translational modification
RNF	RING finger protein
RPA	Replication protein A
SIM	SUMO-interacting motif
SSB	DNA single-strand break
STUbL	SUMO-targeted ubiquitin ligase
SUMO	Small ubiquitin-like modifier
UBD	Ubiquitin-binding domain
UIM	Ubiquitin-interacting motif
USP	Ubiquitin-specific protease
UV	Ultraviolet light
VCP	Valosin-containing protein
XRCC4	X-ray repair cross-complementing protein 4
γH2AX	Phosphorylated histone variant 2AX
53BP1	p53-binding protein 1

1 INTRODUCTION

1.1 GENOME INTEGRITY AND DNA DAMAGE RESPONSE

Genome integrity

Every life form depends on intact DNA for their viability and fitness. Maintaining genomic stability is therefore crucial in order to deliver intact genetic material to the next generation (Jackson and Bartek, 2009). Over the life span, DNA can be subject to different types of damage. These can be of endogenous nature such as reactive oxygen species or DNA aberrations that result from nucleotide mismatches during DNA replication or DNA breaks that arise from defective topoisomerase activity. Exogenous/environmental DNA-damaging sources can be ultraviolet (UV) light, naturally occurring ionizing radiation (IR) from radioactive decay or DNA-damaging chemicals.

In order to keep DNA intact, cellular pathways have evolved that detect and repair DNA lesions. The global detection pathway of DNA lesions is termed the DNA damage response (DDR), which senses damaged DNA in order to trigger the activation of repair pathways, but also signals the induction of cell cycle arrest or apoptosis, if required (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). Depending on the type of DNA damage, different repair mechanisms can be activated. While any insults to the DNA can result in the appearance of mutations leading to a loss of genomic information, double-strand breaks (DSBs) are especially cytotoxic due to the lack of an undamaged strand as the repair template. To date, two main DSB repair pathways have been identified, which are homologous recombination (HR) and non-homologous end joining (NHEJ) (Ciccia and Elledge, 2010; Jackson and Bartek, 2009).

The DNA damage response

The DDR encompasses sensor, transducer and effector proteins that work in a hierarchical cascade to signal the presence of damaged DNA to repair pathways (Marechal and Zou, 2013). Three phosphoinositide-3-kinase-related protein kinases (PIKKs), Ataxia-telangiectasia mutated (ATM), Ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK), recruited through different sensor proteins, transduce the DNA damage signal by initiating phosphorylation events that trigger downstream repair pathways

(and a cell cycle arrest) and the ultimate DNA repair. While ATM and DNA-PK mainly respond to DSBs, ATR is recruited in response to single-stranded DNA and stalled replication forks (Sirbu and Cortez, 2013). The recruitment of these PIKKs occurs by DNA damage sensor proteins and is relatively well studied. ATR is recruited through ATR interacting protein (ATRIP) to replication protein A (RPA)-coated single-stranded DNA. The Ku70-Ku80 complex, recognizing broken DNA ends and a component of the NHEJ repair pathway, recruits DNA-PK to DSBs. ATM binds the MRE11-RAD50-NBS1 (MRN) complex, which senses DSBs (Falck et al., 2005).

Upon recruitment and activation, these PIKKs phosphorylate histone variant H2AX (γH2AX) at Ser139, which in itself marks the establishment of the DDR and triggers signaling cascades in order to activate downstream repair events (Fernandez-Capetillo et al., 2004).

This thesis mainly focuses on DNA damage signaling, specifically the recruitment of components that are involved in the ATM/MDC1-dependent response to DSBs. In the following, I will first provide an overview of the DSB response and then describe individual post-translational modifications (PTMs) and how they function in DNA damage signaling.

The ATM/MDC1-mediated DNA double-strand break response

DSBs are initially recognized by the MRN complex, which triggers the recruitment and the activation of the ATM kinase (Uziel et al., 2003) (**Figure 1**). The ATM sensor kinase phosphorylates histone variant H2AX at Ser139 (Burma et al., 2001; Rogakou et al., 1998), which serves as the DNA damage signal that facilitates the binding of mediator of DNA damage checkpoint 1 (MDC1) through its BRCA1 C-terminal (BRCT) domain (Stucki et al., 2005). MDC1 is a scaffold protein that is phosphorylated by ATM resulting in the recruitment of the ubiquitin ligase RING finger protein 8 (RNF8) through its forkhead-associated (FHA) domain (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007). This event triggers the initiation of a ubiquitylation cascade. RNF8 promotes the recruitment of the RNF168 ubiquitin ligase (Doil et al., 2009; Stewart et al., 2009). One study reports that RNF8-Ubc13 catalyzes polyubiquitin chains on histone 1 (H1)-type linker histones, which recruit RNF168 (Thorslund et al., 2015). RNF168 contains an N-terminal ubiquitin-dependent DSB recruitment module (UDM) 1, which binds RNF8-generated ubiquitylation products (Panier et al., 2012; Thorslund et al., 2015). A second study recently reported that the linker protein is rather likely to be

lethal(3)malignant brain tumor-like protein 2 (L3MBTL2), a putative polycomb group protein, which is recruited to DSBs in an ATM- and MDC1-dependent manner, where it is ubiquitylated by RNF8 and in turn recruits RNF168 (Nowsheen et al., 2018).

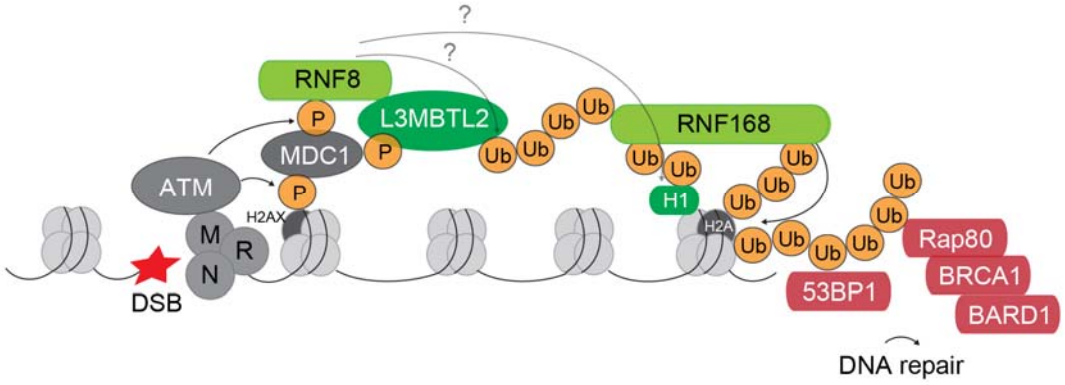

Figure 1. The ATM/MDC1-mediated DNA damage response to DSBs. DSBs are recognized by the MRE11-RAD50-NBS1 (M, R, N) complex, which recruits the ATM kinase. ATM is phosphorylating H2AX, which facilitates the recruitment of MDC1 and in turn RNF8 binds ATM-phosphorylated MDC1. One model suggests that RNF8-ubiquitylated histone 1 (H1) recruits RNF168, while a second model proposes RNF8-ubiquitylated L3MBTL2 to be the recruiter of RNF168. RNF168 ubiquitylates H2A thereby creating a broad ubiquitin binding platform for downstream repair factors. For explanation also see the main text. P, phosphate group; Ub, ubiquitin.

While RNF8 also targets other substrates and catalyzes both proteolytic lysine 48- and non-proteolytic lysine 63-linked ubiquitin chains (Feng and Chen, 2012; Lok et al., 2012; Mallette et al., 2012), the ubiquitin ligase RNF168 typically conjugates lysine 63-linked ubiquitin chains (Bohgaki et al., 2013; Mattiroli et al., 2012). RNF168 ubiquitylates histone 2A (H2A) on Lys13/15 (Mattiroli et al., 2012) and likely other substrate proteins. By recognizing ubiquitylated H2A itself through its C-terminal UDM2 (Panier et al., 2012; Thorslund et al., 2015), RNF168 creates a positive feedback loop and forms a broad recruitment platform for repair factors bearing ubiquitin-binding domains (UBDs). Examples are p53-binding protein (53BP1) (Fradet-Turcotte et al., 2013) or Rap80, which contains an ubiquitin-interacting motif (UIM) and is found in complexes with breast cancer type 1 susceptibility protein (BRCA1) (Kim et al., 2007; Sobhian et al., 2007). The balance between recruited 53BP1 and BRCA1 can modulate the choices for NHEJ or HR repair pathways (Ceccaldi et al., 2016).

While this is only a brief overview of the main events in the ATM/MDC1-mediated phosphorylation- and ubiquitylation-dependent response to DSBs, many more proteins and PTMs are involved, some of which will be introduced below.

1.2 POST-TRANSLATIONAL MODIFICATIONS IN DNA DAMAGE SIGNALING

DNA damage signaling by post-translational modifications

Multiple types of PTMs are involved in signaling around DNA lesions. Their main function is to coordinate the timely and hierarchical recruitment of sensor, mediator and effector proteins involved in the DDR. Prominent PTMs involved in signaling at sites of DNA damage are phosphorylation, ubiquitylation, SUMOylation (SUMO, small ubiquitin-like modifier) and poly(ADP-ribos)(PAR)ylation (Bergink and Jentsch, 2009; Caldecott, 2014; Harper and Elledge, 2007; Schwertman et al., 2016).

The ubiquitin-conjugation machinery

Protein ubiquitylation is a PTM that regulates different cellular processes including protein turnover, intracellular trafficking and DNA repair (Komander and Rape, 2012). An enzymatic cascade composed of a set of enzymes (E1, E2 and E3) regulates the covalent attachment of an 8.5 kD ubiquitin to lysine (Lys, K) residues in substrate proteins by isopeptide bonds. An E1 enzyme activates ubiquitin and transfers it to an E2 enzyme. While E2 enzymes are the ubiquitin-conjugating components, the E3 ubiquitin ligases recognize a variety of substrates and facilitate the conjugation (Bergink and Jentsch, 2009). Whereas there are two E1 and several (ca 40) E2 enzymes, around 600 E3 ubiquitin ligases are encoded by the human genome, which account for the substrate specificity (Bergink and Jentsch, 2009; Schwertman et al., 2016). Substrates can be decorated with one or more mono-ubiquitin modifications, or with polyubiquitin chains as conjugation can occur at any of the seven lysine residues (K6, K11, K27, K29, K33, K48, K63) that are present within ubiquitin. The type of polyubiquitin chain can influence the fate or properties of the target protein. Classically, K48-linked ubiquitin chains target substrate proteins for proteasomal degradation, whereas non-proteolytic K63-linked chains are primarily considered to be involved in signaling. A prominent E2 enzyme that functions in the DDR is Ubc13, which is specific for K63-linked ubiquitin. To our knowledge, K48- and K63-linked ubiquitin chains are the most common ubiquitin signals found at DNA damage sites (Schwertman et al., 2016).

Ubiquitin marks on substrates can be read by proteins that possess a UBD. UBDs can be of diverse nature (more than 20 types are known) and some of them can distinguish between distinct types of ubiquitin chain linkages or length (Dikic et al., 2009). Certain UBDs seem to

be enriched among DDR proteins such as UIMs, motif interacting with ubiquitin (MIU) or ubiquitin-binding zinc-finger (UBZ) (Dikic et al., 2009; Schwertman et al., 2016). Also tandem arrays of UBDs, such as tandem UIMs in Rap80 or ataxin-3 (Sims and Cohen, 2009), are found in DDR proteins, which promote higher binding affinities to polyubiquitin chains of certain linkage types and add a layer of specificity (Schwertman et al., 2016). For example, the spacing of the UIMs in Rap80 mediates the selective recognition of K63-linked ubiquitin chains (Sims and Cohen, 2009).

Ubiquitylation can be reversed by ubiquitin proteases called deubiquitylating enzymes (DUBs), which remove or edit conjugated ubiquitin chains. There are many DUBs that are connected to DNA damage signaling and repair pathways, several of which counteract histone ubiquitylation (Jacq et al., 2013).

Ubiquitin in the DNA damage response

Modification of histones and chromatin-associated proteins with ubiquitin plays an important role in signaling during the DDR. RNF8 and RNF168 are the main E3 ubiquitin ligases that mediate initial histone ubiquitylation upon DNA damage (Doil et al., 2009; Huen et al., 2007; Mailand et al., 2007; Mattiroli et al., 2012; Stewart et al., 2009), which in turn serves as a recruitment platform for downstream repair factors like the BRCA1/Rap80 complex or 53BP1.

While ubiquitylation plays a prominent role in signal spreading in the vicinity to damaged chromatin, ubiquitin can also mark DDR players for protein removal or turnover. This event can facilitate, on the one hand, the replacement of proteins as was shown for L3MBTL1. DNA damage-induced ubiquitylated L3MBTL1 is actively extracted from chromatin by Valosin-containing protein (VCP)-Npl4 to unmask a chromatin-binding site for 53BP1 as both proteins bind the H4K20me2 histone mark (Acs et al., 2011). On the other hand, ubiquitylation of DDR proteins can lead to their turnover to promote disassembly of the damage response signaling cascade. Such an example is MDC1, which was shown to be a substrate of the SUMO-targeted ubiquitin ligase (STUbL) RNF4 (Galanty et al., 2012; Luo et al., 2012; Yin et al., 2012). MDC1 is SUMOylated by PIAS4 (Luo et al., 2012), which allows its recognition by RNF4's SUMO-interacting motifs (SIMs) and the resulting ubiquitylated MDC1 showed decreased chromatin retention times (Galanty et al., 2012). In the absence of RNF4, persistence of γH2AX, MDC1 and 53BP1 ionizing radiation-induced foci (IRIF) was observed, suggesting that RNF4 activity is required for the clearance of repair foci (Galanty et al., 2012; Yin et al., 2012).

As RNF168 is the main E3 ubiquitin ligase responsible for spreading of ubiquitin signaling at sites of DNA damage, the protein and its activity are tightly regulated. One example is its targeting by the E3 ligases Ubr5 and Trip12, which promote RNF168 degradation to limit RNF168 availability and to avoid excessive spreading of ubiquitin signaling into the surrounding undamaged chromatin away from DSBs (Gudjonsson et al., 2012). In addition to regulating RNF168 protein levels, there are also several DUBs suggested to oppose RNF168-mediated chromatin ubiquitylation that will be described below.

Deubiquitylating enzymes in the DNA damage response

Given the importance of ubiquitin signaling in the DDR, it might not be surprising that during the recent years many DUBs have been reported to counteract ubiquitylation in the DDR, many of which act on histones. In a systematic screen, about half of the 94 DUBs expressed in humans were found to localize at laser-induced DNA damage (Nishi et al., 2014). Why are DUBs recruited to sites of DNA damage and what is the outcome of DUB activity at DSBs? DUB activity can lead to a balance and right dosing of chromatin-associated ubiquitylation by opposing ubiquitin ligase activity and reversing histone ubiquitylation. Limiting the spreading of ubiquitin signaling into undamaged chromatin can be important to avoid the excessive transcriptional silencing that occurs in response to DNA damage and to avoid the unnecessary depletion of DDR players. While recruitment mechanisms of DDR proteins are better studied, less is known about limiting factors that prevent superfluous responses or how repair responses are terminated. It has become evident that DUBs can play significant roles in these events. Besides restricting the spreading of histone ubiquitylation, DUBs are recruited to DNA damage sites to regulate the stability of DDR proteins or to fine-tune recruitment signals.

Many DUBs were found to restrict unlimited DNA damage-induced chromatin ubiquitylation by opposing RNF168 activity. DUBs like USP3 act catalytically on RNF168-ubiquitylated histones H2A and H2B. USP3 counteracts RNF168 by deubiquitylating H2A and H2B and the overexpression of USP3 leads to the prevention of 53BP1 and RNF168 accumulation at IRIF (Doil et al., 2009; Nicassio et al., 2007; Sharma et al., 2014). USP44 reverses RNF168-dependent ubiquitylation of H2A (Mosbech et al., 2013). POH1, a DUB residing in the 19S proteasome regulatory particle, was suggested to cleave K63-linked ubiquitin chains at DSBs (Butler et al., 2012). Furthermore, BRCC36, a BRCA-associated DUB, was suggested to edit ubiquitin chains on H2A to facilitate optimal recognition by Rap80 (Shao et al., 2009). Lastly,

the DUB OTUB1 acts in a non-catalytic manner by binding the E2 Ubc13 and making it less available for RNF168, whereby the activity of RNF168 is restricted (Nakada et al., 2010).

In addition to ubiquitylation by RNF168 on K13/15, H2A has also been found to be ubiquitylated by the heterodimeric E3 ligase BRCA1/BARD1 on K127/129, which promotes long-range resection, HR repair and the repositioning of 53BP1 (Densham et al., 2016; Kalb et al., 2014). In a recent study, USP48 was described to counteract the BRCA1/BARD1-mediated ubiquitin mark on H2A, possibly to avoid over-resection and to fine-tune BRCA1/BARD1 catalytic activity (Uckelmann et al., 2018).

In contrast to opposing histone ubiquitylation, rather few DUBs are reported that control the stability or retention of DDR proteins. For instance, USP34 was found to promote the stability of RNF168 as the depletion of USP34 leads to RNF168 degradation and defective recruitment of BRCA1 and 53BP1 (Sy et al., 2013). Another example is the DUB ataxin-3, highlighted in this thesis, that regulates the ubiquitylation status of MDC1 thereby prolonging the chromatin retention time of MDC1, which may be important for reinforcing the DDR cascade during its initiation phase (Pfeiffer et al., 2017) (**paper I**).

The SUMO-conjugation machinery

The covalent modification of proteins with the ubiquitin-like modifier SUMO occurs similarly to the conjugation of ubiquitin with a sequential three-step E1 – E2 – E3 enzymatic cascade. However, to date there is only one known SUMO E1 enzyme (heterodimeric SAE1/SAE2) and a single E2 conjugating enzyme (Ubc9), while there are around a dozen known SUMO E3 ligases (Schwertman et al., 2016). Analogously, SUMO is added to one or more lysines on a target protein (Gareau and Lima, 2010).

Three SUMO isoforms have been reported with the highly related SUMO2 and SUMO3 being indistinguishable by antibodies. While SUMO1, missing a consensus SUMO modification site in the N-terminus (K11), is conjugated as mono-SUMO on to target proteins, SUMO2/3 can be conjugated as poly-SUMO chains (Tatham et al., 2001).

Similar to protein domains that recognize and interact with ubiquitin, consensus motifs have been described for the interaction with SUMO. Such SUMO-interacting motifs (SIMs) are characterized by a short stretch of hydrophobic amino acids and are typically composed of V/I/L-x-V/I/L-V/I/L or V/I/L-V/I/L-x-V/I/L (where x is any amino acid). Unlike UBDs, the diversity of SIMs seems to be more limited (Kerscher, 2007). However, tandem arrays of SIMs

can, similarly to UBDs, promote high binding affinity for poly-SUMO2/3 chains as found in RNF4, which contains four N-terminal SIMs (Tatham et al., 2008). SUMO proteases can reverse SUMOylation, among them sentrin-specific proteases (SENPs) are best characterized in humans (Gareau and Lima, 2010; Hickey et al., 2012).

SUMO in the DNA damage response

In comparison to chromatin-associated ubiquitin signaling in the DDR, the picture about the involvement of SUMOylation is more incomplete. Today, the literature suggests that *in situ* SUMOylation of DDR proteins influences localization, interaction, catalytic activity and protein stability (Sarangi and Zhao, 2015). However, also "group-SUMOylation" in response to DNA damage in yeast has been proposed. This model suggests that SUMO acts as a protein glue to initiate complex building and to accelerate the overall repair (Psakhye and Jentsch, 2012), but it remains unclear if coordinated induction responses occur similarly to histone ubiquitylation signaling. Another study performed in *Saccharomyces cerevisiae* also concluded that DNA damage-induced SUMOylation is widespread and targets a large set of specific proteins, proposing that damage-induced SUMOylation is an integral part of the DDR (Cremona et al., 2012).

All SUMO isoforms, SUMO1 and SUMO2/3, the SUMO E3 ligases PIAS1 and PIAS4 and the E2 conjugating enzyme Ubc9 were found to localize at sites of DNA damage where they promote the response to DSBs (Galanty et al., 2009; Morris et al., 2009). While PIAS1 mediates SUMO2/3 conjugation at DSBs, PIAS4 is required for SUMO1 modifications, but also seems to contribute to SUMO2/3 conjugation (Galanty et al., 2009). Intriguingly, in the absence of PIAS1 and PIAS4, DNA damage-induced ubiquitylation is abrogated (analyzed by immunostaining with an ubiquitin FK2 antibody) and the accrual of RNF168 at DSB requires the presence of PIAS4 (Galanty et al., 2009; Morris et al., 2009).

A couple of proteins involved in the DDR were identified to be SUMOylated though the function of the modification is not always fully understood. BRCA1 was found to be SUMOylated (Galanty et al., 2009; Morris et al., 2009; Vyas et al., 2013), which positively affects the E3 ligase activity of the heterodimer BRCA1/BARD1 (Morris et al., 2009). 53BP1 is modified with SUMO1 and SUMO2 (Galanty et al., 2009; Vyas et al., 2013), but the functional significance is unknown. MDC1 is SUMOylated in response to DNA damage leading to its recognition by RNF4, which promotes the turnover of MDC1 (Galanty et al., 2012; Luo et al., 2012; Vyas et al., 2013; Yin et al., 2012). In this case, the *in situ* SUMO

modification of one protein serves as the recruitment signal for another protein. The modification of MDC1 with SUMO is required for a proper damage response (Luo et al., 2012). Furthermore, HERC2 and RNF168 were identified to be modified with SUMO1 at DSBs promoted by PIAS4 (Danielsen et al., 2012). SUMOylation of HERC2, an E3 ubiquitin ligase and an auxiliary factor for RNF8 (Bekker-Jensen et al., 2010), is necessary for a stable interaction with RNF8-Ubc13. On top, a ZZ-type zinc finger motif in HERC2 was found to act as a SUMO-binding module. It was suggested that SUMOylation of RNF168 by PIAS4 promotes the maintenance of proper RNF168 expression levels as overexpression of RNF168 could compensate for the inability of SUMOylating RNF168 in PIAS4-depleted cells (Danielsen et al., 2012).

Poly(ADP-ribos)ylation and PARP1

While ubiquitin and SUMO are proteinaceous PTMs, PARylation is the covalent attachment of negatively charged PAR moieties to acceptor proteins (Schreiber et al., 2006). PAR chains are generated by PAR polymerases (PARPs), which assemble PAR moieties on substrates from nicotinamide adenine dinucleotide (NAD^+) (**Figure 2**) (Schreiber et al., 2006). The elongation of PAR chains, forming linear or branched polymers of varying length, occurs by glycosidic bonds between ADP-ribose units. In contrast to other PTMs, site-specific PARylation is not fully understood but can occur *e.g.* on glutamic or aspartic acid residues. Also lysine residues in PARP1 were identified as acceptor sites for PARylation (Altmeyer et al., 2009). Recently, PARylation of serine residues in histones and PARP1 in response to DNA damage was reported and is dependent on histone PARylation factor 1 (HPF1), an interactor of PARP1 (Bonfiglio et al., 2017; Gibbs-Seymour et al., 2016). PARylation is reversible and PAR chains are rapidly hydrolyzed by PAR glycohydrolase (PARG) in the DDR, generating free ADP-ribose (Schreiber et al., 2006).

At damaged chromatin, PARP1 is the main PAR polymerase that is responsible for DNA damage-induced PARylation (Ray Chaudhuri and Nussenzweig, 2017; Schreiber et al., 2006). Upon induction of DNA lesions, PARP1 is almost immediately recruited to single- and double-stranded DNA breaks, making PARP1 an important sensor of DNA damage, where it PARylates target proteins like histones and itself (Liu et al., 2017; Schreiber et al., 2006). The quick generation of PAR chains in response to DNA damage is thought to provide an initial recruitment platform for DDR proteins. Recognition of distinct units of PAR chains can occur

through different types of PAR binding domains, which include BRCT domains (*e.g.* in BARD1) or WWE domains (*e.g.* in RNF146) (Liu et al., 2017).

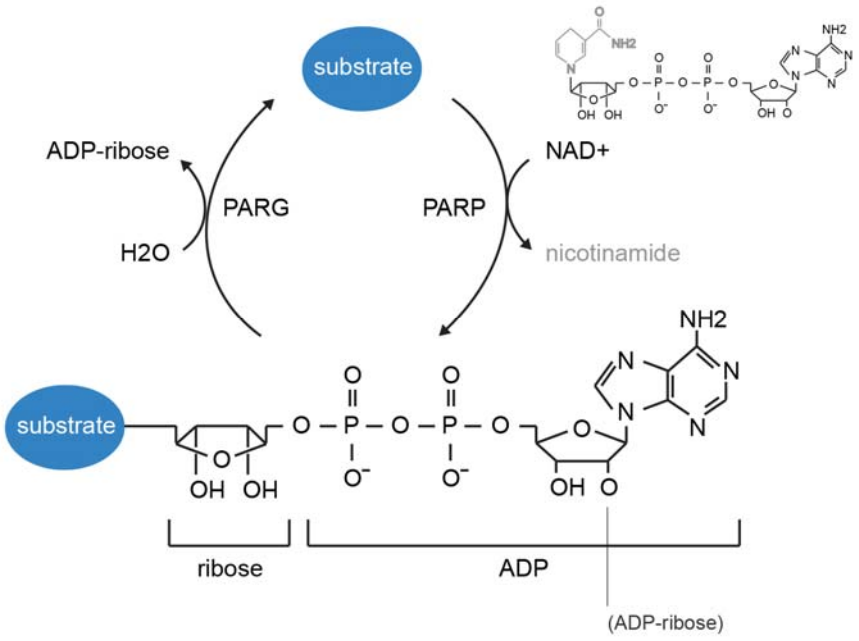

Figure 2. PARylation of substrate proteins. Poly(ADP-ribose) (PAR) polymerases (PARPs) hydrolyze nicotinamide from NAD^+ and transfer ADP-ribose moieties to target proteins. PAR chains be elongated to linear or branched polymers. PARylation is reversible and ADP-ribose is hydrolyzed by PAR glycohydrolase (PARG). ADP, adenosine diphosphate.

PARP1 has an N-terminal DNA-binding domain containing a zinc finger domain and a C-terminal catalytic domain. Under normal conditions, basal levels of PARylation in cells are low. Upon binding to DNA (damage), conformational changes within PARP1 lead to its activation and extensive PARylation of its auto-modification domain (Ali et al., 2012; Langelier et al., 2012). Auto-PARylation of PARP1 initiates its own degradation as it is targeted by RNF146 (see below). PARP1's intrinsic negative feedback loop and the rapid turnover of PAR polymers by PARG make PARylation a rather short-lived signal.

It is also noteworthy to mention that PAR chains and DNA are both negatively charged. This may lead to a negative charge-charge repulsion between DNA and PARylated histones, resulting in a loose chromatin structure (Poirier et al., 1982) and making the chromatin environment at sites of DNA damage more accessible for repair factors. Also the chromatin dissociation of auto-PARylated PARP1 may be promoted by charge repulsion (Muthurajan et al., 2014).

PARP inhibitors, blocking catalytic PAR activity, have emerged as promising therapeutic strategies in cancer treatment. Tumor cells deficient for BRCA1 or BRCA2, which are often found mutated in familial breast cancer and involved in HR repair, are highly sensitive to PARP inhibition (Bryant et al., 2005; Farmer et al., 2005). PARP1 has a key role in recognizing single-stranded breaks (SSBs). It is assumed that SSBs, that stay unrepaired in PARP inhibitor-treated cells, result in DSBs during replication. In BRCA-deficient tumor cells, however, the repair of DSBs, emerged during replication and which require HR repair, is inefficient with the result that DSBs stay unrepaired triggering cell death or are repaired by error-prone NHEJ leading to genomic instability (Helleday, 2011; Shah et al., 2013). This approach exploits the idea of synthetic lethality where PARP1 inhibition in combination with a DNA repair defect has a lethal effect on cancer cells (Javle and Curtin, 2011).

PAR in the DNA damage response

The current understanding is that dynamically regulated PARylation after the introduction of DNA damage is providing a first recruitment wave of DDR proteins prior to downstream retention mechanisms (Liu et al., 2017). For instance, the early recruitment of BRCA1 to DSBs is PAR-dependent through BRCT domain-mediated interaction with PAR of BRCA1's binding partner BARD1 (Li and Yu, 2013). A more stable retention of BRCA1 at DSBs is then facilitated by Rap80-mediated interaction with ubiquitin conjugates (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007).

The E3 ubiquitin ligase RNF146 (also known as Iduna) is an interesting example of functional interaction between DNA damage-induced PARylation and ubiquitylation. RNF146 is translocated to DSBs in a PAR-dependent fashion and binding to PARylated proteins activates its enzymatic activity. One of its substrates is auto-PARylated PARP1 and by ubiquitylating it, RNF146 targets PARP1 for proteasomal degradation (Kang et al., 2011).

Crosstalk between PTMs

It has become clear that PTMs in the DNA damage response not only work in an isolated manner, but are also widely interconnected. Prominent examples, outlined above, are RNF4, which mediates crosstalk between SUMO and ubiquitin, or RNF146, connecting PAR and ubiquitin.

While several examples illustrate the serial interplay between PTMs and how one PTM can be "translated" to another one, it has also become evident that the recruitment of single DDR players can be mediated by dual recognition modes providing spatiotemporal resolution. For instance, 53BP1 is recruited to sites of DNA damage by binding both to di-methylated lysine 20 of histone 4 (H4K20me2) through its Tudor domain (Botuyan et al., 2006) and to RNF168-ubiquitylated lysine 15 of histone 2A (H2AK15ub) through a ubiquitin-dependent recruitment (UDR) motif (Fradet-Turcotte et al., 2013). The recruitment of ataxin-3 to DSBs was identified to be regulated both by DNA damage-induced SUMOylation and PARylation (**paper I** and **paper II**). Also NIPBL (see below, 1.5) recruitment to DNA damage sites is mediated by combinatorial PTMs including H3K9me3-binding heterochromatin protein 1 (HP1) γ and RNF8/RNF168-mediated ubiquitin signaling (**paper III**). These examples highlight that the complex coordination of dynamic events by multiple cooperative PTMs is required to preserve genome integrity.

1.3 REPAIR OF DNA DOUBLE-STRAND BREAKS

The ultimate goal of the DDR is to ensure that repair pathways are activated to restore damaged DNA and maintain genome integrity. Two main DSB repair strategies have been identified, which are HR and NHEJ (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). The cell cycle phase, during which cells encounter DNA lesions, can affect the type of DSB repair. HR is usually carried out during the S/G2 phase of the cell cycle when a sister chromatid is available as a template for the repair. NHEJ, providing much faster repair kinetics than HR (Mao et al., 2008), can occur throughout the cell cycle though it is dominant during the G1 phase when HR repair is more restricted (Ceccaldi et al., 2016). Also the balance of the antagonistic binding of BRCA1 and 53BP1 at DSBs can affect the choice of the repair pathway. While the accumulation of 53BP1 in DSB regions occurs rather during the G1 phase of the cell cycle and inhibits HR by blocking DNA resection, BRCA1 binds more during the S/G2 phase and tends to exclude 53BP1 and promote resection and HR (Bunting et al., 2010; Chapman et al., 2012; Chiruvella et al., 2013).

Non-homologous end joining

NHEJ is a DSB repair pathway that joins two DSB ends by direct ligation without using DNA templates. This makes NHEJ repair an error/mutation-prone mechanism as it may lead to the loss of nucleotides and genetic information (Chiruvella et al., 2013). Components of the NHEJ pathway are the Ku70-Ku80 heterodimer, a DSB-specific end-binding protein and recruiting the catalytic subunit of DNA-PK, and DNA ligase IV, catalyzing the strand ligation together with its non-enzymatic binding partner XRCC4 (Chiruvella et al., 2013).

Homologous recombination

While displaying slower repair kinetics than NHEJ (Mao et al., 2008), the repair of DSBs by HR is the most accurate since it is using an undamaged DNA template (from the sister chromatid) for the repair process (Ceccaldi et al., 2016). HR repair is initiated by DNA end resection surrounding the DSB, which exposes single-stranded DNA that is coated by RPA, followed by RAD51-mediated strand invasion and the introduction of missing nucleotides using a homologous DNA sequence as the repair template (Jasin and Rothstein, 2013).

1.4 ATAXIN-3

Ataxin-3 is a ubiquitously expressed enzyme with deubiquitylating activity (Burnett et al., 2003). The protein itself has an N-terminal Josephin domain containing a catalytic cysteine residue (C14) and a SIM, while the C-terminal fragment harbors three ubiquitin-interacting motifs (UIM1-3), a characterized VCP-binding motif (VBM), and a polyglutamine (polyQ; Q_n) stretch between UIM2 and UIM3 (**Figure 3**). Through its UIMs, ataxin-3 can interact with ubiquitin chains, preferably K48- and K63-linked ubiquitin chains, and disassemble or edit these (Winborn et al., 2008). PolyQ expansion in the C-terminus of ataxin-3 is the cause for the neurodegenerative disorder Machado-Joseph disease (or spinocerebellar ataxia type 3, SCA3) (Kawaguchi et al., 1994).

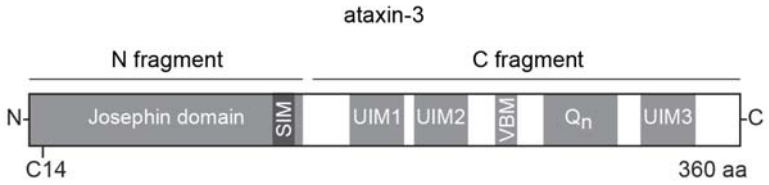

Figure 3. Schematic depiction of ataxin-3. Ataxin-3's N-terminal Josephin domain contains the catalytic cysteine residue (C14) and a SUMO-interacting motif (SIM). The C-terminal fragment harbors three ubiquitin-interacting motifs (UIM1-3), a VCP-binding motif (VBM) and a polyQ (Q_n) stretch.

While ataxin-3's catalytic properties are studied in detail *in vitro*, its cellular functions are more diffuse. Ataxin-3 is found both in the cytosol and in the nucleus where it has various described functions. Cytosolic ataxin-3 is involved in protein quality control. Ataxin-3 interacts with the AAA-ATPase VCP via a characterized VCP-binding motif (VBM) (Boeddrich et al., 2006). By binding to the VCP complex, ataxin-3 has a regulatory role in ER-associated degradation (ERAD) and promotes the transfer of substrates from VCP to the proteasome (Wang et al., 2006). Besides binding to VCP, ataxin-3 was also reported to interact with hHR23, a protein shuttling proteolytic substrates to the proteasome (Doss-Pepe et al., 2003; Wang et al., 2000). One model suggests that ataxin-3 associates with the proteasome where it receives ubiquitylated substrates through the combined action of VCP and hHR23 (Doss-Pepe et al., 2003).

In the nucleus, chromatin-bound ataxin-3 was reported to bind histone deacetylase 3 (HDAC3) resulting in increased deacetylase activity and transcriptional repression (Evert et al., 2006). Recently, ataxin-3 was identified to interact with polynucleotide kinase 3'-phosphatase (PKNP), a DNA end-processing enzyme involved in the repair of SSBs and DSBs (Chatterjee et al., 2015). Through the interaction with ataxin-3, phosphatase and DNA repair activities of PKNP are enhanced. It is, however, unknown whether and how the DUB activity of ataxin-3 influences PKNP functions. Another reported DNA repair promoting function of ataxin-3 is its stabilization of checkpoint kinase 1 (Chk1) (Tu et al., 2017). Chk1 is activated by DNA damage or replication stress to delay cell cycle progression in order to facilitate DNA repair. Ataxin-3 was found to be involved in the regulation of steady-state levels of Chk1. By deubiquitylating Chk1 and thereby preventing it from proteasomal degradation, checkpoint signaling and DNA repair were promoted by ataxin-3 upon exposure to genotoxic stress. Additionally, we have recently reported a stimulatory role of ataxin-3 on the DDR by counteracting RNF4 activity and preventing premature chromatin dissociation of MDC1 (Pfeiffer et al., 2017) (**paper I**).

1.5 THE COHESIN LOADER PROTEIN NIPBL

NIPBL (nipped-B-like protein) is the loading factor of cohesin onto chromatin. The role of the ring-like cohesin complex is to tether sister chromatids after replication to ensure proper segregation during mitosis. Apart from its canonical role in sister chromatid cohesion, the cohesin complex has also been shown, especially in yeast studies, to be recruited to DSBs, to establish damage-induced cohesion and to facilitate DNA repair (Dorsett and Strom, 2012).

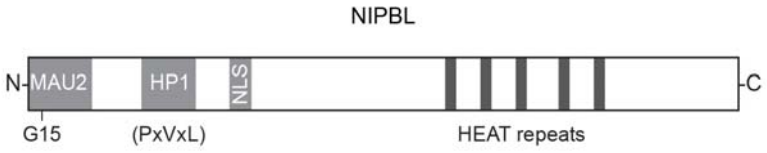

Figure 4. Schematic presentation of NIPBL. The binding site of MAU2 (interrupted by a single mutation of G15) and the HP1 interaction motif (PxVxL) lie in the N-terminus of NIPBL. The C-terminal domain of NIPBL contains several HEAT repeats.

The human NIPBL (**Figure 4**) is a large protein (> 300 kDa) that exists as a heterodimer with MAU2. Mutations in the gene encoding NIPBL are often associated with Cornelia de Lange syndrome (CdLS), a disorder that affects both mental and physical development (Liu and Baynam, 2010). Cells derived from CdLS patients were originally associated with increased DNA damage sensitivity presumably by defective HR repair (Vrouwe et al., 2007), but defects in NHEJ were also reported (Enervald et al., 2013).

Even though depletion or mutations in NIPBL cause defects in sister chromatid cohesion, it is largely unknown how NIPBL is loading cohesin onto chromosomes. In terms of involvement in DNA repair, it was demonstrated that human NIPBL is recruited to DSBs dependent of MDC1, RNF168 and HP1 (Kong et al., 2014; Oka et al., 2011). We have recently confirmed that NIPBL is accumulating at sites of DNA damage by two independent mechanisms, which are influenced by the type of DNA damage. At laser-induced DNA damage, the N-terminus of NIPBL is recruited by HP1, while the recruitment of the C-terminus requires ATM/ATR activity. Additionally, RNF8/RNF168 and ubiquitin signaling are needed and both recruitment mechanisms can compensate each other. On the contrary, NIPBL recruitment to nuclease-mediated DSBs is strictly dependent on HP1 (**paper III**).

1.6 INDUCTION OF DNA DAMAGE

Since this thesis focuses on the recruitment of proteins to sites of DNA damage, I will highlight in this section how DNA damage was experimentally induced in cells. To study the recruitment of proteins to damaged chromatin by fluorescence microscopy, DNA damage was introduced by two different techniques: laser micro-irradiation and FokI nuclease-generated DSBs.

Laser micro-irradiation is an established technique that is widely used to study recruitment and dynamics in the DNA damage response (Berns, 2007; Lukas et al., 2003; Lukas et al., 2004; Paull et al., 2000). For performing laser-inflicted DNA damage, cells were pre-treated with bromodeoxyuridine (BrdU) in order to sensitize the DNA for laser damage. In live cell imaging conditions, DNA damage was locally inflicted by a pulsed nitrogen laser (365 nm, UV-A range). Cells were fixed at a chosen time point, immunolabeling was performed and the accumulation of proteins of interest at the damaged area was analyzed by fluorescence microscopy. This technique is limited to a rather small number of cells in a single experiment and includes the manual definition of sub-nuclear regions that are damaged, which were quantified by a semi-automated method. The phosphorylation of histone 2A.X (γH2AX) by the ATM kinase is a rapid response to damaged chromatin and serves as a standard marker for DSBs (Kuo and Yang, 2008). It was used to label and visualize sites of DNA damage. Despite certain limitations, laser micro-irradiation is a valued method for the introduction of DNA damage as it allows temporal recruitment analysis.

While laser micro-irradiation predominantly causes DSBs, it can also inflict other types of DNA damage (Kong et al., 2009). Therefore, an additional system using DSBs introduced by the FokI endonuclease was used to verify our findings. This system is based on a U2OS cell line that has LacO repeats stably integrated into the genome and an mCherry-LacR-FokI protein fused to a destabilization domain and a modified estrogen receptor (Tang et al., 2013). The last two domains ensure that the fusion protein is only found in the nucleus when the small molecules Shield-1 and 4-OHT are present. In the modified U2OS cells, these compounds add a controllable layer to FokI-induced DSBs as both are required for the fusion protein to accumulate in the nucleus where it tethers to the chromatin via the LacO-LacR interaction and introduces DSBs.

For biochemical experiments, DNA damage was introduced by treating cells with the glycopeptide antibiotic bleomycin, which uses iron to reduce oxygen into superoxide, resulting in the formation of hydroxyl radicals that cause DSBs.

2 AIMS

All three studies included in this thesis focus on investigating how specific DDR players are recruited to DSBs. The individual aims of each paper were the following:

The aim of **paper I** was to identify proteins that are recruited to damaged chromatin and regulate DNA damage-induced ubiquitin signaling. We explored a possible role of ataxin-3 in the DDR based on the following rationale: -i- Ataxin-3 interacts with VCP (Boeddrich et al., 2006; Doss-Pepe et al., 2003; Kobayashi et al., 2002), which is involved in the DDR (Acs et al., 2011; Meerang et al., 2011). -ii- Ataxin-3 is a DUB disassembling Lys48- and Lys63-linked ubiquitin chains (Winborn et al., 2008), which have high implications in histone and chromatin-associated ubiquitin signaling at sites of DNA damage (Dantuma and van Attikum, 2016). -iii- Ataxin-3 is present in the nucleus (Tait et al., 1998). -iv- PolyQ-expanded ataxin-3 can cause a neurodegenerative disorder and links between defective DNA repair and neurodegeneration (*e.g.* ATM) have been described (Rass et al., 2007).

The focus of **paper II** was to investigate how the early recruitment of ataxin-3 to laser-inflicted DNA damage is regulated. Since the retention of ataxin-3 at DSBs was restricted to the early phase of the DDR, which resembles the presence of short-lived PARylation in response to DNA damage, we sought to identify whether PARylation was involved in the recruitment of ataxin-3.

The study presented in **paper III** aimed at investigating requirements for the recruitment of the cohesin loading factor NIPBL to damaged chromatin.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Ataxin-3 consolidates the MDC1-dependent DNA double-strand break response by counteracting the SUMO-targeted ubiquitin ligase RNF4.

The aim for **paper I** was to identify proteins that are recruited to damaged DNA and that regulate chromatin-associated ubiquitin signaling. Based on its interaction with VCP, which is involved in the DDR, we tested whether the DUB ataxin-3 was recruited to sites of DNA damage.

We observed that ataxin-3 was recruited both to laser-induced DNA damage and to DSBs introduced by chromatin-tethered FokI nuclease. The recruitment of ataxin-3 was neither dependent on one of the protein's typical characteristics (*i.e.* catalytic activity, UIMs, VCP-binding motif) nor on chromatin ubiquitylation, ruling out that ataxin-3 retention at DSBs was mediated solely by its UIMs and binding to ubiquitin. This would have been the most likely mechanism based on ataxin-3 being a DUB and possessing UIMs. We, however, identified that a different type of PTM was enabling ataxin-3 recruitment, namely SUMOylation. In the absence of Ubc9 or PIAS4, two enzymes involved in DNA damage-induced SUMOylation, the recruitment of ataxin-3 to laser-induced DNA damage was abrogated. Biochemical analyses showed that ataxin-3 is interacting with SUMO1. Moreover, both interaction with SUMO1 and recruitment to sites of DNA damage was dependent on a predicted SIM (Guzzo and Matunis, 2013) that lies in the C-terminus of ataxin-3's Josephin domain. Furthermore, we found that the *in vitro* DUB activity of ataxin-3 was stimulated by the presence of SUMO1. Interestingly, ataxin-3 was identified by others to be SUMOylated resulting in a stronger association with VCP, while SUMOylation of ataxin-3 had no significant impact on its DUB activity (Almeida et al., 2015). In contrast, ubiquitylation of ataxin-3 enhances its catalytic activity (Todi et al., 2009). In our study, we found that the recruitment of ataxin-3 was independent of VCP but it might still be interesting to investigate whether SUMOylation or ubiquitylation of ataxin-3 have an impact on its recruitment to DSBs or on its role in the DDR.

Considering these findings and trying to identify a substrate of the DUB ataxin-3 in the DDR, we had a closer look at RNF4, which interconnects SUMO and ubiquitin at sites of DNA damage. RNF4 is a SUMO-targeted ubiquitin ligase (STUbL), which is recruited in a SIM-

dependent manner and ubiquitylates SUMO-modified MDC1 in order to reduce its chromatin retention time (Galanty et al., 2012; Luo et al., 2012; Yin et al., 2012). The studies reporting this mechanism proposed that MDC1-targeting by RNF4 (at a late time point; RNF4 accumulation persists for several hours) was an event to promote DSB repair and the eventual disassembly of the signaling/repair cascade. As SUMOylation is quickly detectable after the induction of DNA damage, one open question is how SUMO-recruited RNF4 would only be retained at a „late“ time point since its recruitment signal is already present at early time points or alternatively, how its enzymatic activity is controlled at different times of the DDR. Besides that, we and others (Vyas et al., 2013) have observed that RNF4 readily accumulates directly after the infliction of laser-induced DNA damage. In this scenario, targeting of MDC1 by RNF4 might lead to an inefficient build-up of the signaling cascade as MDC1 is a key element in initiating the DDR. Interestingly, both RNF4 and ataxin-3 accumulated at laser DNA damage directly after its infliction and had opposing effects on the chromatin retention time of MDC1. Subsequently, we found that ataxin-3 is interacting with MDC1 and is also regulating the ubiquitylation status of MDC1. The depletion of ataxin-3 increased ubiquitylated MDC1, which was largely rescued by the ectopic expression of wild-type ataxin-3. Ectopically expressed catalytic inactive ataxin-3 could not reverse the effect on ubiquitylated MDC1 in ataxin-3-depleted cells, demonstrating that its catalytic activity is required and that MDC1 is a substrate of ataxin-3. In contrast to the outcome of ataxin-3 depletion, the knock-down of RNF4 resulted in a decrease of ubiquitylated MDC1 both in control and ataxin-3-depleted cells. These findings led to the conclusion that the DUB ataxin-3 is counteracting the RNF4 E3 ligase on the MDC1 substrate to enhance its chromatin retention time during the initiation phase of the DDR (**Figure 5**).

Our results do not exclude that there might be also alternative functions or mechanisms of ataxin-3 in the DDR. Whether there are more shared targets of ataxin-3 with RNF4 in the DDR remains to be explored. A potential candidate is RPA, which is regulated by RNF4 (Galanty et al., 2012; Yin et al., 2012) and showed decreased accumulation at laser-inflicted DNA damage in ataxin-3-depleted cells. It is also possible that ataxin-3 is fine-tuning RNF4 activity outside the context of DNA damage signaling, as both proteins have described roles in protein quality control (Doss-Pepe et al., 2003; Guo et al., 2014). It should also be mentioned that while ataxin-3 is interacting with MDC1, its recruitment to DNA damage sites is independent of MDC1 and ataxin-3 still interacted with an MDC1 mutant that cannot be SUMOylated or when SUMOylation was impaired in Ubc9- or PIAS4-depleted cells. We therefore speculate that the recruitment of ataxin-3 occurs through DNA damage-induced group SUMOylation or another SUMOylated protein than through specifically SUMOylated MDC1.

In line with our model (**Figure 5**), in which ataxin-3 deubiquitylates MDC1 during the DDR initiation phase in order to prolong MDC1's chromatin retention time, DNA damage-induced ubiquitin signaling (mediated by RNF8 and RNF168) was impaired in the absence of ataxin-3 and affected not only the recruitment of 53BP1 and BRCA1, but also the repair of DSBs by NHEJ and HR. An inefficient repair of DSBs by HR was accompanied by a reduction in the recruitment of RPA and RAD51 to sites of DNA damage and a higher sensitivity to PARP inhibitor treatment in ataxin-3-depleted cells. Notably, also the recruitment of the NHEJ component XRCC4 to DSBs was negatively affected and to some extent regulated by the DUB activity of ataxin-3. Additionally, the co-depletion of RNF4 and ataxin-3 partly restored the impaired recruitment of XRCC4 to laser-induced DNA damage observed upon knock-down of ataxin-3. Also cell viability after exposure to IR was improved in co-depleted cells compared to cells depleted of only RNF4. These findings suggest that the opposing activities of ataxin-3 and RNF4 play a role in the signaling and repair of DSBs.

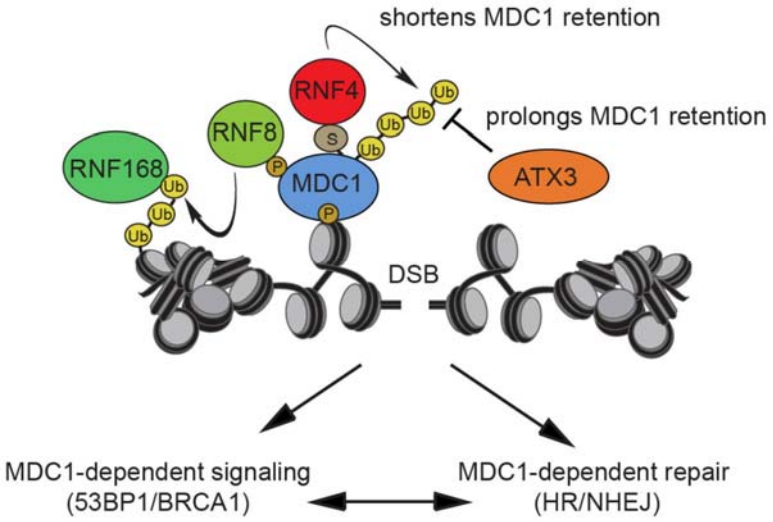

Figure 5. Model of the regulatory role of ataxin-3 in preventing premature removal of MDC1 during the initiation phase of the DDR. The DUB ataxin-3 is counteracting the E3 ubiquitin ligase activity of RNF4 on MDC1 during the early phase of the DDR. By acting on ubiquitylated MDC1, ataxin-3 is prolonging the chromatin retention time of MDC1 to ensure that the DDR is accurately activated. Adapted from Pfeiffer et al., 2017.

We have demonstrated that ataxin-3 and RNF4 behave as a protease/ligase pair that acts to balance RNF4 activity. Ataxin-3 is, however, not the first DUB reported to oppose RNF4-mediated ubiquitylation. USP11 can deubiquitylate RNF4-generated hybrid SUMO-ubiquitin chains to counteract RNF4 and stabilize PML nuclear bodies in response to the DNA-damaging agent methyl methanesulfonate (Hendriks et al., 2015). With that, USP11 and RNF4 also

constitute a balanced protease/ligase pair to establish an equilibrium of RNF4-catalyzed ubiquitylation. USP11 is not the only example of a DUB deubiquitylating hybrid SUMO-ubiquitin chains. USP7, a replisome-enriched DUB, is counteracting ubiquitylation of SUMOylated proteins (Lecona et al., 2016). Whether or not the DUB ataxin-3 also directly acts on hybrid SUMO-ubiquitin chains remains to be determined in more detail. Fine-tuning ligase activity is not specific to RNF4 (as mentioned in the introduction, many DUBs in the DDR were described to counteract RNF168-mediated histone ubiquitylation). It is, however, interesting that the SUMO-targeted ligase RNF4 is acting in concert with counteracting DUBs, as it raises the question how substrate-specific RNF4 is binding to and acting on SUMOylated proteins. Counteracting DUBs may inhibit RNF4 if it is not acting in the right spot at the right time. It is possible that more DUBs might be identified that edit RNF4 activity.

3.2 PAPER II

Poly(ADP-ribos)ylation limits SUMO-dependent ataxin-3 recruitment to DNA double-strand breaks to the early phase of the DNA damage response.

Paper II (manuscript) was based on the observation in paper I that ataxin-3 was recruited early to laser-induced DNA damage. When we analyzed the recruitment kinetics of ataxin-3 to laser-inflicted sites of DNA damage, we observed consistently that the accumulation of ataxin-3 was not only early but also of transient nature. In paper I, we identified that DNA damage-induced SUMOylation, a signal that is present at DNA damage sites for several hours (Galanty et al., 2012), promotes ataxin-3 accumulation at damage sites. However, this alone would not account for a transient recruitment of ataxin-3. Therefore, we aimed at identifying additional recruitment mechanisms that would limit the prompt recruitment of ataxin-3 to DSBs to the early phase of the DDR.

The early and transient accumulation pattern of ataxin-3 is reminiscent of PARylation at damage sites, which occurs instantly after damage induction. PAR chains are relatively quickly hydrolysed by PARG making PARylation a rather short-lived signal. Indeed, we found that the recruitment of ataxin-3 was dependent on DNA damage-induced PARylation as the accumulation at damage sites was abrogated in cells treated with a PARP inhibitor or depleted of PARP1, the main PAR polymerase at DNA damage sites. The PAR-dependent recruitment was mediated by ataxin-3's N-terminal Josephin domain that also facilitated SUMO-dependent retention (paper I). The region within the N-terminal domain that accounts for PAR-dependent recruitment of ataxin-3 remains to be identified.

How is DNA damage-induced PARylation involved in recruiting ataxin-3 to sites of DNA damage? There are different possible scenarios: -i- Ataxin-3 binds directly to PAR chains generated around DNA damage sites; -ii- Ataxin-3 binds (auto-PARylated) PARP1; -iii- Ataxin-3 is PARylated itself, which might promote its retention at DSBs; -iv- Ataxin-3 interacts with a binding partner (other than PARP1) that is PARylated or binds to PAR chains.

Following these potential threads, we did not observe in *in vitro* experiments that ataxin-3 is binding directly to PAR chains. However, we found the N-terminal domain of ataxin-3 to be constitutively interacting with PARP1. The interaction was weaker in the presence of the PARP inhibitor KU-0058948, which suggests that binding of ataxin-3 to PARP1 is promoted by PARP1 activity, although it cannot be excluded that the PARP inhibitor sterically interferes with the interaction between PARP1 and ataxin-3. In addition, both catalytic inactive PARP1

and ataxin-3 still co-immunoprecipitate, demonstrating that the interaction is not strictly dependent on PARylation activity by PARP1. Binding to PARP1 is, however, not sufficient for the recruitment of ataxin-3 to DSBs as PARP1 still accumulates at laser damage in PARP inhibitor-treated cells, a condition in which ataxin-3 retention was impaired. These data suggest that PARP1 activity is required for the recruitment of ataxin-3 to DSBs.

The interaction between PARP1 and ataxin-3 is of a direct nature and was even enhanced in *in vitro* reactions that were performed with previously activated PARP1. We tested whether ataxin-3 itself might be modified with PAR chains. Both *in vitro* and in cells, we identified that ataxin-3 is PARylated. The identity of the PARylation site(s) and a possible role of PARylation of ataxin-3 for its retention at sites of DNA damage remains to be investigated. This would be an intriguing mechanism that is similarly to tyrosyl-DNA phosphodiesterase 1 (TDP1), a repair enzyme for trapped topoisomerase I cleavage complexes. TDP1 directly interacts with PARP1 and is PARylated by PARP1, which enhances its recruitment to DNA damage sites. TDP1 is SUMOylated on a specific Lys residue and together with SUMOylation, PARylation stabilizes TDP1 (Das et al., 2014). Therefore, it would be exciting to probe into the questions of whether PARylation of ataxin-3 was involved in its recruitment or has an effect on the catalytic DUB activity of ataxin-3. One other possibility is that ataxin-3 interacts with an additional unknown binding partner that binds to PAR chains at DNA damage sites and indirectly mediates PAR-dependent ataxin-3 accumulation.

Even though the exact recruitment mechanism has not been identified yet, it has become apparent that in addition to DNA damage-induced SUMOylation (paper I), PARylation promotes the retention of ataxin-3 at DSBs as well. Both PTMs are independent of each other as PAR is still detectable at DSBs in Ubc9-depleted cells and SUMO is still observable in PARP inhibitor-treated cells. Also SUMO-recruited RNF4 is still accumulating at DSBs after cell treatment with a PARP inhibitor. The dual signal (SUMO and PAR) that is needed for ataxin-3 retention at DSBs possibly opens a small time window for ataxin-3 to counteract the catalytic activity of RNF4 (**Figure 6**).

While we showed in paper I, that the absence of ataxin-3 negatively affects the efficient repair of DSBs by NHEJ and also the recruitment of the NHEJ protein XRCC4 to laser damage, we found that XRCC4 recruitment is also impaired by a PARP inhibitor. Treatment with a PARP inhibitor in ataxin-3-depleted cells did not further reduce the accumulated levels of XRCC4 suggesting that the recruitment of XRCC4 might be promoted by PAR-recruited ataxin-3. In our model, the recruitment dependency on early and transient PAR signalling ensures that ataxin-3 is only retained at DNA damage sites during the early phase of the DDR when it

prevents premature removal of DNA repair proteins. During a later phase of the DDR, when the opposing DUB ataxin-3 cannot be retained, RNF4 can act on SUMOylated DNA repair proteins to promote their turnover.

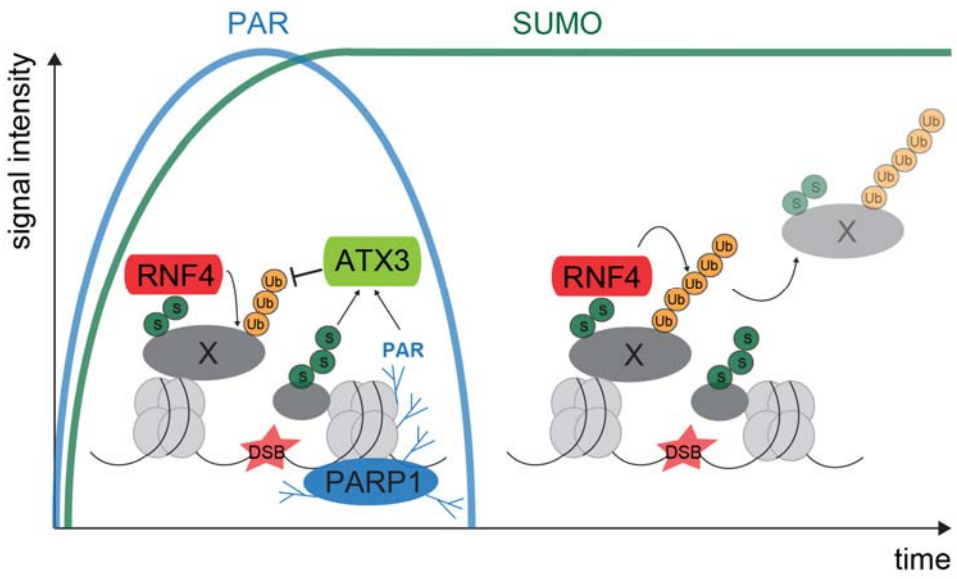

Figure 6. Model of PTM-mediated restriction of ataxin-3 recruitment to the early phase of the DSB response. The recruitment of ataxin-3 (ATX3) to DSBs is dependent on combinatorial DNA damage-induced SUMOylation and PARylation. This dual signal only exists during the early phase of the DDR and is thereby limiting the time window for ataxin-3 recruitment. While ataxin-3 can counteract RNF4-mediated ubiquitylation during the initiation phase of the DDR, ataxin-3 is not retained at DSBs when PAR signaling ceases. This limits RNF4-mediated ubiquitylation and potential chromatin removal of substrate proteins (X) to a late phase of the DDR when the counteracting DUB is not present. S, SUMO; Ub, ubiquitin; PAR, poly(ADP-ribose).

3.3 PAPER III

Independent mechanisms recruit the cohesin loader protein NIPBL to sites of DNA damage.

Cohesin, a ring-like complex that has the ability to tether replicated sister chromatids, was shown in yeast and mammalian cells to be enriched at sites of DNA damage and to promote DNA repair (Caron et al., 2012; Kim et al., 2002; Potts et al., 2006; Strom et al., 2004; Unal et al., 2004). Yeast studies have shown that in response to DNA damage, cohesin is SUMOylated (McAleenan et al., 2012) and *de novo* cohesin complexes are loaded to sites of damaged chromatin (Strom et al., 2007; Unal et al., 2007), meaning that the establishment of cohesion is not restricted to the replication event. Whether DNA damage-induced cohesion also takes place in mammalian cells is presently less understood. Loading of cohesin to chromatin is assisted by NIPBL. However, little is known how it functions as a cohesin loader and to which extent it is involved in DNA damage responses. Therefore, this study aimed at understanding recruitment mechanisms of NIPBL to damaged DNA. Such knowledge may help to understand how potential *de novo* cohesion at DNA damage sites facilitates DNA repair in mammalian cells.

Since we experienced low transient transfection efficiencies of NIPBL, stable HEK293 cell lines were generated that express inducible GFP-tagged NIPBL. Two human isoforms of NIPBL, NIPBL-A and NIPBL-B, were tested and found to accumulate at laser-inflicted DNA damage and at FokI-induced DSBs. The accumulation of NIPBL at sites of DNA damage was independent of interacting with its heterodimeric binding partner MAU2 (though MAU2 itself was recruited to laser DNA damage). NIPBL contains an HP1 binding motif and mutation of this motif abrogated the recruitment of NIPBL to FokI-inflicted DSBs, consistent with an earlier study (Oka et al., 2011). Interestingly, the HP1-binding mutant was still recruited to laser-introduced damage that can principally give rise to different types of DNA damage. Also a C-terminal truncation mutant of NIPBL, missing the HP1-binding motif, but containing several HEAT repeats, was recruited to laser-induced DNA damage sites, but not to DSBs introduced by FokI. HP1-mediated accumulation at laser damage was restricted to the N-terminal fragment of NIPBL suggesting that a second recruitment mechanism of the remaining C-terminal fragment exists that can compensate for the absence of or interaction with HP1.

Interestingly, we found that the recruitment of the C-terminal truncation fragment of NIPBL to laser DNA damage required ATM/ATR kinase activity. Combinatorial treatment with an ATM and ATR inhibitor impaired its recruitment, while a DNA-PK inhibitor had no effect on the

retention of NIPBL. This result was analogous to findings in budding yeast where cohesin loading is dependent on ATM and ATR activity. How ATM/ATR kinase activity facilitates NIPBL recruitment remains to be investigated. The NIPBL protein could be a target of phosphorylation promoting its recruitment and retention at DNA damage sites or, alternatively, could interact with binding partners at damaged chromatin that depend on ATM/ATR signaling.

Since another study had shown NIPBL recruitment to DNA damage to be dependent on the ubiquitin ligase RNF168 (Oka et al., 2011), we probed into the question how RNF8/RNF168 activity and ubiquitin signaling might be involved in NIPBL accumulation. At laser damage, the recruitment of both N-and C-terminal truncation mutants of NIPBL were impaired in cells depleted of RNF8 or RNF168. Also cell treatment with the proteasome inhibitor MG132, which can deplete the nuclear pool of ubiquitin (Dantuma et al., 2006; Mailand et al., 2007), abrogated the accumulation of both NIPBL truncations. How these findings relate to the recruitment of NIPBL requires further experimental investigation. Does NIPBL bind to chromatin-associated ubiquitin marks? Is NIPBL ubiquitylated? Is it recruited through other binding partners whose retention at DNA damage sites is RNF8/RNF168/ubiquitin-dependent?

These findings led to the conclusion that there are two independent recruitment mechanisms of NIPBL and their relative contribution is influenced by the type of DNA damage (**Figure 7**). While the N-terminus of NIPBL is recruited through interaction with HP1, the C-terminus requires active ATM/ATR for recruitment and both mechanisms are dependent on RNF8/RNF168 and ubiquitin signaling. However, the recruitment to “clean” DSBs introduced by FokI is strictly dependent on binding to HP1 at the DSBs.

HP1 is a protein that promotes transcriptional silencing of heterochromatin and binds a methyl mark on histone 3 (H3K9me3). The recruitment dependency of NIPBL on HP1 and presence of RNF8/RNF168/ubiquitin is intriguing in the light that other DDR proteins have been shown to be recruited by combinatorial PTMs (*e.g.* 53BP1, see introduction). It is possible that chromatin-associated ubiquitylation at DNA damage sites sets a specific signal for NIPBL recruitment in addition to general HP1-linked binding to an epigenetic methyl mark (H3K9me3).

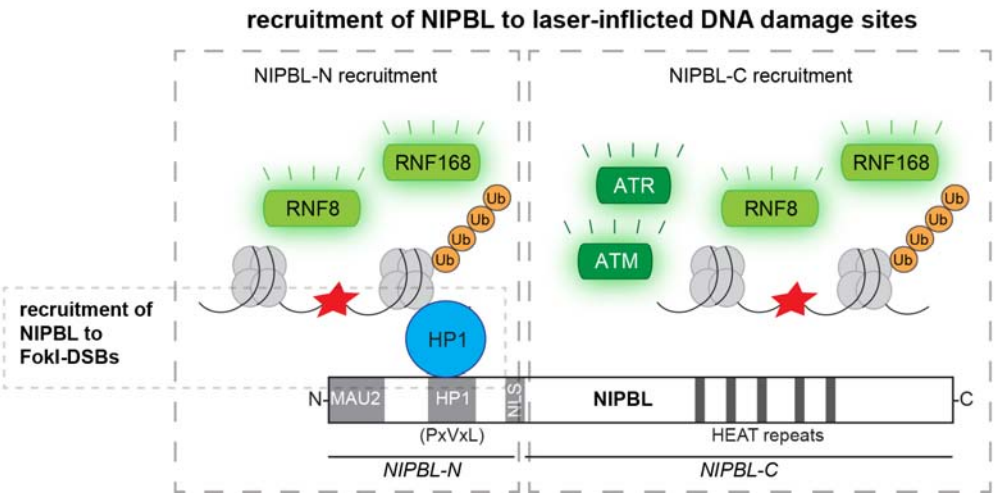

Figure 7. Model of NIPBL recruitment to sites of DNA damage. An N-terminal fragment of NIPBL is recruited to laser-inflicted DNA damage sites in dependency of HP1 and RNF8/RNF168 and ubiquitin signaling. The remaining C-terminal fragment of NIPBL requires ATM/ATR activity and RNF8/RNF168 and ubiquitin in order to be retained at damage sites. Both recruitment mechanisms are compensatory. Recruitment of NIPBL to FokI-induced DSBs occurs strictly by HP1.

Other studies have suggested that cohesin/NIPBL recruitment to damaged chromatin is cell cycle-dependent (G2/M). However, we found NIPBL recruitment to laser-induced DNA damage throughout the cell cycle. This is in line with findings describing CdLS patient-derived cells carrying NIPBL mutations to have defects both in HR and NHEJ repair (Enervald et al., 2013; Vrouwe et al., 2007).

4 CONCLUDING REMARKS

The DUB ataxin-3 is stimulating the DNA damage response

We have described that the DUB ataxin-3 is recruited to sites of DNA damage in a SUMO-and PAR-dependent fashion. The dual recruitment signal presumably limits the time window for ataxin-3 during the DDR initiation phase to counteract the ubiquitylation activity of RNF4 on MDC1. Reversing ubiquitylation on MDC1 prolongs its chromatin retention time, which ensures that sufficient chromatin-bound MDC1 is present to activate the MDC1-dependent response cascade to DSBs.

It is not unlikely that there are additional targets in the DDR, on which RNF4 and ataxin-3 act as a balancing ligase/protease pair, and RPA might be one of them. Ataxin-3 interacts with PARP1, which is SUMOylated in response to DNA damage (Zilio et al., 2013). We also observed that the *in vitro* DUB activity of ataxin-3 was stimulated by the presence of SUMO. Interestingly, one study reported that PARP1 is SUMOylated by PIAS4 after exposure to heat shock and that SUMOylated PARP1 is targeted by the STUbL RNF4 to regulate the stability of PARP1 (Martin et al., 2009). Whether SUMOylated PARP1 is also regulated by RNF4 in response to DNA lesions would be an exciting question to address. It would open the possibility that ataxin-3 might counteract RNF4 on PARP1 during the early phase of the DDR and that PARP1 is another shared substrate of this ligase/protease pair. Since ataxin-3 is PARylated by PARP1 it might initiate a positive feedback loop for its transient accumulation, especially if it turns out that PARylation of ataxin-3 is required for retention at DSBs. Even though this is still speculative, it opens interesting questions to follow up on in further experiments. Besides counteracting RNF4, it is also possible that ataxin-3 can reverse ubiquitylation mediated by other E3 ubiquitin ligases. The PAR-recruited ubiquitin ligase RNF146 targets PARP1 for degradation, which might be a potential target of ataxin-3 activity.

The relevance of the involvement of ataxin-3 in the DDR was demonstrated in our study by increased sensitivity of ataxin-3-depleted cells to IR and impairments of HR and NHEJ repair. Additionally, the loss of ataxin-3 sensitized cells to treatment with a PARP inhibitor. These findings would make the screen or development of an ataxin-3 inhibitor attractive, as ataxin-3 inhibition combined with IR or PARP inhibition could potentially be used to induce synthetic lethality in cancer cells. Whether the catalytic inhibition of ataxin-3 has the same cellular effect like the depletion of ataxin-3 would be essential to test especially given that low ataxin-3 expression levels have been reported in human gastric cancer (Zeng et al., 2014).

Ataxin-3 causes the neurodegenerative disorder Machado-Joseph disease (MJD; or SCA3) when its polyQ tract is expanded. PolyQ-expanded proteins tend to aggregate making the protein and/or binding partners unavailable for their cellular functions resulting in loss-of-function. By determining cellular functions of ataxin-3, we might get a better understanding of factors that could contribute to the development of MJD in the presence of polyQ-mutant ataxin-3. Interestingly, an increase of DNA damage is present in SCA3 patient brain sections and SCA3 mouse brain (Gao et al., 2015). PolyQ-expanded ataxin-3 is accumulating at laser-induced DNA damage and at FokI-introduced DSBs (personal observations), but whether it is executing the same functions in the DDR as described in this thesis for wild-type ataxin-3 remains to be explored.

What is the significance of NIPBL recruitment to sites of DNA damage?

Cohesin has been shown to be enriched at damaged chromatin and we have confirmed that the cohesin loader NIPBL is recruited to sites of DNA damage in HEK293 cells. What is the significance of this finding? It may support the idea that there is *de novo* DNA damage-induced cohesion in mammalian cells, which usually is established after replication to tether sister chromatids. What roles do cohesin and NIPBL have in the response to DNA damage and in DNA repair? Based on its canonical function, it is assumed that cohesin assists HR repair during the late S/G2/M phase of the cell cycle by keeping broken DNA ends and templates in close proximity. To this end, the pre-existing cohesin already loaded onto chromosomes after replication may be considered to be sufficient. Several PTMs of cohesin were described in response to DNA damage, among them is ATM-mediated phosphorylation (Yazdi et al., 2002). Therefore, it is possible that pre-loaded cohesin, modified in response to DNA damage, might be sufficient for promoting DNA repair, raising the question why NIPBL is recruited to sites of DNA damage.

We found surprisingly that the cohesin loader protein NIPBL is recruited to damaged chromatin throughout the cell cycle and mutations in NIPBL were also associated with impaired NHEJ repair (Enervald et al., 2013). It can be therefore speculated that NIPBL-assisted *de novo* damage-induced cohesion also exists in mammalian cells (as shown in yeast) and that it is also important in other cell cycle phases than G2/M. It cannot be excluded though that NIPBL might have an additional function upon the recruitment to sites of DNA damage that is cell cycle-independent and distinct from cohesin loading. In that light it would be interesting to determine what impact the absence of NIPBL would have on cohesin at DNA damage sites (in different

cell cycle phases) and on DNA repair. Is the presence of NIPBL actually required for damage-induced cohesion (*i.e.* loading of additional cohesin) throughout the cell cycle? Far from our understanding is how DNA damage-recruited NIPBL might mechanistically load *de novo* cohesin to damaged chromatin sites.

While it was described that the recruitment of cohesin is limited to the immediate vicinity of DSBs in human cells (contrary to yeast studies) (Caron et al., 2012), it is not known how the spreading is restricted. On the contrary, the loader NIPBL is recruited to DNA damage by ATM and RNF8/RNF168 activity, which all result in spreading of phosphorylation (γH2AX) and ubiquitylation, respectively, into undamaged chromatin and presumably spreading of NIPBL. It will be intriguing to examine the spatial recruitment pattern of NIPBL and how it relates to the proximal or distal recruitment of cohesin.

We found two independent recruitment mechanisms of NIPBL, which seem to be influenced by the type of DNA damage. There are other examples in the literature describing proteins to be recruited to different types of DNA damage by distinct parallel events (*e.g.* SLX4 is recruited to DSBs dependent on SIM-SUMO interaction while it is binding to ubiquitylated FANCD2 at DNA inter-strand crosslinks) (Dantuma and van Attikum, 2016). Future work will be required to identify to which extent the type of DNA damage is dictating the mode of recruitment or whether the dual recruitment mechanism of NIPBL is redundant in order to insure its retention at sites of DNA damage.

There are many question marks regarding the recruitment and function of NIPBL and DNA damage-induced cohesion in mammalian cells and addressing their answers will give us additional mechanistic insight into the tight regulation of sensing and repairing DNA lesions.

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