Understanding the mechanisms of histone 2A ubiquitination

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Understanding the mechanisms of histone 2A ubiquitination

De mechanismes van histone 2A ubiquitinatie

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LIST OF ABBREVIATIONS

Å	Angstrom (1 Angstrom = 0.1 nm)
APC/C	Anaphase promoting complex / Cyclosome
ATM	Ataxia telangiectasia mutated kinase
ATR	Ataxia telangiectasia and Rad3 related kinase
BER	Base excision repair
CRL	Cullin-RING ligase
Da	Dalton
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DUB	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
F	Full-length construct
FP	Fluorescence polarization
GFP	Green fluorescent protein
GST	Glutathione S-transferase
H2A	Histone 2A
H2AK119ub	Ubiquitinated H2A on K119
H2AK13-15ub	Ubiquitinated H2A on K13-15
H2AX	Histone H2A.X
H2B	Histone 2B
H2BK120ub	Ubiquitinated H2B on K120
H3	Histone 3
H4	Histone 4
HECT	Homologous to the E6-AP carboxyl terminus
ICL	Interstrand crosslink
IR	Ionizing radiation
K119	Lysine on position 119
K13-15	Lysines on position 13 and 15
MIU	Motif interacting with ubiquitin
NER	Nucleotide excision repair
PcG	Polycomb
PCNA	Proliferating cell nuclear antigen
PRC	Polycomb repressive complex
PRR	Post-replicative repair
R	RING domain construct
RING	Really interesting new gene
RMSD	Root-mean-square deviation
RNF	Ring finger protein
SAXS	Small-angle X-ray scattering
SCF complex	Skp, Cullin, F-box containing complex
SPR	Surface plasmon resonance
TLS	Translesion synthesis
UV	Ultraviolet
WT	Wild-type

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INTRODUCTION

Protein ubiquitination

Ubiquitination refers to the covalent attachment of a small protein, ubiquitin, to the ε-amino group of a lysine residue on the target protein, with the purpose of regulating the fate, the activity or the localization of this target.

Ubiquitin is an 8.5 kDa protein, found in eukaryotic cells and highly conserved in aminoacid sequence through evolution. The cellular process that yields ubiquitin conjugation has been well studied in the past decades and involves three distinct enzymatic reactions. First, the C-terminus of ubiquitin is activated in an ATP-dependent manner by an E1 enzyme. Then the ubiquitin is transferred to a cysteine residue on an E2 conjugating enzyme which will interact with an E3 ligase that will allow the final ubiquitin conjugation step to a lysine of the substrate protein[1-2]. To counteract and regulate protein ubiquitination in cells, a large set of deubiquitinating enzymes (DUBs) are present (Figure 1a). These enzymes are largely cysteine-proteases capable of cleaving off the ubiquitin moiety from the lysine on targets[3]. Their function in cells is crucial to balance the cellular signaling of the ubiquitination events[4]. While only a handful of E1s and few tens of E2s are found in humans, the complexity of the system is highlighted by the hundreds of E3s described today, and the almost hundred DUBs. Moreover, a number of ubiquitin-like molecules (e.g. SUMO, NEDD8, FAT10) have been identified that are functioning in a parallel but distinct fashion.

In general, a target protein can undergo monoubiquitination (one ubiquitin moiety is conjugated to one lysine residue on the substrate), multiple monoubiquitination (multiple monoubiquitination events on one protein) (Figure 1b). Furthermore, because ubiquitin itself contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) it can act as a substrate and be ubiquitinated, forming ubiquitin chains. The formation of these chains on a substrate protein is known as polyubiquitination (Figure 1b). Additionally, ubiquitination on the N-terminal amino group of the polypeptide chain of a protein or of ubiquitin itself have been reported[5].

Ubiquitin E3 ligases and target selection

Although E3-independent ubiquitin conjugation may occur[6], in general E3 ligases are required to confer substrate selectivity and efficiency to the ubiquitination reaction. There are two main families of ligases known so far: Ubox/RING(Really Interesting New Gene)- and HECT(Homologous to the E6-AP Carboxyl Terminus)-type E3 ligases, named after the domain present on the protein to confer them the ligase function (Figure 1a).

The HECT ligases function via an active cysteine residue which receives ubiquitin from the E2 and through a covalent bond it is then able to transfer it to the lysine on the target protein[7].

On the other hand, the RING domains are small structural units where 8 cysteine or histidine residues are involved in the coordination of two Zinc cations[8]. RING and the structurally related Ubox E3 ligases are unable to covalently bind ubiquitin but they act as bridging platform where the charged E2 (E2 carrying the ubiquitin) and substrate protein are brought together and ubiquitin is directly transfered from the E2 to the lysine on the substrate[9] (Figure 1c).

In human the majority of the ligases are RING-type E3s and members of this family are the main focus of this thesis. Their

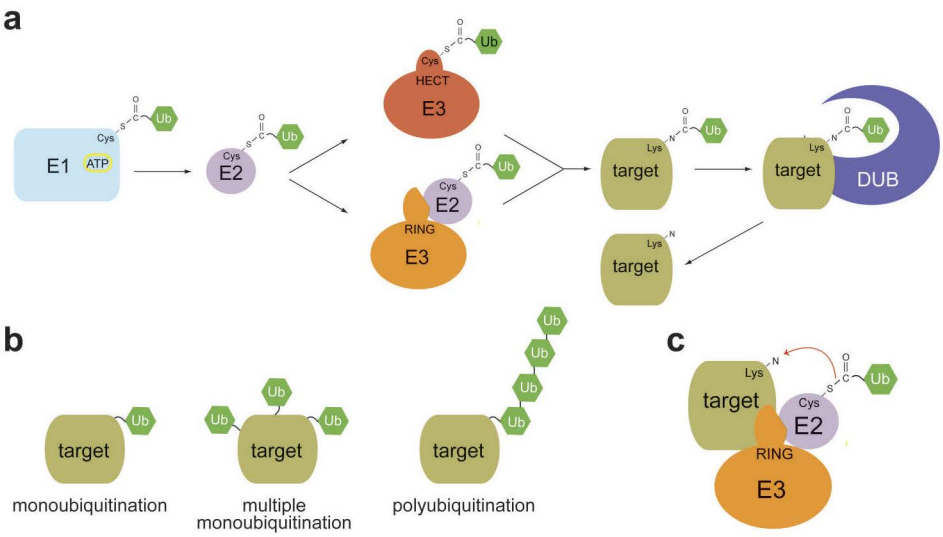

Figure 1. **a**) Schematic representation of the enzymatic reactions involved in ubiquitin conjugation and deconjugation. The C-terminus of ubiquitin is activated in an ATP-dependent manner by an active cystein (Cys) residue on an E1 enzyme. Ubiquitin is then transferred to a cysteine on the E2 conjugating enzyme. Two classes of enzymes can catalyze the transfer of ubiquitin to the target protein. HECT ligases also function through an active cysteine, Ubox/RING ligases allosterically activate the E2 enzyme. Ubiquitination takes place on a lysine (Lys) residue of a substrate protein and it can be reverted by the function of DUB enzymes, that cleave the modification and release the target protein unmodified. **b)** Representation of monoubiquitination, multiple monoubiquitination and polyubiquitination that can occur on target proteins. **c)** Representation of the transient complex that is formed during RING-dependent catalysis. The RING domain bridges the charged E2 enzyme and the target to activate the release of ubiquitin from the cysteine on the E2 to the lysine on the substrate.

function in the ubiquitination cascade is at this time the most intriguing one, since they lack a catalytically active site. While recent work has shown that RING domains allosterically activate the release of ubiquitin from the E2[10-12], their way to select the substrate for ubiquitination in most cases still remains unknown.

A large sub-group is composed of Cullin-RING ligases (CRLs) with roles in a large number of cellular pathways[13]. CRLs are multisubunit complexes where the elongated Cullin protein bridges a RING–containing subunit on its C-terminal end to the target specificity module on the N-terminal side[14]. Interestingly, the function of these ligases is regulated by the modification with the ubiquitin-like modifier NEDD8, which appears to be required for their activation[15]. The RING unit on CRLs is a small protein whose role is purely the activation of the E2~ubiquitin complex, while the rest of the complex function to select and position the target protein correctly for its ubiquitination[13].

Otherwise, RING E3 ligases can function as monomers or dimers and are found in cells either in isolation or in the context of larger complexes[8]. A subset of RING and Ubox proteins homo- or

heterodimerize through the contribution of their flanking regions, an overview of the structural aspects of the function of these E3s is presented in chapter 2, with special focus on DNA damage related processes[16].

In this class of ligases, target specificity can be achieved on one hand by the involvement of a different subunit of their complex or a different domain on the E3 that directly recruits the substrate or relocalizes the E3 in its proximity[17-21]. On the other hand, it appears that *in vitro* the isolated RING domain of some E3s is sufficient to achieve target specificity[22-24], suggesting that this domain itself can contain the molecular determinants for target selection. In chapter 4 and 5, we present our data on the identification and analysis of a region involved in substrate recognition in the RING domains of RING1B, RNF168 and RNF8, the charges at this site located C-terminally to the core domain determine the ability of these E3s to target nucleosomal H2A.

Functions of ubiquitination

Ubiquitin modification needs to be recognized in order to function as a cellular signal, a large number of proteins containing ubiquitin binding modules (e.g. UBD, UBZ, UIM or MIU motives) have been described to date and those are the "readers" that translate the ubiquitination into further cellular signaling[25]. Although the enzymatic steps required in the ubiquitin conjugation and deconjugation pathway have been described, the readers and the subsequent functions of most ubiquitination events remain elusive, with few exceptions.

The best described cellular system where ubiquitin plays a major role is the signaling arising from the formation of K48-linked ubiquitin chains on substrates. This polyubiquitination label target proteins for degradation by the proteasome. The proteasome is a large multimeric complex that degrades the proteins ubiquitinated with such chains whilst recycling the ubiquitin moieties. In fact this was the first function associated with ubiquitination in the early 1980s and awarded the Nobel Prize in Chemistry in 2004[26]. The proteasome contains a large number of ubiquitin receptors, the readers that bind K48 ubiquitin chains, to direct the proteins targeted for degradation to the proteases present in the complex[27-31].

A well known example of an important pathway where ubiquitination is used for degradation purposes is the cell cycle. In this highly sensitive system timing and order of protein degradation is crucial for healthy progression through the cycle. The CRLs APC/C and SCF are the central players that orchestrate the degradation of cyclins and other proteins crucial for mitosis progression and DNA replication[32-33]. While SCF binds the E2 CDC34 to catalyze K48-linked polyubiquitin chains, APC/C works differently[13,34]. APC/C employs two distinct E2s, it initiates multiple monoubiquitination of its substrates with UbcH10 and then it extends K11-linked ubiquitin chains with the E2 Ube2s[35-36]. Interestingly both signals will target the substrate proteins for degradation by the proteasome[36-37].

Currently also other types of polyubiquitin chains, e.g. K29 and K63, are proposed to signal for degradation[38-39] although the specific ubiquitin receptors that will integrate these signals haven't yet been identified. The well known K63-linked polyubiquitin chains do not normally target proteins for degradation. This chain has been studied in great details in the past years and our knowledge

of its structural properties allowed the development of specific biochemical tools now widely used to analyze its functions *in vitro* and *in vivo*[44-45]. We currently know that this polyubiquitin chain functions as recruitment or localization signal in DNA damage, endocytosis and many other signaling pathways[40-42]. In the NF-kB pathway K63 ubiquitin chains have also been described to function as unanchored chains[43]. A great number of readers for this polyubiquitin chain have been identified in all these pathways, nevertheless in most cases we do not yet fully understand if the attachment site where the chains are conjugated has functions in the signaling.

The study of the role of other types of polyubiquitin chains and of specific monoubiquitination signals is hampered by the lack of experimental tools for research (e.g. antibodies, recombinant ubiquitinated proteins).

To date, one of the best understood ubiquitination targets is PCNA (Proliferating Cell Nuclear Antigen) thanks to contributions of *in vitro* and *in vivo* studies[46-48]. PCNA is the processivity clamp for DNA polymerases in eukaryotic cells. It encircles the DNA, thus creating a topological link between the replication machinery and the DNA molecule. In situations of DNA damage, PCNA is monoubiquitinated on K164 by the E2/E3 pair RAD6/RAD18 and then it can be polyubiquitinated by a set of E3s (HLTF, SHPRH) and the E2 Ubc13/Mms2, specific for K63 chains. The molecular function of the monoubiquitination signal has been resolved. Monoubiquitinated PCNA allows the switch from the canonical DNA polymerase to the specialized Translesion Synthesis (TLS) polymerases (e.g. polη) required for replication of damaged nucleotides. These polymerases contain a conserved ubiquitin binding domain (UBZ) and a PCNA interacting peptide (PIP) that allow a selective dual binding to the ubiquitinated form of PCNA[49]. On the contrary, the function of the polyubiquitination on PCNA still requires further investigations. We know that this modification activates the template switch pathway and recruits component of the homologous recombination (HR) machinery to the fork. This ensures maintenance of correct genetic information by using the other newly synthesized DNA strand as template for the DNA polymerase. Nevertheless, the protein readers of the polyubiquitin chain on PCNA are not yet known. This molecular signaling are counteracted by the activity of the DUB USP1[50].

Another interesting system where roles for monoubiquitination signals are known is the internalization of some receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) and the platelet derived growth factor (PDGF) receptors. In the modulation of extracellular signaling, endocytosis and subsequent lysosomal degradation of these receptors are regulated by the function of the E3 ligase c-Cbl. Strictly the multiple monoubiquitination of the receptors, rather than the polyubiquitination is the driving force for the internalization[51-52]. These ubiquitin moieties serve as a binding platform for adaptor proteins required to initiate endocytosis such as Eps15[53-54]. Nevertheless, to complicate the picture, K63 chains also seem to have a role for other receptors as well as in later stages of the endocytic process where members of a family of HECT ligases are active[55-56].

For the other myriad of ubiquitination events we still don't have a clear picture

of the molecular mechanisms that the cell employs for signaling. This thesis is focused on the understanding of the ubiquitination steps that lead to the modification of the very first ubiquitinated protein ever identified in 1977: histone 2A (H2A)[57-58].

H2A ubiquitination

H2A is one of the four histone proteins that constitute the proteinaceous core of the nucleosomes with H2B, H3 and H4. Nucleosomes are composed of an octameric complex of the four histones around which a stretch of 147 bp DNA is wrapped[59] (Figure 2a). These units are then organized in higher ordered structures to form chromatin fibres, which allow a functional and organized packing of the long DNA molecules inside the cell nucleus. Histones are small proteins (~12 kDa) with a structurally conserved core domain that allow the formation of the histone octamer and highly accessible C- and N-terminal tails that protrude out of the core nucleosome. In fact, these histone tails are targets for a large number of post-translational modifications including ubiquitination that strongly affect all aspects of chromatin biology[60].

From an evolutionary point of view, H2A is a target for ubiquitination only in higher eukaryotes, plants and the worm *Caenorhabditis elegans*[61-62], whereas *Saccharomyces cerevisiae* lacks this ubiquitination mark as well as the E3 ligases that are responsible for histone 2A modification. This limits the experimental opportunities to use yeast as model organism.

In human cells, 5-15% of H2A is monoubiquitinated on K119 and this prevalent modification is the product of the activity of the RING E3 ligases present in the Polycomb Repressive Complex 1 (PRC1)[23,63-65]. H2A is also ubiquitinated in response to DNA damage (DDR) in mammals, where RNF168 and RNF8 are the main RING E3 ligases involved[24,66-72]. We and others have recently described that this modification takes place on a different site, the N-terminal lysine residues K13-15[24,73]. Our detailed analysis of the *in vitro* and *in vivo* findings of this novel ubiquitination site is presented in chapter 4 (Figure 2b).

In cells, H2A ubiquitination is controlled by specialized DUBs. To date USP16[74-75], PR-DUB in *Drosophila* and its human homolog BAP1/ASXL1[76-77], USP21[78-79], USP3[80], USP22[81-82], 2A-DUB[83], USP12 and USP46[84] have been suggested to have a role. Because of the very recent characterization of the K13-15 ubiquitination site, the relative contribution of these DUBs to the two different signalings needs to be elucidated. In chapter 5, we discuss some aspects of this specificity.

Polycomb signaling and H2A ubiquitination

Epigenetics refers to modifications on chromatin that do not involve a change in the nucleotide sequence of DNA but that are capable of changing the activity of specific genetic regions and that can be transmitted through cell division. This includes direct modifications on the DNA molecule (e.g. methylation) as well as modifications of the histone tails on nucleosomes[60].

Polycomb (PcG) signaling is a well known example of epigenetic regulation of specific genetic loci[85-86]. PcG proteins were initially discovered in *Drosophila melanogaster* to be involved in the regulation of morphogenesis by repressing homeotic (Hox) genes[87]. Now PcG proteins are found in vertebrates, plants and *C.elegans*[61-62] and our knowledge of their repertoire of targets has been greatly expanded by a series of

genome-wide studies. These have identified hundreds of new target genes that encode for transcription factors and key players in developmental pathways, placing PcG signaling at the helm of the regulation of multicellular development, stem cell biology and tumorigenesis[85].

The first link between PcG and cancer came from the observation that the PcG protein BMI1 promoted B- and T-cell lymphomas in collaboration with MYC[88-89]. This collaboration involves the regulation of the *CDKN2A* locus, one of the most important PcG targets[90-91]. This genetic locus encodes for the tumour suppressor proteins p16 and p19, key regulators of cell cycle control and senescence in situations of aberrant mitogenic signaling. Tight regulation of these genes is essential for the healthy survival of the cell[92]. This locus partially contributes to the oncogenic potential in situations of deregulation of PcG proteins, but it's clear that additional mechanisms are also important. To date deregulation of several PcG proteins have been associated with neoplastic development and in some cases these were found to be *CDKN2A*-independent[93]. Moreover, given that PcG proteins promote stem cell maintenance through repression of lineage-specific genes[94-95], it has been proposed that aberrant PcG signaling in cancer causes the establishment of a more primitive differentiation state that is characteristic of many tumours and generally correlates with their clinical aggressiveness[93].

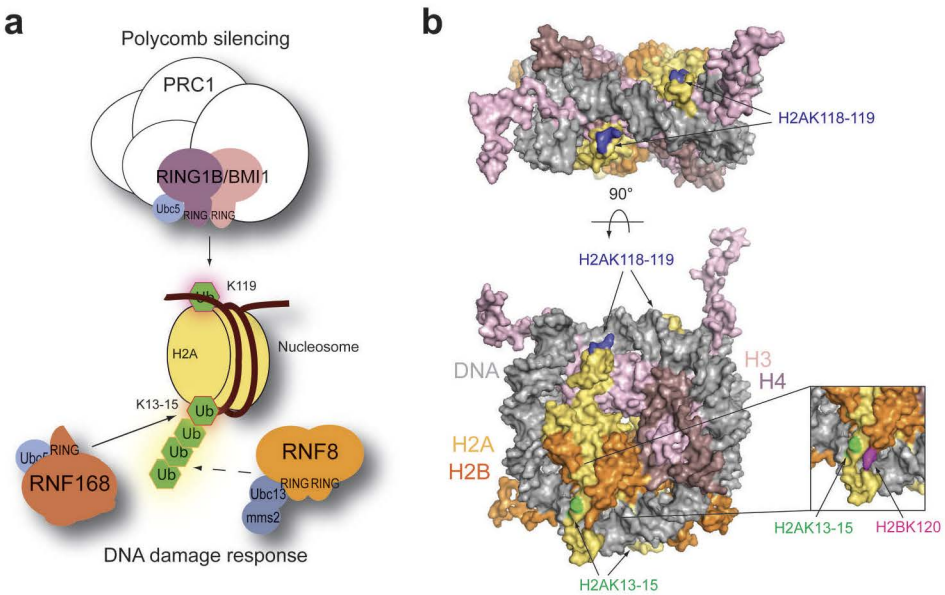

Figure 2. a) Distinct pathways target K119 or K13-15 on H2A in nucleosomes. The Polycomb Repressive complex 1 (PRC1) is the main ligase to targeting H2A on K119. In DNA damage response RNF168 targets K13-15 to initiate H2A ubiquitination where subsequently K63 polyubiquitin chains are extended, possibly by RNF8. **b)** Surface representation of the nucleosome structure (1KX5). H2B is shown in orange, H2A in yelloworange, H3 in lightpink and H4 in dirtyviolet. K118-119 on H2A are highlighted in blue and K13-15 are in green. The close up shows the proximity of H2A K13-15 in green with H2B K120 in magenta. Images prepared using Pymol.

PcG proteins exert their function through the assembly of two distinct complexes, PRC1 and PRC2. The complexity of the PcG signaling is augmented by the presence in human and mice of families of paralog proteins, originated during evolution from gene duplication of single PcG genes found in *Drosophila*. Recent work highlighted that PRC2 and most prominently PRC1 are present in multiple versions in cells. Not only different PcG subunit compositions confer different functions to the complexes but also some PcG proteins are found to interact with proteins not initially identified as Polycomb related factors, e.g. the dRAF complex in *Drosophila*, and its human homolog BCOR complex[96-100].

PRC2 has four core subunits and its core activity is to methylate K27 on histone H3. Tri-methylation of the lysine is the functional signature for the repressive Polycomb signaling as it mostly overlaps with PcG distribution in genome-wide studies[101-104]. Nevertheless, this mark is also present in the so called bivalent domains on asymmetric nucleosome together with the transcriptionally active marks of H3K4 or H3K36 methylation[105-107]. The methyltransferases in human PRC2 are EZH1 and EZH2 and recent studies have highlighted major differences in their activity. A first difference lies in their relative expression in different cellular contexts: EZH1 is highly expressed in adult tissues and non-dividing cells, while EZH2 predominates in proliferating cells and during embryogenensis[108-109]. Additionally their catalytic activity within PRC2 is drastically different: the complex containing EZH1 is much less active than the one containing EZH2, but it can compact polynucleosomes whereas EZH2-containing PRC2 can not[108]. This presents an interesting biochemical case, as the proteins are 65% identical and they complex with the same subunits within the PRC2.

PRC1 is a much more diverse complex, composed of proteins belonging to different families. Its core activity is the monoubiquitination of H2A at K119[63-65,99]. The E3 ligase paralogs present in the human complex are RING1B or RING1A. For proper function, they heterodimerize with the PCGF (Polycomb group RING finger) proteins, e.g. BMI1 or MEL18, via their respective RING domains. Additional PcG families compose the PRC1 complex: the Polycomb family (e.g. CBX4, CBX7, etc), the Polyhomeotic proteins (PH1, PH2), the RYBP and YAF2 proteins and additional substoichiometric components[99].

Recent work has shown the diversity in composition of the mammalian PRC1[96,99-100], associated to this alternative composition there is diversity in functions between the PRC1 variants. This supported the diverse models put forward by several groups aimed at understanding the mechanism by which PRC1 represses target genes. It appears that PRC1 is capable of stalling RNA polymearse II during transcript elongation[110] and this is currently thought to at least partially explain its repressive function. Nevertheless, two different molecular mechanisms have been proposed, one involves H2A ubiquitination[76,110-111], the other is independent on the the E3 ligase function and involves direct chromatin compaction[112-114]. Interestingly, a recent study proposed a combinatorial function of these two mechanisms[115]. Using mouse ES cells where both *RING1A* and *RING1B* genes have been knocked-out, this group analyzed the contribution of the E3 ligase

activity of PRC1 for its repressive function and its stem cell maintenance capacity. Using an inactive version of RING1B mutated on the E2-E3 interaction surface (I53A and I53S) they find that the H2A ubiquitination activity of PRC1 is dispensable for its target binding and its activity to compact chromatin at Hox loci, but is indispensable for efficient repression of target genes and thereby for ES cells maintenance[115]. This work nicely presents a validation of the importance of H2A ubiquitination in the Polycomb signaling. Moreover, this study shows that H2A ubiquitination occurs only on a subset of the genes bound by RING1B[115]. This finding suggests that only a subset of PRC1 variant may function through H2A ubiquitination, while the role of other variants may be independent on this catalytic activity. This is in line with the observation that dKDM2 in *Drosophila* is required for bulk H2A ubiquitination by the dRAF complex that contains dRING/PSC, the fly counterparts of RING1B/BMI1[100]. This suggests that in flies this complex rather than canonical PRC1 accounts for most ubiquitination events on H2A.

A study has proposed an additional E3 ligase for K119 on H2A involved in transcriptional repression, 2A-HUB[116]. This E3 in the context of the N-CoR complex can inhibit transcription elongation by ubiquitinating H2A on a subset of gene promoters, interfering with the action of the histone chaperone FACT[116].

Lastly, a recent study has characterized the first binding protein for ubiquitinated H2A, ZRF1. This factor is important for de-repression of Polycomb genes and after binding to modified histone, it can function by displacing PRC1 and recruiting the H2A DUB USP21 to chromatin to allow the reversion of the ubiquitination and the activation of the target genes[78].

Steric inhibition of transcription elongation and ZRF1 binding are the first molecular modes of action proposed for ubiquitinated H2A in the Polycomb signaling, but additional yet unknown effects may also play important roles.

DNA damage response and H2A ubiquitination

The survival of organisms depends on the accurate transmission of genetic information from one cell to its daughters. Such correct transmission requires not only accuracy in DNA replication but also the ability to survive spontaneous and induced DNA damage while minimizing the number of mutations. To achieve this fidelity, cells have evolved surveillance mechanisms that detect the occurrence of DNA damage and coordinate repair and cell cycle progression. This complex system, referred to as DNA damage response (DDR) activates a transcriptional program to ensure DNA repair and activation of checkpoints that pause cell cycle progression and give the cell time to repair the damage before continuing to divide. In case of unresolved damage the DDR is also responsible for the induction of cell death by apoptosis[117-118]. In cases of deregulation of these response pathways, the cell can bypass the checkpoints and undergo cell division, which will lead to aberrant transmission of genetic information which, if it is not enough to kill the cell, in many cases it will transform it to a potential cancer cell[119].

The master regulators of DDR in human cells are the sensor protein kinases ATM and ATR. Their crucial importance is highlighted by the severe phenotypes observed in case of mutations of either of

these kinases. ATR is essential for cell and organism survival, suggesting a role for this protein not only in DDR but also is basal cellular processes[120-121]. Lack of detectable ATM protein in human is associated to the severe AT syndrome displaying among other hypersensitivity to Ionizing Radiation (IR), immune deficiencies and predisposition to cancer[122-123]. ATM and ATR activate the complex signal transduction network that coordinates repair and cell-cycle control[117-118,124].

Recent work has shown that DDR networks widely rely on ubiquitin-related processes[16,125]. Only recently we began to unravel the complex involvement of this modification in these pathways. A big involvement of ubiquitin resides in its degradative role. This is emphasized by the effects of proteasome inhibition that is sufficient to hamper these pathways[126-127]. In fact, tight control of the life span of the proteins involved in these pathways is essential to ensure efficient and timely actions. A clear example of the importance of this role of ubiquitination is the regulation of the tumor suppressor p53. p53 is a transcription factor engaged by the cell in response to a variety of genotoxic stresses, whose transcriptional program ensures repair, cell cycle control and apoptosis. p53 protein stability is crucial for its function and it is strictly regulated by its ubiquitination state mainly dictated by the balance between the activity of the E3 MDM2/MDMX and the DUB USP7[128-129].

These and other DDR effects occur in response to a variety of genotoxic stresses, but the cell has evolved specialized pathways triggered by specific types of damages and ubiquitin-dependent signaling is required for proper function of virtually all of them. Mismatch repair (MMR) corrects errors of DNA replication and recombination that result in mispaired but undamaged nucleotides. Base excision repair (BER) takes care of recognizing and replacing damaged DNA bases. Nucleotide excision repair (NER) normally functions in response to UV-induced damage, where lesion recognition can occur both through transcription-coupled NER (TC-NER) or Global Genome NER (GG-NER) and where a short single-strand stretch of the DNA surrounding the lesion is replaced in a common fashion. Post-replicative repair (PRR) ensures the correction of lesions encountered by the DNA replication machinery. Interstrand cross-linkages (ICLs) are corrected by the Fanconi Anemia proteins. Finally, the deleterious double-strand breaks (DSBs) are repaired either by homologous recombination (HR) that ensures maintenance of the genetic information or non-homologs end joining (NHEJ) which refers to a error-prone repair mechanism.

In BER, the activity of the E3 ligases ARF-BP1/Mule and CHIP towards DNA polymerase β when not involved in actual repair[130-131]. Moreover, CHIP is connected to the degradation of the BER factor XRCC1[130], and Mule regulates the level of DNA polymerase λ according to its phosphorylation state[132]. Interestingly, these two E3 ligases seem to work in a concerted manner on DNA polβ where the HECT Mule E3 initiates ubiquitination on three N-terminal lysines on DNA polβ and the Ubox CHIP ligase extend the chains to target it for degradation[131]. This is counteracted by the action of the DUB USP47[133].

In NER, ubiquitination has both degradative roles as well as regulatory

functions[134-136]. In this pathway CRLs assembled with the DDB1/2 and CSA/B adaptor subunits have crucial roles. Degradation of DDB2 in GG-NER and CSB in TC-NER are required for signaling. In TC-NER the degradation of RNA polymerase II is important to remove the transcription machinery and allow assembly of the NER proteins for repair. Non-degradative ubiquitination signals are found on the XPC, required for its binding to the proteasome-associated ubiquitin receptor HR23A/B and important for recruitment and binding of XPC to the damaged region. Interestingly histone proteins are also found to be ubiquitinated in these processes, the CRL complexes are not the only E3s responsible for these activities, but also the the Polycomb protein RING1B and the DNA Double-strand Break factor RNF8 were proposed to have a role through the induction of ubiquitination of H2A[137-140]. The function of H2A modification in this pathway still awaits clarifications.

In the late stages of NER, PCNA ubiquitination was shown to be important, suggesting a shared signaling cascade between this damage pathway and PRR signaling[141]. In fact, PCNA is the central player of the PRR cascade, its ubiquitination is tightly regulated and controls different stages of this repair signaling. The mechanisms of its modification are described above. It appears that PCNA ubiquitination functions as a more general player in the later stages of repair for different types of damage that require DNA polymerases action[47].

This also includes the repair pathway of ICLs[142-143]. In response to ICLs, the large multimeric Fanconi Anemia complex is activated and it is required for repair[143]. This complex contains a RING E3 ligase subunit (FANCL) that targets the FANCD2 and FANCI heterodimer for specific monoubiquitination on K561 nad K523 respectively. The modified complex localized on damaged chromatin acts as a platform for the recruitment of the nuclease FAN1 that binds directly via its UBZ4 domain to initiate repair of the lesions[144-148].

At last, DSBs account for the most deleterious lesions that can occur on our genome, their severity is given by the high risk of loosing genetic information during the repair process. The early steps of the molecular response to these lesions have been recently characterized and they largely involve non-degradative ubiquitination, in addition to other post-translational modifications such as sumoylation and phosphorylation[149-151]. DSBs are detected by the MRN complex (Mre11, RAD50 and NBS1) that binds the kinase ATM. Upon activation, ATM phosphorylates S139 on the histone variant H2AX (γH2AX) and other targets, e.g. MDC1 and CHK2. Phosphorylated MDC1 bound to γH2AX recruits to the site of damage the ubiquitin ligase RNF8 via its FHA domain[68-70]. This ligase is the first E3 recruited at the lesion and the integrity of its RING domain is required for the subsequent recruitment of RNF168, a second RING E3 ligase[71-72]. The latter contains MIU ubiquitin binding motifs that are responsible for its accrual to damage sites, this suggested a model where RNF168 binds RNF8-dependent ubiquitin modifications. The kinetics of the accumulation of these factors to the lesion is in the order of minutes (RNF8 in ~3 minutes, RNF168 in 3-5 minutes)[71].

A serie of substrates have been described for RNF8 and RNF168[152-155], but so far

the primary targets for non-degradative ubiquitination are histone H2A and its variant H2AX. In particular, these ligases catalyze the polyubiquitination of these histones in an unexpected manner according to their order of recruitment. In chapter 4, we show that RNF168 is the first ligase to target H2A, implying a yet unknown target for the initial RNF8-dependent modification that recruits RNF168. We propose a two-step mechanism to achieve polyubiquitination of H2A/H2AX, where RNF168 is involved in the priming monoubiquitination and RNF8 may have roles in the K63 chain extension step. Further analysis *in vivo* is required to validate this hypothesis. Importantly we find that H2A/X are ubiquitinated on a novel N-terminal site, K13-15[24,73]. The importance of the functions of the ligases RNF8 and RNF168 was demonstrated by the observation that mutations in the *RNF168* gene in humans cause the RIDDLE syndrome, a radiosensitivity and immunodeficiency disorder[72,156]. Additionally, depletion of either of these ligases in mouse models recapitulates this phenotype and gives predisposition to cancer[157-159].

Additional ubiquitin-related proteins are known to play roles in this pathway, these includes DUBs, OTUB1[160] and USP3[80], and E3s, RAD18[161-163], RNF169[163-165], HERC2[166], UBR5 and TRIP12[167]. Nevertheless, the RNF8/RNF168 ubiquitination cascade is central to the pathway, as it is required for the recruitment of the BRCA1 complex and 53BP1 at the sites of damage. These proteins are crucial players in the selection of the mechanism employed by the cell to repair the damage: BRCA1 promotes HR, while 53BP1 favors NHEJ[168-172].

BRCA1 is a RING E3 ligase itself, it heterodimerizes with BARD1 and is part of different complexes[173], its function at damage sites is crucial to control both repair and checkpoint activation[172,174]. Nevertheless, BRCA1 function as an E3 ligase at DSBs has not yet been fully understood, in particular its physiologically relevant targets. A recent study has shown that ectopic expression of H2A fused to ubiquitin rescued the transcriptional de-repression of heterochromatic satellite DNA repeats caused by loss of BRCA1[175]. This study highlights a neat molecular link between this histone mark and the heterochromatic state of these regions, proposing a direct function of its ligase activity for H2A *in vivo*. BRCA1 recruitment at DSBs is linked to the binding to K63 chains at the lesion of the UIM domain of the adaptor RAP80 protein, one of the components of the BRCA1-A complex[70,176-178]. Our data presented in chapter 4 suggest that the presence of K63 chains *per se* at the site of damage is not sufficient for BRCA1 and RAP80 recruitment[24], challenging this model and complicating the picture of the biochemical requirement for BRCA1 recruitment at DSBs.

The recruitment of 53BP1 is even more enigmatic. This protein is not thought to be part of larger complexes and it doesn't contain characterized ubiquitin binding domains, nevertheless its accrual is dependent on the activities of the E3 ligases RNF8/RNF168[24,67-69,71-72]. Because we know that 53BP1 binds to H4K20 methylation at DSBs[179], it has been proposed that ubiquitin-related processes allow the methylation mark to get exposed and induce 53BP1 binding[152,154], but this hypothesis still awaits full validation. Interestingly, a study observed the spatial separation in nuclear DNA damage foci between the ubiquitin-

related players RNF8-RNF168-BRCA1 and γH2AX-MDC1-53BP1[180], the functional meaning of this observation may help in the understanding of the complex set of signals taking place during the early response to DSBs.

Because some of the players of these early events of the DDR to DSBs (e.g. RNF8, BRCA1) have been found to play roles in other DNA repair pathways, such as NER[138,181] and the Fanconi Anemia pathway[182-183] as well as telomere damage signaling[184-185], it seems plausible that these represent a general molecular network not specifically connected only to the response cascade to DSBs but potentially involved in shared aspects of the DDR.

In addition to the RNF8/RNF168-dependent H2A ubiquitination, Polycomb-dependent ubiquitination in response to DSBs is also playing a role[186]. RING1B and BMI1 have been both found to accumulate to sites of damage in different studies[187-194]. To date, the mechanism that triggers the recruitment of these factors at lesions is not understood, although the kinetics of their accumulation resembles the ones of early players in DSB response, such as RNF8[189]. Although multiple PcG proteins are found to be recruited at the site of damage[187], it's still unclear which PRC1 complex is important to function at DSB sites. The presence of PRC1 components at lesions has suggested the possibility of a transcriptional regulation program induced by the DDR to silence genes in proximity of the lesion[195].

Two flavors of H2A ubiquitination

H2A ubiquitination can occur in two different flavors, the monoubiquitination on K119 (H2AK119ub) catalyzed by Polycomb proteins and the K63-linked polyubiquitination on K13-15 (H2AK13-15ub) by RNF168/RNF8 (Figure 2a). The structural differences of these two modifications not only reside in the number of ubiquitin attached to the histone, but also on their relative location within the nucleosome. In fact, K119 of both copies of H2A in the nucleosome lie in proximity of the dyad, while K13-15 are located on the diametrical opposite site of the octamer (Figure 2b).

Interestingly, as described in chapter 4, the finding that RNF168-dependent H2A/X ubiquitination is required for DSB signaling while formation of K63 chains is not sufficient for signaling[24] indicates that K13-15 on H2A are not simply serving as a anchoring site for the formation of K63 chains, but ubiquitin conjugation at this site has important functions *per se*[24].

The location of K13-15 within the nucleosome suggests few hypotheses for its potential roles in the signaling, in fact K13-15 on H2A are closely positioned to the C-terminal K120 on histone H2B tail (Figure 2b). This lysine is also a known target for a specific monoubiquitination (H2BK120ub) by the RING E3 ligase RNF20/RNF40 in humans and BRE1 in yeast[196-198] and the function of H2BK120ub has been widely studied and linked to several effects. First, a neat reconstitution study *in vitro* shows that nucleosome stretches containing ubiquitinated H2B retain a more open arrangement than the unmodified fibers[199]. This effect in opening chromatin regions was confirmed *in vivo* and the specificity for the ubiquitination signal was validated by the substitution of the ubiquitin moiety with the structural homolog Hub1[199]. Interestingly H2BK120ub crosstalks with other histone modifications, in particular it

stimulates the trimethylation of K4 and K79 on histone H3, catalyzed by the COMPASS and the DOT1 methyltransferases respectively[200-203]. These observations are not simply associated to a steric effect, because mono- and dimethylation by these enzymes still can occur in absence of ubiquitination[204] and they appear to be again specific for ubiquitin, and not other ubiquitin-like molecules[199,205]. Finally, it appears that H2BK120ub assists the histone chaperone FACT in facilitating the passage of the transcription machinery[206] in contrast to the inhibitory effect that was observed for H2AK119ub[116].

H2AK13-15ub structurally will closely resemble H2BK120ub and therefore some effects of these modifications may be shared. Nevertheless the observations that H2B ubiquitination occurs in later steps of the DSB signaling[207-208] and RNF168 doesn't target the H2B tail[24] suggest that distinct regulation of these ubiquitination events exists and therefore diverse effects could stem from these two.

K13-15 modification during DNA damage is an early event, the need to rearrange and open chromatin around the site of damage is a likely requirement for the recruitment and assembly of the many large complexes that are involved in the repair steps. In addition, the involvement of active remodeling at DSBs by ATP-dependent enzymes is becoming very apparent[209-210]. The p400/Tip60 complex[211], the NuRD subunit CHD4[212-213], the INO80 complex[214], the SMARCAD1 protein[215-216] and the SNF2H ATPase, whose recruitment at DSBs is mediated by H2BK120ub[208], have all been implicated in the response to DSBs. In relation to some of the remodeling events, histone acetylation seems also to be important[217-218], including acetylation of H4K16 that may correlate with RNF8 activity[219-220]. The potential role of H2AK13-15ub in regulating these events still requires further analysis.

H2AK119ub function at DSBs is still a matter of debate, PcG proteins are recruited at the sites of DSBs, but whether their ubiquitin ligase function is strictly required will need further investigation. Nevertheless, the importance of transcriptional silencing of chromatin regions neighboring DSBs has been proposed by different studies[195,221-223]. One study, which precedes the characterization of H2AK13-15ub, shows that transcriptional inhibition *in cis* occurs when a DSB is induced in proximity of a reporter gene, and this is dependent on H2AK119ub, shown by using H2A K118-119R mutant[195]. It would be interesting to see the effects of a K13-15R mutation in this reporter assay to address the possible differences of the two H2A ubiquitination events.

To further support our hypotheses on the differences in signaling arising from H2AK119ub or H2AK13-15ub is an *in vitro* study that analyzes the effects of nucleosomes reconstituted with H2AK119ub or H2BK120ub, the latter being structurally very similar to H2AK13-15ub, in their cross-talk with DOT1L or PRC2[224]. They show that the presence of H2BK120ub in nucleosomes stimulated methylation of H3K79 by DOT1L, as previously shown, while H2AK119ub did not influence DOT1L activity. In contrast, H2AK119ub inhibited PRC2 methylation of H3K27, but H2BK120ub did not influence PRC2 activity.

Taken together, these observations confirm how the position of monoubiquitination

in the nucleosome affects the specificity and direction of cross-talk with enzymatic activities on chromatin and therefore likely initiate distinct signaling cascades.

Relevance of these studies

Studying the mechanistic details of how the E3s catalyze the ubiquitination of H2A is of crucial importance for our understanding of both Polycomb and DDR signaling. Determining target lysines and mode of interactions of the ubiquitination machinery aid in the definition of the molecular requirements for the functioning of those pathways. Moreover, some of these findings may provide explanations for more general modes of action of this class of enzymes outside these specific cellular pathways.

The complexity of the ubiquitin system has delayed the possibility of exploiting it for targeted therapy. Nevertheless, in recent years a series of drugs have been developed able to target it at different levels[225]. In addition to the recently described E1[226], E2[227],E3[228] and DUB[229-230] inhibitors, the success of the proteasome inhibitor Bortezomib (Velcade®)[231] in cancer therapy well illustrates the importance of this ubiquitin-dependent signaling for the cell. H2A ubiquitination is important for both Polycomb and DDR, and these pathways are crucial for the healthy survival of the cell as their deregulation is associated with several diseases. Understanding the biochemical picture of how H2A is ubiquitinated in these pathways provides new opportunities in thinking and developing new therapeutic strategies to target them in instances of these diseases.

OUTLINE OF THIS THESIS

The work presented in this thesis is aimed at the understanding of the mechanisms by which Polycomb- and DNA damage-associated E3 ligases ubiquitinate H2A.

In **chapter 2** we review the structural aspects of multi-domain RING/Ubox E3 ligases involved in DNA repair. We present an overview of the different role of these ligases in the various DNA repair pathways, including their known substrates and focusing on the structural and mechanistic analysis.

In **chapter 3** we report our data on the *in vitro* reconstitution of the E3 ligase activities present within the PRC1 complex. We show that the different E3 ligase dimers can be purified and retain activity towards H2A in oligonucleosomes. We initiated studies on the effects of post-translational modifications of this E3. Finally, in an effort to reconstitute *in vitro* a more functionally relevant reaction including other members of the PRC1 we show a method to produce four-component complexes from insect cells.

In **chapter 4** we present an extensive analysis of the mechanistic aspects of H2A ubiquitination by RNF168 and RNF8 during DNA damage signaling. With a combination of *in vitro* and *in vivo* data, we clarify the order of activity of these ligases; we show that this modification takes place on a novel site on H2A and H2AX, K13-15. Last, we validate that this mark is essential for the integrity of the DNA Double-strand Break signaling pathway.

In the light of the finding of this novel ubiquitination site on H2A, in **chapter 5** we analyze the diversity in target recognition used by the E3 ligases and DUBs for the two

ubiquitination sites on H2A. Moreover, we investigate the role of the nucleosome in directing and activating the RING E3 ligases targeting H2A.

In **chapter 6** we summarize and highlight the main findings presented in the chapters 2 to 5 and we discuss their implications for future research.

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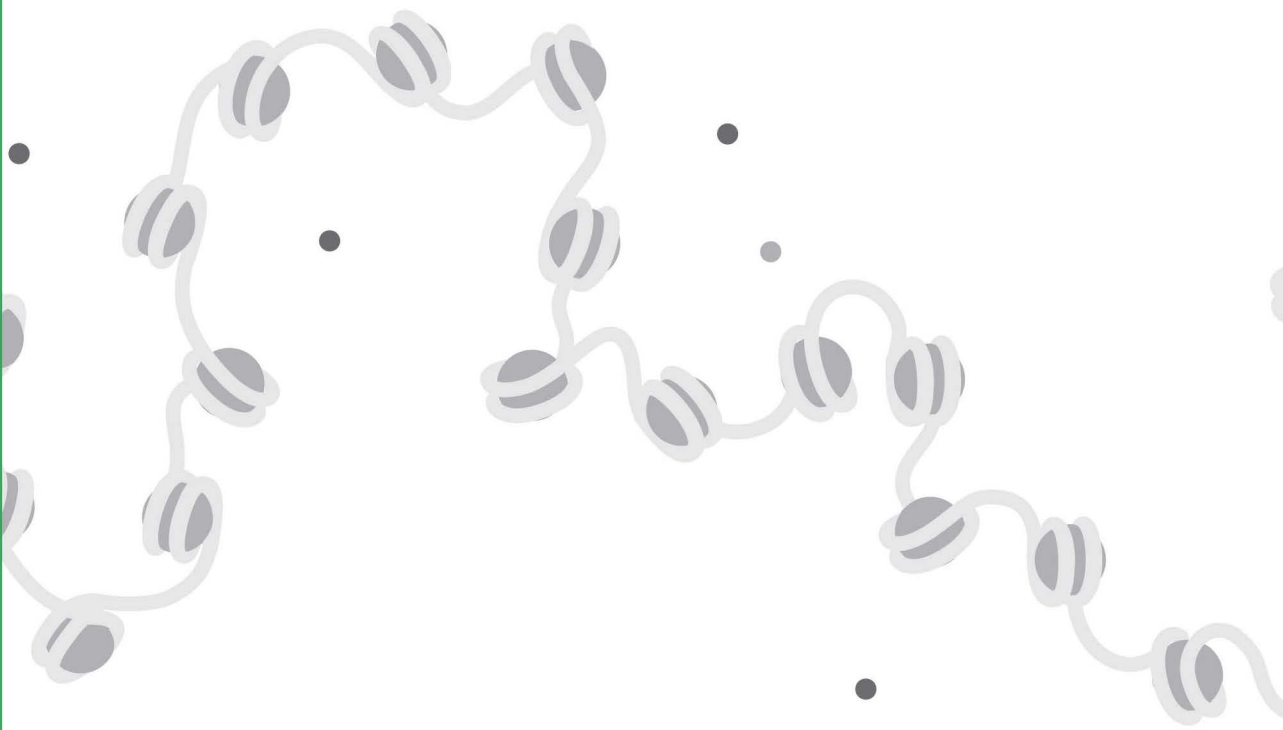

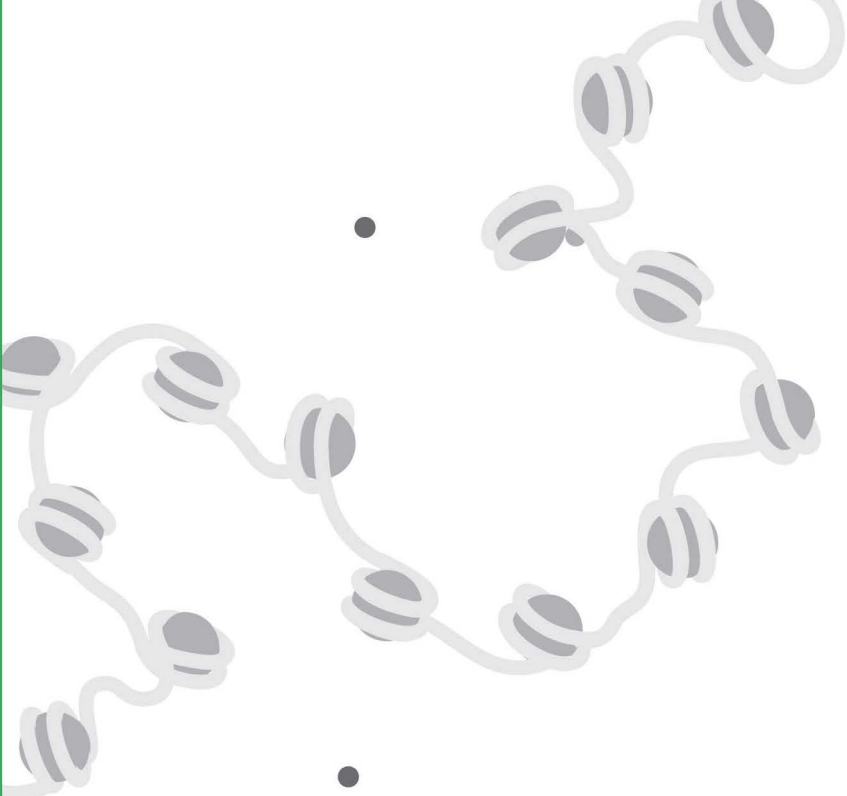

2

STRUCTURAL ASPECTS OF MULTI-DOMAIN RING/UBOX E3 LIGASES IN DNA REPAIR

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ABSTRACT

Ubiquitin conjugation plays critical roles in virtually all DNA repair pathways. This review provides an overview of the known multi-domain RING/Ubox E3 ligases and their domain structures. An analysis of known RING/Ubox X-ray and NMR structures leads to a discussion of the effects of dimerization. Structural and mechanistic data relating to the E3 ligase preferences for E2 interaction and chain-type specificity are reviewed and the role of the E3 ligases in regulation of the repair pathways is discussed.

INTRODUCTION

In many cellular pathways, including DNA repair, the fate and function of proteins are modulated by mono- or polyubiquitination. The basic mechanism for ubiquitin conjugation is well established. After an initial activation by an E1 activating enzyme, the C-terminus of ubiquitin is transferred to an E2 conjugating enzyme. The E3 ligase then catalyzes the transfer of ubiquitin from the E2 to a lysine residue on the target. Lysine residues on the ubiquitin itself can be further ubiquitinated by the same mechanism to form ubiquitin chains. The two major classes of E3 ligases contain the structurally related RING/Ubox domains and the HECT domains. HECT domains directly bind to ubiquitin via a thiolester bond, while RING domains never form a covalent bond to the ubiquitin, but rather act as the docking station for the target and the E2 enzyme.

While in some cases, E2 enzymes play a role in chain-type specificity, the E3 ligases confer the majority of the target recognition to the reaction and are able to productively interact with only a selective subset of existing E2s. These crucial roles of the E3 ligases in determining E2 and substrate specificity underlines their pivotal importance in the regulation of ubiquitin conjugation processes. Nevertheless, the mechanism by which the selectivity is achieved is still a matter of debate. Interestingly, most of the RING/Ubox containing proteins are multi-domain proteins, with the exception of the Cullin–RING Ligases (CRL). In the CRLs a small RING protein, RBX1, is complemented with a larger Cullin and a target specific protein, while in other E3 ligases, the RING/Ubox domain protein contains one or more other domains in the same polypeptide sequence (Figure 1). Since the CRL-type RING domains have been discussed elsewhere[1], we focus in this review on the role of the principal multi-domain E3 ligases in DNA repair, analyzing their structural and mechanistic aspects.

RING/UBOX E3 LIGASES IN DNA REPAIR

In almost all DNA repair pathways multi-domain RING-containing ubiquitin E3 ligases have recently been identified. Often they are components of multi-protein complexes such as FANCL in the Fanconi anemia core complex or P44 in TFIIH. Even when a role for a RING E3 ligase has been identified within a pathway, it can be challenging to identify the target of ubiquitination. For some systems both the target of ubiquitination and the down-stream effect of this modification

are well understood, but in most cases the mechanistic aspects still need to be resolved. First we review the known RING/Ubox E3 ligases in DNA repair and what is known of their role in the process.

Nucleotide excision repair (NER)

The NER repair process is often utilized following exposure to ultraviolet light. In NER a small amount of DNA surrounding the site of damage is removed and replaced. TFIIH is a multi-protein complex required for NER as well as transcription. The core TFIIH is composed of at least five subunits in humans, including the RING protein P44, a homolog of yeast Ssl1[2,3]. The E3 ligase activity of Ssl1 is not required for NER directly, but seems to affect DNA repair through transcriptional activities on genes with roles in DNA damage pathways[4]. The TFIIH core complex interacts with a CDK-activating kinase complex that contains three proteins, including RING protein MAT1 in humans or Tfb3/Rig2 in yeast, but ubiquitin conjugation activity for this component has not yet been studied. In addition, a role for various Cullin/RING complexes, such as DDB2[5,6], CSA[7] and RAD16 complexes[8] has been described in NER, but these are outside the scope of this review.

Base excision repair (BER)

Single bases that have been chemically damaged, for example by alkylation or deamination, are recognized, removed and replaced by the proteins in the BER pathway. While no direct involvement of a RING protein in BER pathways has been described to date, the details of a proteasome-dependent degRADation mechanism of BER proteins are relatively well understood. This involves the Ubox E3 ligase CHIP, which regulates degRADation of XRCC1 and DNA polβ when not involved in actual repair[9]. Newly produced BER proteins are thus constantly degRADed, but only when not in use. This serves as a mechanism to respond quickly to increased levels of damage. Undoubtedly additional E3 ligases will be found that have such a proteasome-dependent role in the various DNA repair pathways and hence indirectly modulate the DNA repair process.

Non-homologous end-joining (NHEJ) and homologous recombination (HR)

NHEJ and HR are the main pathways used to repair DNA double-strand breaks (DSB). The BRCA1/BARD1 E3 ligase complex is essential in DSB repair, playing a crucial role in orchestrating the cross-talk of the DNA repair pathways with different cellular processes (both NHEJ and HR). The precise role of the ubiquitination activity of the breast cancer susceptibility gene, BRCA1, is only slowly being resolved. BRCA1 heterodimerizes with BARD1, and these RING proteins participate in different larger complexes to coordinate their function. Although many targets have been suggested, none has yet explained the role of the ubiquitination events[10,11].

Representative BRCA1-containing complexes include the MRE11-RAD50-NBS1 (MRN) complex together with CtIP[12], which is involved in both HR and NHEJ as well as mediating cell cycle checkpoint signaling. MRE11 carries nuclease activity while RAD50 binds DNA in an ATP-dependent manner and probably holds the two extremities of the DNA close together when DNA repair takes place. The NBS1 protein plays a role in recruitment of kinases and other factors that mediate the signaling to the ATM/ATR-dependent checkpoints.

Another complex with BRCA1 is the BRCA2(FANCD1)-RAD51 complex[13,14], which mediates HR. While the exact function of BRCA2 is not clear, the RAD51 protein binds to single- and double-stranded DNA and exhibits DNA-dependent ATPase activity, playing a role in unwinding DNA.

BRCA1 is also part of the BACH1(FANCJ)-TopBP1 complex[15,16], which regulates the intra-S-phase checkpoint. BACH1 exhibits DNA-dependent ATPase and DNA helicase activity and TopBP1 functions in recruiting and activating components of the checkpoint machinery.

Finally, BRCA1 is a member of the recently characterized ABRA1-RAP80 complex[17-19]. This complex is tightly connected to the ubiquitin pathway, since it also involves the recently discovered RING E3 ligase, RNF8[20-22]. The recruitment of RNF8 to the site of DSB leads to H2A/H2AX polyubiquitination with K63-linked ubiquitin chains, catalyzed by the E2 enzyme Ubc13 and the RING finger of the RNF8 E3 ligase. These chains are recognized by the UIM (Ubiquitin Interacting Motif) domains of RAP80, which functions as an adaptor protein for the binding of ABRA1. ABRA1 in turn recruits the BRCA1/BARD1 ligase in a phosphorylation-dependent manner.

Another RING E3 complex that is proposed to regulate HR is Slx5/Slx8. These proteins are required for genome stability in budding yeast[23] and slx5 and slx8 mutant strains showelevated gross chromosomal rearrangements[24]. RNF4 is the only human homolog for this E3 ligase, and it can substitute the known essential functions of either Slx5 or Slx8[25], implying that itmay function as a homodimer. Interestingly, Slx5/Slx8 and RNF4 are SUMO-targeted ubiquitin ligases, needing a sumoylation event on the substrate before the ubiquitination can take place. The few targets that have been proposed have a role in DNA damage sensing and repair pathways, RAD60[25,26], RAD52[58], Ddc2 and the kinase RAD53[24]. Recently it has been shown that Slx5/Slx8 interacts with Nup84, a component of the nuclear pore complex, and this complex associates with irreparable DSBs that are relocated to the pores for repair[27].

The RAD6 pathway (see PRR, below) also seems to have a role in double-strand break repair. In yeast, the E3 ligase RAD5 functions via the Mre11/RAD50/Xrs2 (MRX) complex and physically interacts with processed double-strand breaks[28]. Ubiquitination controls the pathway of damage repair, with monoubiquitination of PCNA by the E2/E3 enzymes RAD6/RAD18 promoting NHEJ, while subsequent polyubiquitination by Ubc13/Mms2/RAD5 suppresses it in favor of HR[29].

Post-replicative repair (PRR)

When DNA damage prevents complete replication by stalling the replication fork, several PRR pathways exist to allow replication to continue. The central role of ubiquitin conjugation in these DNA damage avoidance pathways during replication is relatively well understood[30,31]. In this pathway, the E2/E3 enzymes RAD6/RAD18monoubiquitinate PCNA on K164 at stalled replication forks[32] in an RPA-dependent manner[33]. This monoubiquitination serves to make a polymerase switch from the replicative polymerase delta to one of the translesion polymerases[34,35]. The translesion polymerases contain a ubiquitin binding domain[36] and a PCNA-interaction motif (PIP), both of which are required for the polymerase

switch, following ubiquitination of PCNA[37]. Interestingly, these mechanisms play a role in immunoglobulin hypermutation to generate diverse immune system responses[38,39]. Monoubiquitination of PCNA at K164 by RAD6/RAD18 leads to recruitment of translesion polymerases, which give rise to the majority of the mutations of template A/T[40].

In PRR, the monoubiquitinated PCNA can be a target for K63- linked ubiquitin chains by a second pair of E2/E3 enzymes. In yeast the E3 ligase is RAD5, with Ubc13/Mms2 as E2 enzyme[41]. The pathway is conserved in mammals, although two different E3 ligases have been described to make K63-linked chains on PCNA; SHPRH and HTLF[42-45]. However, how the K63-linked chains on PCNA cause a switch to homologous recombination is not yet understood in molecular terms[46].

Interstrand cross-link (ICL) repair

The cellular response to ICL was highlighted via a group of proteins that are mutated in Fanconi anemia (FA), a rare genetic disease. Some of these proteins constitute the FANC core complex, which shows E3 ligase activity. The role of this complex in DNA cross-link repair and in HR is intensely studied[47,48]. In this large multi-subunit complex the FANCL protein contains a RING domain and conveys the ubiquitination activity. The target is the FANCD2 protein, which is apparently modified by monoubiquitination. Although the exact function of its modification is not well clarified, there is evidence that monoubiquitinated FANCD2 interacts with the BRCA2 (FANCJ)-RAD51 complex, leading to the recruitment of BRCA1[49]. Monoubiquitinated FANCD2 is targeted to chromatin, and localization was retained when monoubiquitination was substituted with a C-terminal fusion of ubiquitin in DT40 cells. When the Ile 44 in the interaction surface of the fused ubiquitin was mutated, the localization was lost, implying that recognition of the monoubiquitin by a ubiquitin binding domain is likely to be central in the process[50]. A link with the RAD6 pathway (PRR) has been established, where RAD6 indirectly regulates FANCD2 monoubiquitination[51]. All these data suggest multiple roles for the FANCD2 molecule that still need to be further elucidated. Interestingly, monoubiquitination of another protein, FANCI, a paralog of FANCD2, is also required for correct DNA repair activation. The E3 ligase for this modification of FANCI is not yet known, but there is an intriguing inter-dependence between FANCI and FANCD2 monoubiquitination events, suggesting that a dual ubiquitination mechanism regulates the DNA repair machinery[52].

The Ubox protein Pso4/Prp19 also plays a role in interstrand cross-link repair, functioning via the WRN helicase that is mutated in Werner syndrome[53]. It is also required for effective DSB repair[54], though its function as an E3 ligase has not yet been established in either of these pathways.

RECONSTITUTION OF E3 LIGASES IN DNA REPAIR *IN VITRO*

The different E3 ligases have widely variable domain structures, in which the RING or Ubox can be surrounded by many distinct domains. In Figure 1 the sizes and domain structures of the different RING/Ubox E3 ligases are displayed. In addition, these multi-domain proteins usually exist in higher order complexes.

In practice these proteins are biochemically not very tractable. *In vitro*

analysis of their activities, their structures and their mechanistic properties has mostly been carried out on individual domains, such as the RING/Ubox domains or their dimers. Some proteins, including CHIP[55], RAD18[56,57] yeast Slx5/Slx8[58-60], its mammalian homolog RNF4[61] and the BRCA1/BARD1 complex[62], have been expressed recombinantly as full-length proteins. For others only RING-domains or slightly longer versions are available *in vitro*.

Various ubiquitination reactions have been reconstituted *in vitro*, using recombinantly expressed proteins. In these assays ubiquitin, E1, E2 and E3 proteins in the presence of ubiquitin and Mg^{2+}/ATP are followed over time. *In vitro* ubiquitination assays have been reported for RAD18 against its target PCNA[57,63,64], for yeast Hex3/Slx8 on RAD52[58] and for RNF4 on PML[65]. In most other cases a target is either not known or not sufficiently expressed *in vitro*. Therefore ubiquitination activity is traced by studying the formation of unanchored ubiquitin chains or the autocatalytic modification of the E3 enzyme.

Crystal and NMR structures of RING/Ubox E3 ligases

Crystal and NMR structures of RING/Ubox domains of a number of E3 ligases in DNA repair have been solved (Table 1). The

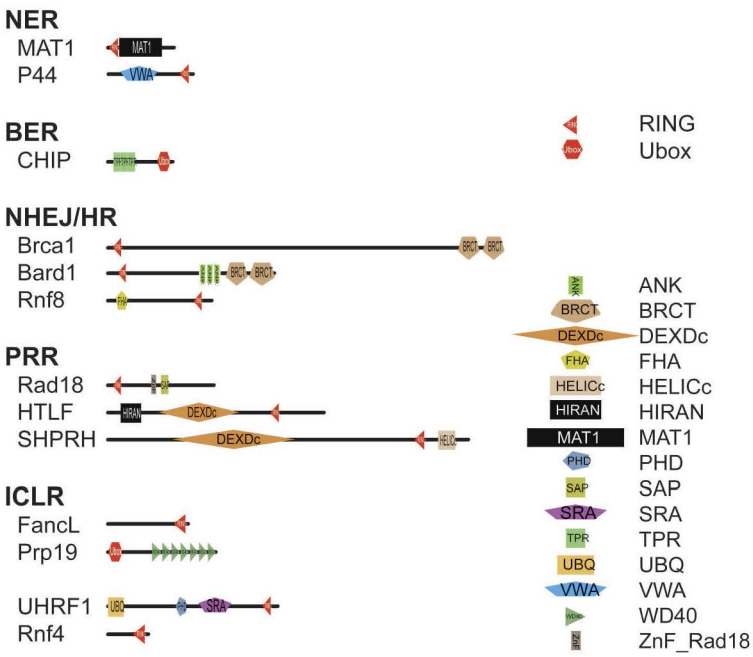

Figure 1 Domain structures of RING-containing multi-domain E3 ligases in DNA repair. Human proteins are drawn to scale and sorted according to the DNA repair pathways in the text. Domain architecture was retrieved for each protein using Uniprot (www.uniprot.org/), Ensembl (www.ensembl.org) and SMART (http://smart.embl-heidelberg.de), with SMART abbreviations used for domain names, except that the C4-RING domain in P44 (Ssl1) is shown as RING domain. RING and Ubox domains are highlighted in red.

Table 1 Known structural data on multi-domain E3 ligases in DNA repair.

Protein	Domain	PDB Entry	Method	Resolution (Å)	Chain	Positions
BRCA1	RING	1JM7	NMR	-	A	1-112
	Brct	1JNX	X-ray	2.50	X	1646-1859
	Brct	1N5O	X-ray	2.80	X	1646-1859
	Brct	1OQA	NMR	-	A	1755-1863
	Brct	1T15	X-ray	1.85	A	1646-1859
	Brct	1T29	X-ray	2.30	A	1646-1859
	Brct	1T2U	X-ray	2.80	A	1646-1859
	Brct	1T2V	X-ray	3.30	A/B/C/D/E	1646-1859
	Brct	1Y98	X-ray	2.50	A	1646-1859
	Brct	2ING	X-ray	3.60	X	1649-1859
	Brct	3COJ	X-ray	3.21	A-G	1648-1859
BARD1	RING	1JM7	NMR	-	B	26-142
	Brct	2NTE	X-ray	1.90	A/B	568-777
	Brct	2R1Z	X-ray	2.10	A/B	569-777
	Brct	3C5R	X-ray	2.00	A/B	425-555
RNF4	RING	2EA6	NMR		A	122-183
RNF8	FHA	2CSW	NMR	-	A	8-139
	FHA	2PIE	X-ray	1.35	A	13-146
CHIP	All	2C2L	X-ray	3.30	A/B/C/D	24-304
	Ubox	2C2V	X-ray	2.90	S/T/U/V	227-304
	Ubox	2OXQ	X-ray	2.90	C,D	207-278
	Ubox	2F42	X-ray	2.50	A	127-282
MAT1	RING	1G25	NMR	-	A	1-65
P44	C4-RING	1Z60	NMR	-	A	328-386
RING1b	RING	2CKL	X-ray	2.00	B	1-159
	RING	2H0D	X-ray	2.50	B	15-114
BMI1	RING	2CKL	X-ray	2.00	A	1-108
	RING	2H0D	X-ray	2.50	A	5-101
UHRF1(NP95)	Ub-like	2FAZ	X-ray	2.00	A/B	1-76
	Tudor domains	3BD3	X-ray	2.40	A	126-285
	Tudor domains	3BD4	X-ray	2.40	A	126-285
	SRA	2PB7	X-ray	1.90	A	405-643
	SRA	3BI7	X-ray	1.70	A	413-617
	SRA	3CLZ	X-ray	2.20	A/B/C/D	413-617
	SRA	2ZKD	X-ray	1.60	A/B	404-613
	SRA	2ZKE	X-ray	2.60	A	404-613
	SRA	2ZKF	X-ray	2.55	A	404-613
	SRA	2ZKG	X-ray	1.77	A/B/C/D	404-613
	SRA	2ZO0	X-ray	2.19	B	419-628
	SRA	2ZO1	X-ray	1.96	B	419-628
	SRA	2ZO2	X-ray	3.09	B	419-628
UHRF2(NP95)	Ub-like	1WY8	NMR	-	A	1-76
	RING	1Z6U	X-ray	2.10	A/B	672-802
	PHD	2E6S	NMR	-	A	326-395
Yeast Prp19	Ubox	2BAY	Xray	1.50	A/B/C/D/E/F	1-56
	Ubox	1N87	NMR	-	A	1-56

typical RING/Ubox domain is characterized by a cysteine- and histidine rich sequence motif of around 40 amino acids. While for RING domains this sequence forms the binding site for two zinc ions, in Ubox motifs this is not the case, although the overall folding of the polypeptide is highly similar. The RING fingers display the so-called "cross-brace" arrangement for the zinc ion coordination in several variations of cysteine and histidine ligands, named, *e.g.* C3HC4, RING-H2, C4C4 and C4H4-RING domains.

The RING/Ubox-containing proteins fragments and complexes that have been elucidated to date are summarized in Figure 2. The RING/Ubox domains exist as either monomers or dimers. Monomer structures include the NMR structure of the RING domain of human MAT1 (a homolog of Tfb3/Rig2) in TFIIH, which structurally resembles most other RING domains[66], monomeric Prp19[67] and RNF4. In addition the C4C4 domain of P44 (Ssl1) has been solved by NMR and resembles the c-NOT4 C4H4 structure[68]. Since the P44 and RNF4 structures were solved by structural genomics consortia, these structures are available in the database, but have not been analyzed in the literature.

More complex dimeric structures of multi-domain RING proteins include the hetero- and homodimers of the RING/Ubox domains of BRCA1/BARD1[69], Prp19[70], CHIP[55,71] (Figure 2). Figure 3 elucidates the position of the RING/Ubox in some of these multi-domain structures.

There are two additional structures that we feel are useful for comparative purposes, those of UHRF1 and a part of the polycomb repressive complex1 (PRC1), RING1B/BMI1. The first of these, the NP95-like protein ICBP90/UHRF1, functions primarily in epigenetic control, maintaining methylation via interaction with DNMT1, which methylates newly synthesized DNA during S phase[72,73]. Recent structural studies have highlighted the role of the SPA domain of UHRF1 in recognizing hemimethylated DNA[74-76]. UHRF1 also seems to promote resistance to many DNA damaging agents, which affect multiple damage response pathways[77], and an interaction with the EME1/MUS81 endonuclease complex requires a functional RING domain[78]. The RING domain of the closely related UHRF2 has been crystallized, also by a structural genomics consortium (Figure 2f).

The polycomb proteins RING1B/BMI1 are part of a larger PRC1 complex and contain E3 ligase activity for Histone 2A. The complex functions primarily in repression of transcription[79], but has recently been implicated in NER pathways[80]. The structure of the RING domains has been solved[81,82] and provides an interesting structural and biochemical template as a RING–RING heterodimer with a known target.

Only for the Ubox protein CHIP more detailed structures are available. An almost full-length crystal structure of the CHIP homodimer has been determined, lacking only the extreme N-terminus. This shows the arrangement of the TPR domains with respect to the Ubox homodimer (Figure 2i and 3c)[55]. In addition, two separate crystal structures have been resolved of the complex between an E2 enzyme and the Ubox homodimer of CHIP (Figure 2j and k)[55,71].

Dimerization of RING/Ubox proteins

Many E3 ligases dimerize (for a recent review see[3]), but the arrangement of the monomers within the dimer can vary. The

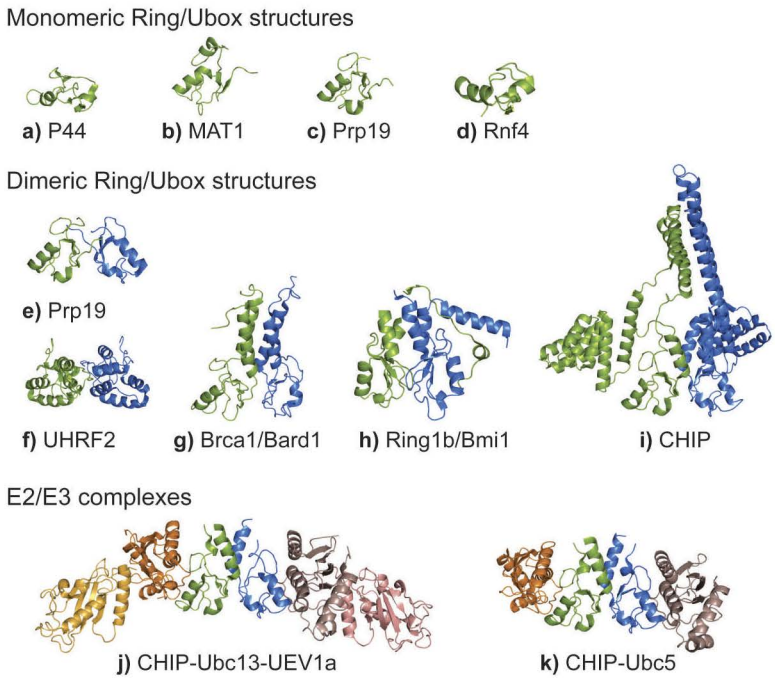

Figure 2 Gallery of RING and Ubox NMR and crystal structures from DNA repair proteins with coordinate files with the following PDB codes. a) 1Z60[68] **b**) 1G25[66] **c**) 1N87[67] **d**) 2EA6 **e**) 2BAY[70] **f**) 1Z6U **g**) 1JM7[69] **h**) 2CKL[81] **i**) 2C2L[55] j) 2C2V[55] **k**) 2OXQ[71]. See Table 1 for details.

available structures show the importance of both N- and C-terminal flanking regions of the RING/Ubox domain in stabilizing the dimer (Figure 3a and b).

Several of the RING/Ubox proteins withDNA repair-related functions resolved so far, such as the BRCA1/BARD1 heterodimer, the CHIP homodimer and the Prp19 homodimer, form similar dimers. Figure 2e–i shows that the orientation of the RING/Ubox domains is similar when the dimers are superimposed. These RING/Ubox domains form a specific subclass of RING/Ubox dimers, with a conserved arrangement of RING domains that can be distinguished from other RING/Ubox dimers such as MDM2/MDMX, or the RAG1 homodimer[83]. In CHIP and BRCA1/BARD1 the N- and C-terminal RING-flanking regions forming the dimer interface make a 4-helical bundle. Figure 3a and b shows how this helical bundle forms the dimerization interface for the RING domains of BRCA1/BARD1 and for the isolated Ubox domains and full length CHIP. A crystal structure of Prp19 shows a dimer with the same arrangement, despite the absence of the flanking helices. Here the dimer is stabilized by a pair of hydrogen bonds to a backbone amide in a beta-hairpin as well as hydrogen bonds between residues close to the N- and C-termini of the fragment.

The crystallographic homodimer formed by the NP95-like RING domain has a different arrangement of the monomers, with a two-fold axis that is orthogonal to

the two-fold in the other structures. The NMR structures of MAT1 and RNF4 are not dimeric, but it is possible that the choice of construct for NMR studies would not allow such dimerization, since possible flanking regions were not included. For RAD18 and RAD5 no structure is known, but RAD18 homodimerizes in solution[41,57,84,85], and the dimerization in RAD18 has been mapped to the RING domain[57] (RGH and TKS, unpublished results).

Asymmetry in dimers

In principle a RING or Ubox dimer contains two RING units that each could interact with an E2 protein. Surprisingly, this does not always happen. In the heterodimeric BRCA1/BARD1 dimer NMR analysis

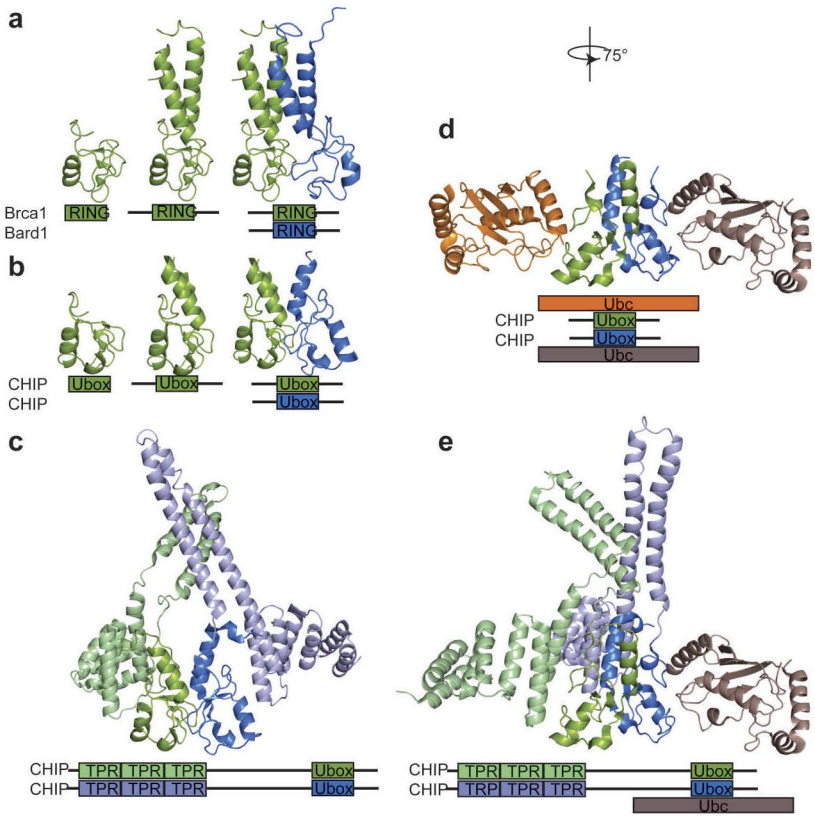

Figure 3 Three-dimensional arrangement of RING dimers. Dimerization and E2 interaction of RING and Ubox domains. **a)** BRCA1 RING domain, RING domain with flanking helices and BRCA1/BARD1 heterodimer. **b)** CHIP Ubox domain, Ubox domain with flanking helices and Ubox homodimer. **c)** An almost full-length construct of CHIP shows an asymmetric homodimer. **d)** The isolated CHIP Ubox homodimer interacts with two molecules of E2. **e)** Model showing full-length CHIP homodimers can only interact with a single UbcH5, owing to the asymmetry in the full-length structure. The following coordinate files were used in the representation: BRCA1/BARD1 1JM7; CHIP with TPR repeats, 2C2L; CHIP/Ubc5 2OXQ.

showed that only the BRCA1 subunit interacts with E2 and not BARD1[86]. An extensive analysis of all known E2 molecules did not reveal a single E2 that interacts with BARD1[87]. Mutational analysis indicates that this may also be true for RING1B/BMI1[81], since only mutations in the E2 interaction site of RING1B and not BMI1 altered the activity of thecomplex for histone 2A modification[81]. Similarly, when Slx5 and Slx8 were studied, only Slx8 showed activity, but this was enhanced by the presence of Slx5[58].

Even more surprising is the asymmetry in the homodimeric CHIP protein. In this homodimer, the crystal structure revealed that only one of the two Ubox domains is used for E2 binding[55], presumably because the other RING domain is rendered inaccessible by the TPR repeats (Figure 3e). Apparently this is not due to crystal contacts, since isothermal calorimetry analysis showed that in fact only one E2 molecule binds per CHIP dimer. There is no intrinsic difference in the Ubox regions, because in the absence of the TPR domains, when only the Ubox domain is cocrystallized with the E2, each Ubox in the homodimer binds an E2 (Figure 3d). Thus, the asymmetry is entirely due to the N-terminal domains (Figure 3e)[55].

It will be of great interest to identify the stoichiometry of other RING and Ubox dimers with their respective E2 enzymes. Thus it will be of interestwhether both Ring domains in the RAD18 homodimer are active. Also, it is conceivable that the observed inactivity of the BARD1 or BMI1 E3 ligase is due to the lack of the correct E2 and target. Along similar lines, it could be imagined that some kind of regulatory process changes the CHIP conformation, such that both Ubox domains become accessible for an E2. This would be an effective dose increase that would provide a very rapid response to an external challenge.

E2 INTERACTION

E2/E3 complex structures

Structures of E2/E3 complexes remain relatively scarce. An early complex of UbcH7 with c-Cbl[88] has long served as a model for other complexes, together with an NMR analysis of the UbcH5c c-NOT4 complex[89]. The recent structure solution of the CHIP Ubox domain with two different E2 enzymes, UbcH5a[71] and Ubc13/Uev1a[55] has validated the use of these models and shown that indeed the primary E2/E3 contacts are highly conserved (Figure 4).

Moreover, the structure of the c-Cbl/UbcH7 complex shows that a second region on the E3 ligase is involved in close contacts with the E2; the linker helix between the RING domain and the TKB domain of c-Cbl hydrogen bonds with the helix α1 of UbcH7[88]. There is no second interface in the E2 complexes of the CHIP complexes, but it may be missing from the CHIP Ubox fragment that was used for these crystallizations. However, a second E2 interface is found in other E3 ligases; for instance, in RAD18 the contacts between the E2 and E3 are located both in the RING domain as well as in a more C-terminal region[56,57]. A better understanding of the differences/similarities in this second interface might underline new aspects of selective E2/E3 recognition.

E2 preference

Different E3 ligases show activity with only a subset of E2 conjugating enzymes. In the DNA repair pathways, there is a high degree of specificity between RAD18 and RAD6,

and between RAD5 and Ubc13. Similarly, only Ube2T has been found to interact with FANCL[90], but no details of the interaction have been described. Prp19 is active *in vitro* with Ubc3 but was inactive with seven other E2 ubiquitin conjugating enzymes tested, including UbcH5c and UbcH6[91], both of which are relatively promiscuous[92]. Another group of E3 ligases, including BRCA1[87], CHIP[55,93,94] and RNF8[95,96], can interact with several different E2 enzymes. Furthermore, there issomeindication of an overlap in the E2s recognized by RNF8 and BRCA1, since both bind to Ube2e2 and Ubc13, but further analysis is required to assess the importance of these interactions *in vivo*.

The detail of this specificity between E2 and E3 is presumably defined by the E2/E3 interaction surface and in the substrate specificity between them. Interestingly, not every E2 that binds to an E3 shows activity. UbcH7 is not active with BRCA1[86], and E2-25K (UbcH1) is not active with RING1B[81], despite the fact that these E2s bind very well to the RING domains.

The crystal structures of the CHIP Ubox domain reveal that the helix and loop L1 are primarily involved in the E2 contacts (Figure 4). On loop L1 several studies[55,71] have established the importance of an isoleucine residue in ubiquitination activity of the ligase. Mutation of this residue in BRCA1 resulted in loss of E3 ligase activity[86], and the same is true for RING1B[81] and CHIP[55,71]. On the E2 side of the complex, the main contacts are given by loop L4 and loop L7. The CHIP complexes confirmed the importance of the sequence of three amino acids, SPA, on loop L7 of the E2 in contacting the E3. Although the overall binding mode is the same in the two crystal structures of CHIP in complex with UbcH5a and Ubc13/Uev1a, there are small differences (Figure 4). For example, the orientation of D229 differs between the two structures, forming a salt-bridge with R14 of Ubc13, which overlays with D12 of Ubc5. M64 in Ubc13 superimposes on F62 of Ubc5, but mutational analysis showed that F62 is important for activity of Ubc5 with CHIP, whereas M64 in Ubc13 is not[71]. These subtle differences lead to a difference in affinity, and they could be important for regulating the processivity of the ubiquitination.

Systematic analysis of the E2 interactions with BRCA1 using a 2-hybrid approach showed a subset of 8 E2s that could interact with the BRCA1/BARD1 RING complex[87]. All of these contain an alanine in the interacting L7 loop, and mutation of this residue leads to loss of activity. Interestingly, all but two of these E2s contain a serine and a proline preceding this residue, thus forming a SPA sequence[71]. Those two that do not contain the serine and proline are inactive in ubiquitin conjugation, again underlining the relevance of these tripeptides in effective E2/E3 interaction. In RAD6 the sequence in this region is SPT rather than SPA. This difference may contribute to the high degree of specificity of RAD18 for RAD6.

Chain-type specificity

E3 ligases can either be specific or promiscuous with respect to the type of ubiquitin modification that they produce. Some E3 ligases preferentially create monoubiquitination at one or multiple sites, while others have a prevalence for chain formation. In the PRR damage avoidance pathways, the model is simple: the monoubiquitination of PCNA occurs by the E2/E3 pair RAD6/RAD18, and this modification is a prerequisite for subsequent

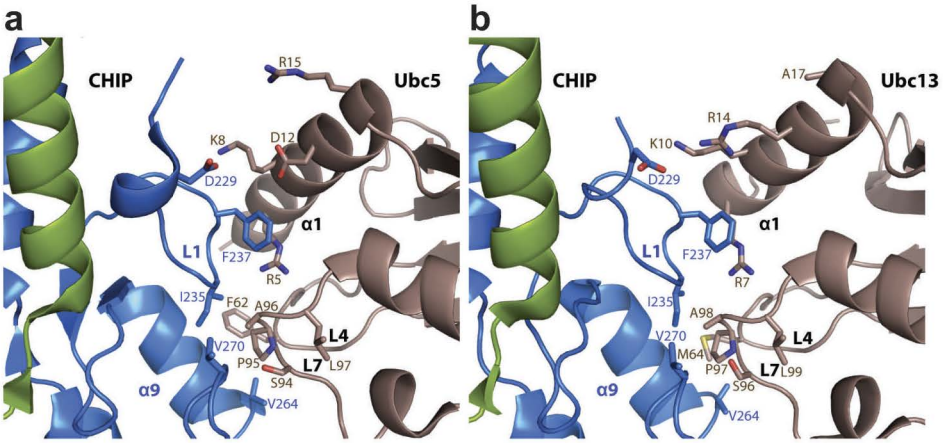

Figure 4 Interaction between E2 and RING/Ubox domains. **a)** Interaction of the CHIP Ubox with UbcH5. **b)** Interaction of the CHIP Ubox with Ubc13. An isoleucine in Loop 1 is a highly conserved at the E2 interaction site on RING/Ubox domains, while an SPA motif in Loop7 of the E2 contributes to the E2 selectivity of the E3. Additional contacts are found between a helix outside the RING domain of c-Cbl and residues in the C-terminus of helix-1 of UbcH7 in the c-Cbl-UbcH7 structure, resulting in a larger buried surface area than these complexes. Only side-chain atoms of selected residues are shown, and numbering is according to the coordinate files 2OXQ (**a**) and 2C2V (**b**). Numbering is according to human proteins.

K63-linked chain formation by the Ubc13/Mms2/RAD5 E2/E3 combination[32]. However, this simple picture may not always be followed.

It is becoming clear that a single E3 ligase can vary its chain-type specificity depending on the E2. Recently it even became clear that atypical ubiquitin chains may have biological relevance[97]. Both BRCA1[98,99] and CHIP[93] can make chains with variable chain specificity with UbcH5 isoforms. For instance, it is clear that BRCA1 is able to make K6 ubiquitin chains[98,99], but also K48 and K29 chains[87]. However, in the presence of Ubc13/Mms2 both BRCA1 and CHIP can also make K63 chains[55,87,100].

Biochemically, Ubc13-dependent chain formation by BRCA1 only occurs in the presence of an E2 that positions the first monoubiquitin, Ube2w, similar to the two-step model for PCNA modification[87]. This mechanism does not seem to be required for CHIP, however, which can form unanchored K63 chains in a single step. RNF8 seems to have the K63 chain formation ability as seen in RAD5. K63 chain formation is relatively well understood, partially due to the role of Ubc13/Mms2 in selecting only the target K63 for conjugation[101]. This chain type is relatively frequently described in DNA damage pathways, and indeed a role for Ubc13 in DSB repair through HR became clear from Ubc13$^{-/-}$ DT40 cells; Ubc13-deficient cells are largely unable to repair DNA damage caused by IR, UV, H2O2, DNA cross-linking and DSB-causing chemicals[100].

FANCL in the Fanconi anemia complex is primarily known to cause monoubiquitination of its known substrate

FANCD2 using Ube2T as E2. However, it remains to be resolved whether this ligase could polyubiquitinate other substrates, possibly in the presence of other E2s.

REGULATION OF UBIQUITIN CONJUGATION

Considering that E3 ligases perform a crucial role in DNA repair, it is important to understand how their function is regulated. In general the initiation of repair pathways seems to be regulated by the DNA damage response, which activates the ATR and ATM kinases[102]. For the most part, ATM functions at DNA double-strand breaks, while ATR functions at a far more diverse range of DNA damage sites[103,104]. These kinases phosphorylate target proteins, leading to their recruitment to sites of DNA damage.

Such phosphorylation-dependent activation could follow different regulatory mechanisms. Primary activation could be achieved by phosphorylation of the E3 ligase: this could directly activate the enzyme, but it could also lead to recruitment to the site of damage. Another option is the selective phosphorylation of an adaptor protein that will then interact with the E3 ligase and will either activate it or target its activity towards specific substrates. RNF8 activity is regulated by phosphorylation in this manner. Upon DSB induction, the ATM kinase activates a cascade of phosphorylation events, which brings phosphorylated MDC1 into proximity of the damage site via direct binding to γH2AX. Phosphorylated MDC1 is recognized by the N-terminal FHA (Fork-head Associated) domain of RNF8, which allows the recruitment of RNF8 to the site of damage[22,93,105]. This localization will probably induce the E3 ligase activity of RNF8 towards the close target H2A/H2AX. Similarly, the E3 ligase activity of FANCL on FANCD2 requires functional ATR, although the relevant mechanism remains to be elucidated[106].

The activation of BRCA1 in different functions seems to be orchestrated by the ability of the two BRCT domains, located at the C-terminus of the protein, to bind phosphorylated substrates. Once phosphorylated, CtIP, BACH1 and ABRA1 can mutually exclusively bind to BRCA1, leading to the recruitment of the different complexes and putatively to different target specificity for the BRCA1/BARD1 E3 ligase. However, none of the suggested targets of the BRCA1/BARD1 dimer are present in these complexes. Furthermore, the E3 ligase activity of BRCA1/BARD1 dimer *in vitro* is enhanced by autoubiquitination[62], but the mechanism of such stimulation still needs to be analyzed.

The function of RNF4 may depend on phosphorylation at different stages of the pathway. Studies in yeast showed that nuclear relocation of unrepairable DSBs required functional homologs of ATR/ATM upstream of the interaction with Slx5/Slx8[27]. Human RNF4 targets polySUMO-modified proteins for ubiquitin-mediated proteolysis. RNF4 has multiple SUMO interaction motifs at its N-terminus which interact with SUMO chains to recruit the C-terminal active RING domain towards the target[65].

An alternative mechanism of recruitment of an E3 ligase to a site of damage involves interaction with Replication Protein A (RPA)-coated single-stranded DNA. RAD18-dependent ubiquitination of PCNA requires the presence of RPA but is independent of the S phase checkpoint. RAD18 physically interacts with RPA,

providing a mechanism for recruitment of RAD18 to stalled replication forks[33]. Interestingly, recent studies showed that the E3 ligase FANCL is also associated with replication forks in an RPA-dependent manner[107], but the role of FANC proteins in this pathway is still unclear.

Activation of the E3 ligase may also require a direct interaction with the DNA. RAD18 is a single-stranded DNA binding protein[56] that recognizes the DNA via its SAP domain[57]. A number of mutants that interfere with this have been described. Mutants at either end of an interhelical loop show reduced affinity *in vitro*, with those around G269 showing the biggest effect[57]. The effect of SAP domain mutants on DNA binding was confirmed recently[84], but mutants within the helices had a greater effect. These leucine to proline point mutants, however, were in the core of the domain and therefore likely to act indirectly by destabilizing or unfolding the domain. Mutants close to G269 in the SAP domain of RAD18 render yeast strains more sensitive to DNA damaging agents[33], presumably highlighting the significance of the DNA interaction in activating RAD18, but the mechanism of the activation remains to be established. The interaction with single-stranded DNA and with RPA could both be required for recruitment of the RAD18 to sites of damage. PCNA has been shown to be more readily ubiquitinated when loaded onto DNA, but the role of the DNA binding properties of RAD18 in this process have not been established[63].

Further regulation of ubiquitination can also take place by the deubiquitinating (DUB) enzymes, which are gRADually emerging as important modulators of these pathways. Thus, the presence a DUB (BRCC36) in the ABRA1-RAP80 complex could be crucial for RNF8-mediated signaling and recruitment of BRCA1[18,21]. A better understanding of the functions of these enzymes in DNA repair will contribute to unraveling the network of ubiquitination events that regulates these important pathways for correct genome maintenance and cell fate.

CONCLUSION

This review describes the different roles for multi-domain RING/Ubox-type E3 ligases in DNA repair pathways. So far a proteasome-dependent regulation of the DNA damage response has been clearly resolved in the base excision repair pathway, where CHIP regulates the levels of repair proteins. It is likely that such regulation is also present in other DNA repair pathways.

Other RING/Ubox ligases seem to have more specific roles, often involving K63 chains or monoubiquitination. The best-described pathways are the modification of PCNA and H2A with monoubiquitin and K63-linked chains. Both the monoubiquitination of PCNA and the K63 chains on H2A seem to result in the recruitment of other factors in the repair process. Therefore the role of ubiquitin in recruitment of other players in the pathway seems to be important in DNA repair.

There seems to be a predominance of K63 chains in the DNA repair pathways from the available data. This could be real, but it is possible that other chains are equally or even more important, since at this time it is simply easier to study K63 chains. It would be of great interest, for instance, to find suitable systems to study the role of the K6 chains in the BRCA1-dependent pathways. It will therefore be a

challenge to find out how the other chain types play a role in DNA repair.

Structures of the RING domains show that they often form homo- or heterodimers, where the flanking regions determine the interaction. The asymmetry of these dimers is intriguing and the necessity to form dimers where only one RING domain seems to be active is curious and not yet understood. The challenge will be to express and purify longer versions of these multi-domain RING proteins and resolve their structure, in isolation or in the context of their repair complexes.

Similarly, the specificity for the different E2 enzymes is still underdetermined in the different repair pathways. More attention to the E2/E3 interaction and the possibility for switching between specific E2 enzymes will be important to resolve the different roles that a single E3 ligase can play, either within one or between different DNA repair pathways.

In the past few years the understanding of the details of ubiquitin conjugation in DNA repair has made great strides. Nevertheless, it is also clear that many questions still exist about the role and regulation of ubiquitin conjugation in DNA repair.

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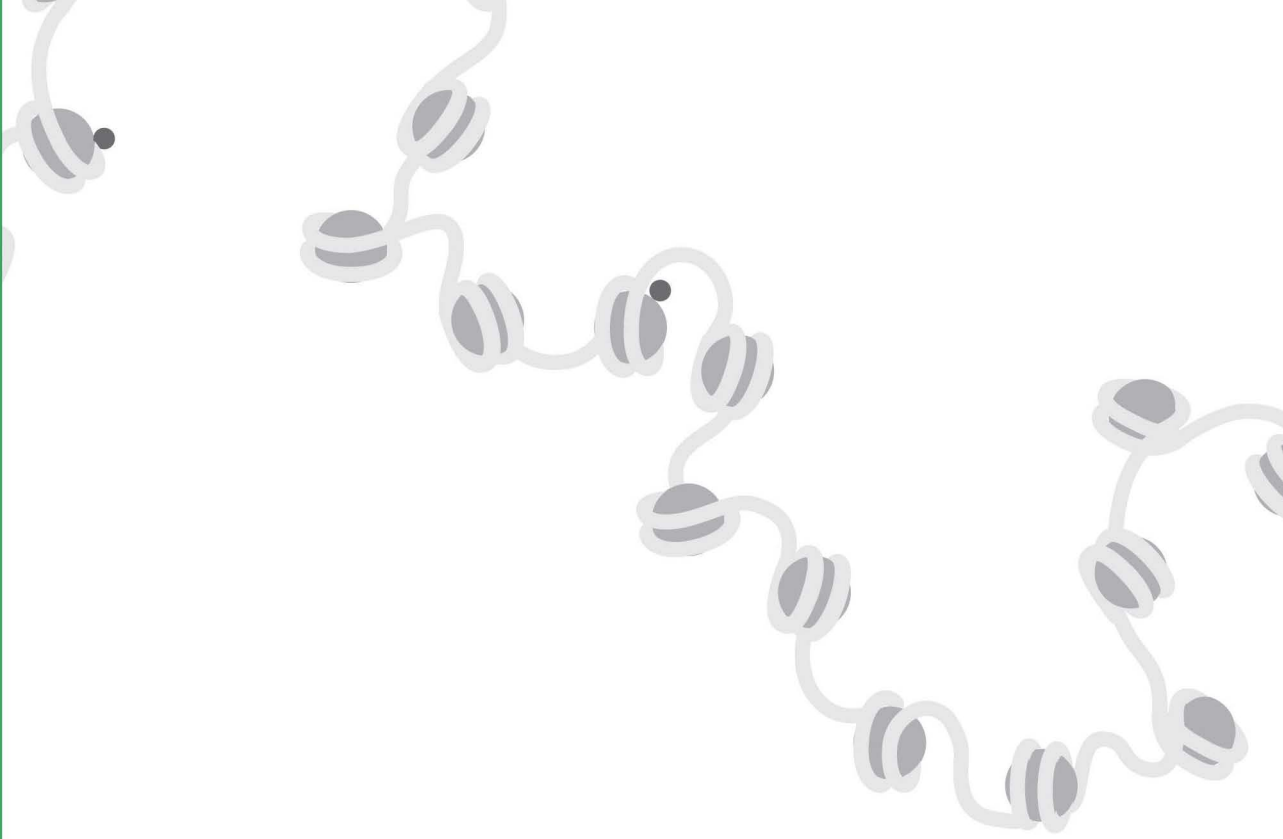

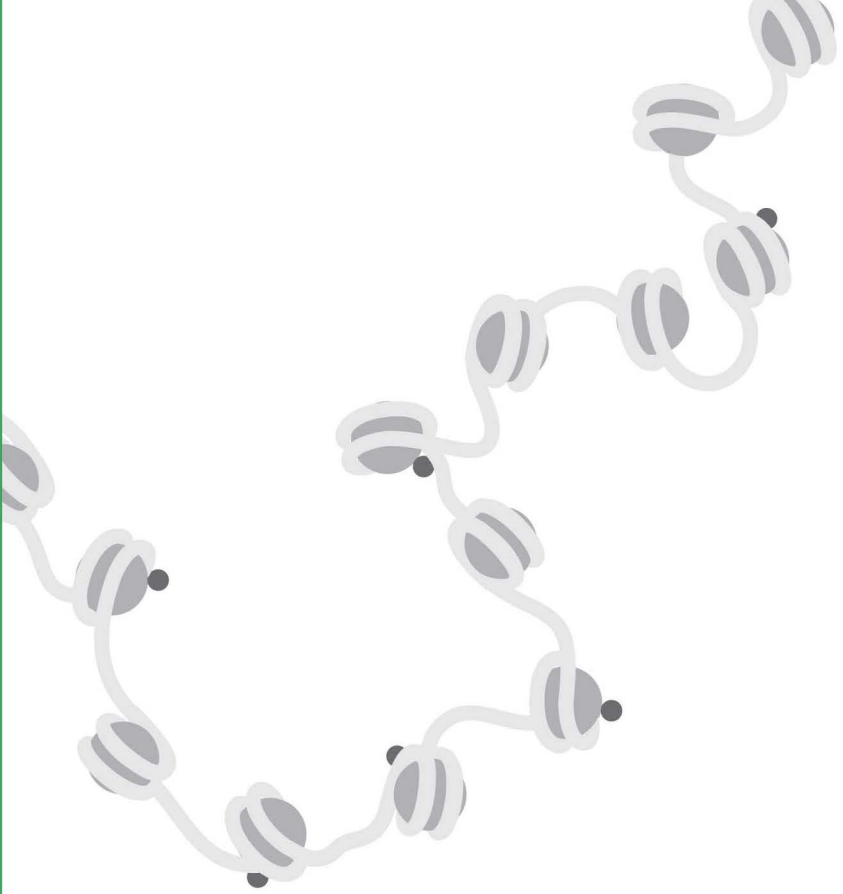

3

BIOCHEMICAL CHARACTERIZATION OF H2A UBIQUITINATION BY THE POLYCOMB REPRESSIVE COMPLEX 1 (PRC1)

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ABSTRACT

The Polycomb Repressive Complex 1 (PRC1) is a large multi-subunit complex involved in transcriptional regulation of crucial genes involved in development and cell homeostasis. PRC1 contains a ubiquitin E3 ligase activity given by its RING domain containing subunits. These E3s specifically target K119 on histone H2A for monoubiquitination. To study the biochemical and functional details of the E3 ligase activity of PRC1, here we present a characterization of the E3 dimers activity composed of Ring1B or Ring1A and Bmi1 or Mel18. Additionally we present a strategy for production of a four-component PRC1 core complex in insect cells that includes the ligases and additional polycomb proteins. These results represent a basis for structural and biochemical studies to achieve understanding of the E3 ligase activity of PRC1.

INTRODUCTION

Polycomb group (PcG) proteins were first identified in *Drosophila melanogaster* for their role in transcriptional regulation of Hox genes during development. In the past decades, PcG proteins have been described in several organisms ranging from plants to human and a large number of additional target genes have been identified. These studies have highlighted the crucial importance of PcG proteins in epigenetic programming of gene expression during multicellular development, stem cell biology and tumorigenesis[1].

PcG proteins are known to form multimeric complexes, where Polycomb repressive complex 1 (PRC1) and PRC2 are the best characterized[2-3]. In *Drosophila*, these complexes are composed of a number of distinct single proteins[4-6]. In humans, these have evolved to families of paralogs proteins as product of gene duplication (Figure 1a). Recent work has pointed out that there are multiple versions of PRC1 or PRC2 in cells with likely different functions[7-13]. Each version is composed of a different combination of paralog proteins from the different families, which in most cases are mutually exclusive in each individual complex.

PRC1 and PRC2 are known as chromatin modifiers involved in transcriptional repression. PRC2 is involved in the initial phase of the repression by methylating K27 on histone H3. Subsequently this mark is important for the recruitment of PRC1 which has roles in the maintenance of the repressive state and specifically ubiquitinates H2A on K119[14-17]. Moreover, a recent study has shown that PRC1 can function independently of PRC2 and its methylation mark, suggesting a novel level of regulation of polycomb signaling[12].

The biochemical details required for the achievement and the maintenance of gene repression by PRC1 are partially accounted for by their role in stalling RNA polymerase II during transcript elongation[18]. Two different molecular mechanisms for PRC1 repression function have been proposed, one involves the regulation of H2A ubiquitination[18-20], the other is independent on the the E3 ligase function and involves chromatin compaction[21-23]. However a more recent study proposed a composite function of these two mechanisms[24], this study shows that H2A ubiquitination occurs only on a subset of the target genes

bound by Ring1B where transcription is repressed. The capacity of PRC1 to compact chromatin is indeed independent on the ligase function, while H2A ubiquitination is required for target genes repression[24].

PRC1 contains two subunits that confer the E3 ligase function: the actual ligase Ring1A or its paralog Ring1B (homologs of the fly dRing protein) and the PCGF subunits (homologs of the *Drosophila* PSC protein, e.g. Bmi1 or Mel18) (Figure 1a-b). The members of these two families interact through their N-terminal RING domains, that confer the ligase activity, to form a functional heterodimer[7,14,16,25] (Figure 1c). The relative role of the different paralogs is yet unclear. Regarding Bmi1 and Mel18 for example, although *in vivo* studies have shown that loss of either of these genes in mice cause partially similar phenotypes, it also appears that these genes are involved in distinct molecular functions, since Mel18 has tumor suppressor activity and Bmi1 has been described as an oncogene[26-29].

In addition, the function of the different E3 dimers is controlled by post-translational modifications[30]. Phosphorylation is described as a prime regulator of different PRC1 ligases, the C-terminus of both Mel18[7] and Bmi1[31-34] are phosphorylated. While for Mel18 this modification was mainly studied *in vitro* and the kinase responsible has not yet been described[7], the phosphorylation of Bmi1 is the result of the kinase AKT[33-34]. In addition, ubiquitination of the PRC1 E3 ligases has also been proposed to modulate their activity[14,35-36] and association of the PRC1 ligases with additional E3s may have functional consequences[36-37]. Finally, sumoylation has also been associated to PRC1 activities, in particular the subunit CBX4 was described to be a SUMO E3 ligase[38], nevertheless the full understanding of this aspect still requires further investigations.

Our aim is to study the mechanistic details of how the E3 ligases within the PRC1 ubiquitinate histone H2A, with attention to the factors that influence this activity such as paralogs choice, post-translation modifications and presence of other PRC1 members.

RESULTS

The E3 ligase dimers within PRC1 and the modulation of their activity

To understand how the E3 ligases in PRC1 monoubiquitinate K119 on H2A, we first set out to reconstitute an *in vitro* ubiquitination reaction using the core RING E3 dimers.

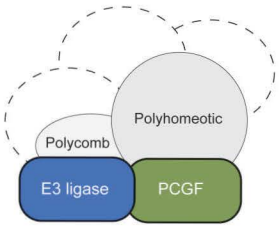
We focused on the ligases Ring1A and Ring1B and their partners Bmi1 and Mel18. We cloned the full-length murine cDNAs into vectors suitable for insect cell expression and we expressed and purified the four different heterodimers formed by these proteins (Figure 2a). All four dimers appear stoichiometric and could be purified in reasonable amounts (yield ~1mg protein/ Liter of insect cell expression) (Figure 2a).

To set up a suitable assay to study their function, we prepared stretches of oligonucleosomes purified from human cells using a partial digestion with Micrococcal Nuclease, since this represents a physiologically relevant context for the substrate of the reaction, histone H2A. We then mixed the different E3s, the substrate oligonucleosomes with recombinant E1, E2 UbcH5c, ubiquitin and ATP and we examined the reactions using Western Blotting techniques with antibodies specific for H2A.

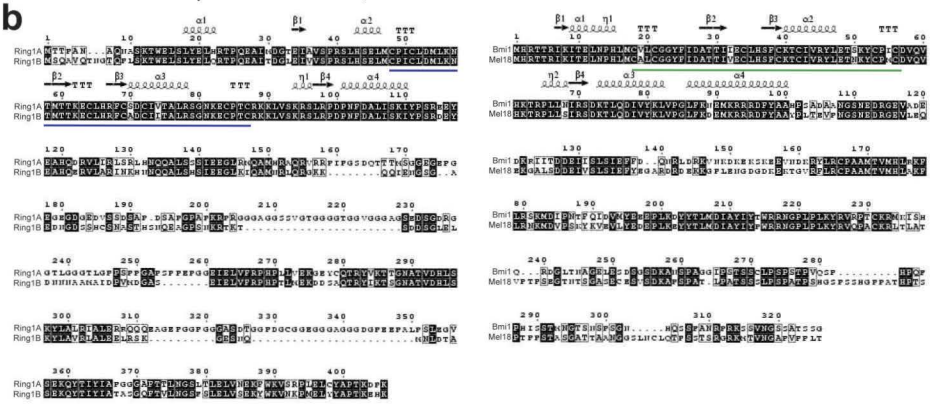
We found that the E3 complexes are active in ubiquitinating H2A in nucleosomes

a

Family	Drosophila protein	Human Paralogues
E3 ubiquitin ligases	dRing	RNF1 (RING1A), RNF2 (RING1B)
Polycomb group RING finger	Posterior Sex Combs (Psc)	PCGF1 (NPSC1), PCGF2 (MEL18), PCGF4 (BMI1), PCGF3, PCGF5, PCGF6
Polycomb	Pc	CBX2 (PC1), CBX4 (PC2), CBX6, CBX7, CBX8 (PC3)
Polyhomeotic	Ph	PH1, PH2
	-	RYBP, YAF2
Sex comb on midleg-like	Scml	SCML1,SCML2, SCML4
Deubiquitinating enzyme	Calypso	BAP1
Additional sex combs	Asx	ASXL1
H3K36me2 demethylase	dKDM2	KDM2B



b



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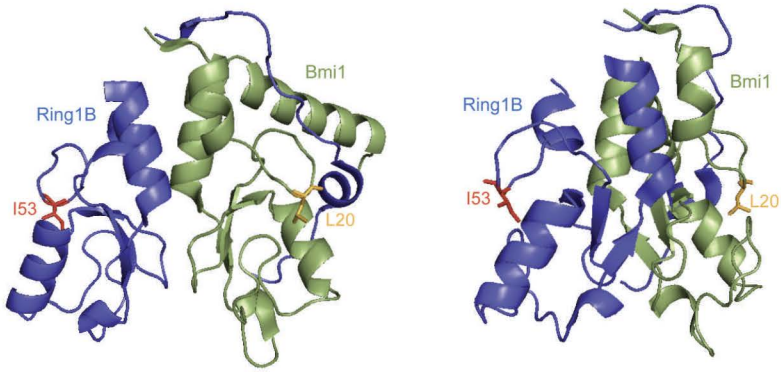

Figure 1. **a**) Table indicating the known protein members of the PRC1 complex in *Drosophila* and human. Schematic representation of the canonical PRC1 complex, where the four core family components are indicated. **b)** Sequence alignment of the human RING1A and RING1B proteins, as well as as the BMI1 and MEL18 proteins. The secondary structure elements in the alignments corresponds to the ones in the crystal structure 2CKL[14] of the RING domain of Ring1B/Bmi1. The horizontal line indicate the core Zn-binding region of the RING domain. **c)** Crystal structure of the RING domain of Ring1B/Bmi1[14]. Ring1B is shown in blue and Bmi1 in green. The residues mutated to inactivate the E2/E3 interaction are indicated in red for Ring1B (I53) and in yellow for Bmi1 (L20).

(Figure 2b). Notably, differences in the activities of the four different full-length E3 dimers are observed (Figure 2b). Future experiments should exclude the possibility that these are due to differences in protein preparation quality. Intrestingly, these differences were not present when we compared the activity of the short dimer constructs containing only the RING domains (Figure 2c). Moreover, we observed that these short constructs are more active than the full-length proteins, suggesting that the E3 ligase activity in the full-length proteins is differently regulated than in the isolated RING domains.

The Ring1B/Bmi1 dimer is the best studied ligase within the PRC1 and the structure of the RING domain complex is known (Figure 1c)[14,16,25]. Ring1B/Bmi1 resembles known RING dimers and contains two putative E2 interaction sites; one on each RING component. In the context of isolated RING domain dimer, mutation of a single residue (I53A) on the RING domain of Ring1B was shown to be important for the activity of the complex, while the structurally equivalent mutation on Bmi1 (L20A) still resulted in a fully active short complex[14](Figure 1c). This finding is in accordance with a recent structure of the complex between UbcH5c and the RING domains of Ring1B/Bmi1, where the E2 interacts only with the Ring1B subunit[25].

So far, it has not been verified whether the E2 interaction of the full-length complex is also dependent uniquely on the Ring1B subunit. Using purified full-length Ring1B/Bmi1 complexes purified from insect cells carrying a mutation on either I53 on Ring1B or L20 on Bmi1, we could show that also in the full-length context only the E2-interaction surface of Ring1B is required for activity (Figure 2d). Our data confirms that Ring1B is the active component in the E3 dimer to target H2A.

Next we wanted to study the influence of phosphorylation on the activity of the Ring1B/Bmi1 full-length complex. We obtained a bicistronic expression vector for the human complex in bacteria (PROTEOLOGICS). Because insect cell expression results in phosphorylated proteins, we set out to compare the activity of the complex expressed in insect cells and the one bacterially expressed. In these experiments, we could not detect any substantial difference in activity (Figure 2e). Nevertheless, it has been shown that treatment of the insect cell expressed Ring1B/Bmi1 complex with Alkaline Phosphatase (AP) showed reduced activity towards H2A compared to the untreated sample[33]. In addition, in accordance with *in vivo* evidence that AKT modulates BMI1 phosphorylation levels to regulate its function, we observed that addition of active AKT in our assays stimulated H2A ubiquitination by Ring1B/Bmi1 while inactive AKT was unable to do so (Figure 2f).

These data suggest that phosphorylation affects function of Ring1B/Bmi1, nevertheless the exact molecular definition of this modification and the mechanism of its effects await further explanations.

Production of the PRC1 core complex

In addition to the E3 ligase dimers, the canonical PRC1 complex contains among others, members of the polyhomeotic (Ph) and the polycomb (Pc) families. To study the effects of these other PRC1 core components on the E3 ligase activity of Ring1B/Bmi1 we set out to produce a four-component complex from insect cells, using PH1 (Ph family) and CBX7 (Pc family).

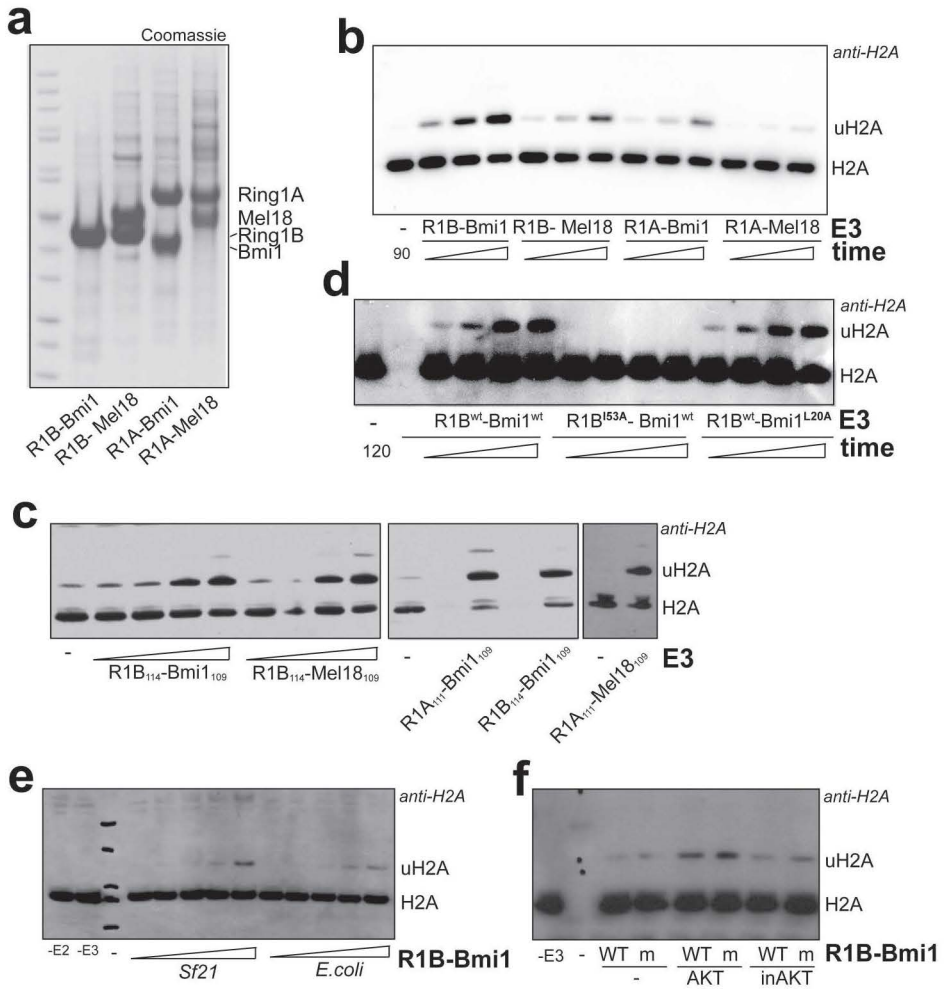

Figure 2. **a**) SDS-PAGE of the final samples of the full-length PRC1 E3 dimers: Ring1B/Bmi1, Ring1B/Mel18, Ring1A/Bmi1, Ring1A/Mel18. **b)** Time course assay (10-30-90 minutes) to assess the activity of the PRC1 dimers towards H2A in oligonucleosomes.**c)** Concentration series (65-130-650-1300 nM) of Ring1B(1-114)/Bmi1(1-109) and Ring1B(1-114)/Mel18(1-109). Assay to show activity of Ring1A(1-111)/Bmi1(1-109) and Ring1A(1-111)/Mel18(1-109). **d)** time course experiment (15-30-60-120 minutes) to study the influence of the E2-E3 interaction surface of Ring1B (I53A mutation) or Bmi1 (L20A mutation). **e)** Concentration series of full-length Ring1B/Bmi1 purified from insect cells or *E.coli* (0.1-0.25-0.5-1-2 μM). UbcH5c was used at 250 nM. **f)** Assay performed in presence of active or inactive (inAKT) AKT kinase. WT Ring1B/Bmi1 full-length protein was used. m stands for a mutant complex where S251,253,255 were mutated to alanine[33]). Samples were run for 15 minutes, with 2 μM E3.

Initial attempts to produce such complex using four individual viruses were unsuccessful, so we moved to the MultiBac technology developed by Imre Berger at EMBL[39]. This system allows the construction of a single polycystronic bacmid carrying the expression cassettes of several cDNAs, permitting the expression of different proteins from a single viral particle.

Starting from four individual plasmids carrying the four different cDNAs, after few cloning steps taking advantage of the different origin of replication, we obtained the final product that was used to transform DH10Bac cells for the transposition into the bacmid (Figure 3a). Virus was produced and amplified and a test expression was carried out on Sf21 cells. Protein expression was monitored on SDS-PAGE from cell lysates harvested at different time points after infection (Figure 3b, dpa stands for day after proliferation arrest, see experimental procedures). Small scale purification was carried out using the His-tag on Bmi1 to validate the formation of a four-component PRC1 complex (figure 3b). While Ring1B and Bmi1 proteins were easily identifiable on SDS-PAGE, the presence of the other PRC1 proteins was validated by Western blot using anti-PH1 and anti-CBX7 (Figure 3b).

These data show that PH1 and CBX7 co-purify with the Ring1B/Bmi1 proteins, however their presence appear to be substoichiometric. To obtain a stoichiometric and pure four-component complex, we could introduce a second copy of these subunits in the MultiBac bacmid. In addition, we obtained a vector carrying only three subunits of the PRC1 core complex, with either PH1 or CBX7 present, and preliminary data suggest that these complexes also are expressed succesfully.

In parallel, based on a more recent method from the Berger lab[40], we designed and cloned the four PRC1 proteins into a monocistronic vector. In this system named PolyProtein, the vector contained at the 5' of the Open Reading Frame the cDNA of TEV (Tobacco Etch Virus) protease and a TEV-specific cleavage site and on the 3' the CFP protein. The codon-optimized cDNAs of the PRC1 proteins were inserted in frame but separated by TEV cleavage sites to produce a long single mRNA molecule (Figure 3c). The system is based on the assumption that in cells after translation of the mRNA TEV would start cleaving in between each subunit to yield separate protein molecule translated from the same mRNA molecule. The presence of a CFP at the end was used to measure fluorescence during expression as an indication of protein production. Preliminary test expression carried out in the Berger lab showed that the proteins were expressed and properly cleaved by TEV, but further analysis is required to assess complex formation and stochiometry (Figure 3d).

Overall our data serves as basis for future work intending to establish the purification of three- or four-component core PRC1 complex for both structural and functional studies. Additional work needs to be done to assess the influence of the PRC1 protein members to the E3 ligase activity of Ring1B/Bmi1. The flexibility of the expression systems allows easy substitution of these proteins with any of their family paralogs to study possible differential contribution of each PRC1 protein.

DISCUSSION

Here we presented a biochemical analysis of the E3 ligases present in the PRC1 complex.

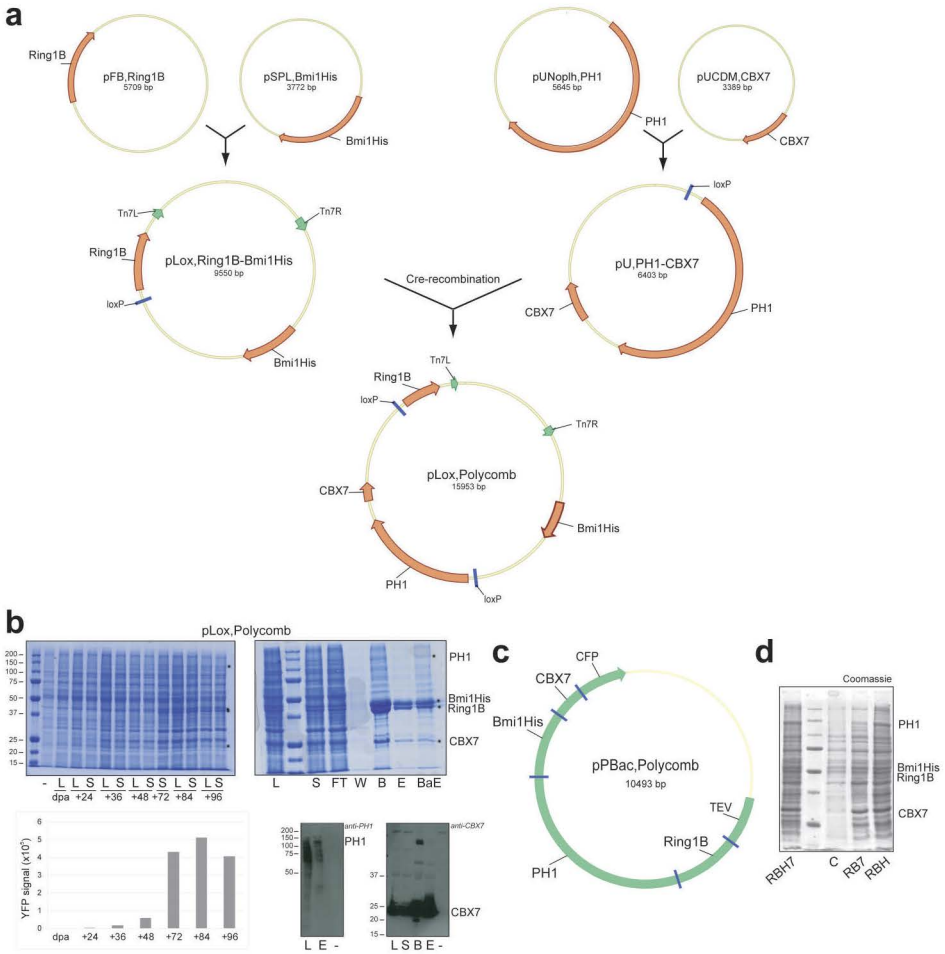

Figure 3. **a**) Overview of the cloning strategy to construct pLoxPolycomb, a single vector containing the expression cassette for Ring1B, Bmi1 , CBX7 and PH1. **b)** YFP signal measured during test expression, SDS-PAGE gels showing lysate from test expression and small scale purification on Nickel beads of the four-component PRC1 core complex. Western blot to validate the expression of PH1 and CBX7. L = lysate, S = supernatant, FT = flow-through, W = wash, B = beads, E = elution, BaE = beads after elution. **c)** Overview of the designed expression cassette used for the PolyProtein approach. VectorNTI was used to generate figures. **d)** SDS-PAGE gel of lysates isolated from expression tests in Sf21 cells for the PolyProtein expression viruses. RBH7 stands for the four-coponenet complex, RBH stands for the three-component complex lacking CBX7 and RB7 refers to the three-component complex lacking PH1.

We were able to express and purify full-length and RING domain constructs of the four E3 dimers formed by Ring1B or Ring1A and Bmi1 and Mel18.

We show that all dimers are active. Interestingly we observe differences in activity between the different full-length ligases, while the RING domain constructs show little variation. In particular, the presence of Bmi1 or Mel18 in full-length dimers seems to affect the activity of the E3 towards nucleosomal H2A, while in the context of the RING domains this difference is not observed. This suggests that the regions outside the RING domain in these proteins may contribute to differentially regulate the activity of the E3s. On the basis of this observation, future work should address this point and investigate the differential contribution of the different paralogs.

Our work validates that Ring1B is the active component of the dimer in the context of full-length Ring1B/Bmi1, where mutation on its E2-interacting interface is sufficient to inactivate the dimer. This is in line with what is observed for the isolated RING domains[25] and suggests that the E2 interacting subunit within the dimer is consistently the RING domain of Ring1B. A similar analysis could be done for the Ring1A paralog.

An additional level of regulation of the activity of the full-length proteins could come from post-translational modifications. Our data supports a role of phosphorylation by AKT in activating the Ring1B/Bmi1 activity. Future work should address the molecular details of this activation: which phosphorylation events are able to stimulate the ligase activity? It is also important to exclude that phosphorylation of other components of the reaction (e.g. E2 or nucleosomes) is responsible for this activation.

At last, an important contribution to the ligase activity of the E3s within PRC1 is likely to come from the other components of the complex. Here we show that production of three- or four-components complexes is possible from insect cells. Future work should aim on one side at the optimization of the purification of the complexes, on the other at the understanding of the effects towards the ligase activity.

EXPERIMENTAL PROCEDURES

Cloning, expression and purification of E3 dimers

Murine cDNAs of Ring1B, Ring1A, Bmi1 and Mel18 were cloned into pFastBac vectors (Invitrogen). In addition to untagged constructs, an uncleavable His-tag was cloned at the C-terminal end of Ring1B and Bmi1 while Ring1A and Mel18 were cloned also into pFastBacHT vectors to yield N-terminal cleavable His-tagged proteins. Viruses were prepared and amplified according to the Bac-to-Bac system (Invitrogen). Sf9 or Sf21 cells were infected with two viruses (normally ~1.5 ml each virus in 300 ml culture at 1-2M cells/ml), one carrying the E3 ligase (Ring1B or Ring1A) and one carrying the partner protein (Bmi1 or Mel18). Ring1B/Bmi1 complex was also expressed from the pLox, Ring1B-Bmi1His shown in figure 3a. Cells were harvested ~3 days after infection. At each co-expression only one protein was expressed with a His-tag. The ligase dimers were purified using Ni-sepharose beads in 30 mM Hepes 8.0, 500 mM NaCl, 10mM imidazol, 10% glycerol, 1 μM $ZnCl_2$, 1 mM TCEP. The protein was eluted with buffer containing 500

mM imidazol and then loaded on S200 16/60 column in 20 mM Hepes 8.0, 400 mM NaCl, 10mM imidazol, 10% glycerol, 1 μM $ZnCl_2$,1 mM TCEP. Fractions containing the complex were pooled and concentrated. Bicistronic vectors containing codon optimized cDNA for Ring1B and Bmi1 for expression in bacteria were obtaind by PROTEOLOGICS. In this case Ring1B was expressed with a N-terminal HA-tag and a C-terminal His-tag and Bmi1 with N-terminal GST- and myc-tag. Purification was done performing first a Nickel beads pull-down in presence of 10 mM imidazol and then after elution with 500 mM imidazol a GST pull-down. The complex was then eluted with 30 mM glutathione and the GST-tag as cleaved using 3c protease. Finally the sample was injected in a S200 column in tandem to a GSH column (GE Healthcare) in the gel filtration buffer (above). RING domain constructs were cloned into a modified pGEX6p vector containing two ribosome binding sites on a single mRNA transcript to allow expression of the two proteins. Contructs included residues 1-114 for Ring1B, 1-111 for Ring1A, and residues 1-109 for both Bmi1 and Mel18. These dimers were purified using the GST-tag on the PCGF subunit in 50 mM Tris pH 7.5, 100 mM NaCl, 5% glycerol, 1 μM $ZnCl_2$, 2 mM DTT. After cleavage on the column, the proteins were eluted and injected into a S75 column (GE HEalthcare) and concentrated before shock freezing them in liquid nitrogen.

In vitro ubiquitination assays

Purified human Uba1 at 0.2 μM was mixed with UbcH5c (0.5 μM), the E3 complexes (1 μM), ubiquitin (20 μM), ATP (3mM) and 10 μM of H2A in oligonucleosomes. The reactions were incubated at 32 °C in buffer 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 1 μM ZnCl2, 1 mM TCEP. Assays similar to the one shown in figure 2e where run with 600 nM E1, 1μM UbcH5c, 2 μM E3, 500 μg of AKT variants (Cell Signaling Technology), 20 μM ubiquitin, 10 μM H2A in oligonucleosomes and 5 mM ATP.

MultiBac cloning

MultiBac vectors (pSPL and pUCDM) were obtained from Imre Berger[39]. We first cloned Bmi1-His into the pSPL vector. By blunt-end digestion and ligation (PmeI for pSPL,Bmi1-His and SnaBI for pFastBac,Ring1B) we obtain a pLox, Ring1B/Bmi1-His. We cloned CBX7 into the pUCDM vector. We modified the pUCDM vector (pUNopolh) to create a helper vector to facilitate the cloning of the large PH1 cDNA. To do this we cleave off a fragment on pUCDM between the NruI and the HpaI sites. We then insert PH1 using the PmeI and SpeI sites on pUCDM. To create a vector carrying both CBX7 and PH1, we cleaved pUCDM,CBX7 and pUNopolhPH1 with AgeI and ClaI and combined the two halves containing the cDNAs. To create the final plasmid with the four-component complexes we use a Cre recombinase reaction. See Figure 3a.

PolyProtein cloning

The vector was obtained from Imre Berger (pPBac,TEV-CFP)[40]. Codon-optimized cDNAs of Ring1B, Bmi1, PH1 and CBX7 containing the TEV cleavage sites were purchased from ATG:biosynthetics GmbH. Additionally Bmi1 was tagged with a 10xHis C-terminal tag and PH1 was tagged on the N-terminus with a CBP (Calmodulin Binding Protein)-tag and a Thrombin cleavage site. First, a fragment containing Bmi1 and Ph1 was inserted into pPBac using BstEII and RsrII. Then, Ring1B will be cloned using the directional BstEII site. Finally CBX7 was inserted using RsrII. See Figure 3c.

PRC1 core complex expression

Vectors containing 3 or 4 core PRC1 subunits were used to transform DH10Bac or EMBacY *E.coli* cells, to allow the transposition of the expression cassette to a viral bacmid. Transfection was carried out using Fugene HD (Roche) on adherent Sf21 cells. First virus batch (P0) was harvested after 60 hours. P0 was used to infect 25 ml Sf21 at 0.5 M cells/ml in suspension. 24 hours after infection we check cell density and we keep monitoring every 12 hours, when the concentration of cells increases above 1 M cells /ml we dilute back to 0.5 M/ml. In parallel we measure YFP (if EMBacY cells were used) or CFP for PolyProtein approach to monitor protein expression (YFP: Excitation 488 nm, Emission 515-600 nm, Length 85 nm, step size 1 nm, integration 0.2 sec) (CFP: Excitation 400 nm, Emission 450-560 nm, Length 110 nm, step size 1 nm, integration 0.2 sec). We harvest P1 virus 24 hours after the Day of Prolifaration Arrest (Dpa+24). For protein expression, cells were culture at 0.5-1 M/ml until the YFP or CFP signal reached saturation.

PRC1 core complex pull-down

Cells were harvested and lysed by sonication in 50 mM TRIS pH 7.9, 200 mM NaCl, 10% glycerol, 10 mM imidazol, 5 mM β-mercaptoethanol. Nickel sepharose beads were incubated with the soluble fraction of the cell lysates, unbound sample was kept for analysis (FT). After a washing step (W), the proteins were eluted using 400 mM imidazol. Samples were prepared for SDS-PAGE analysis.

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RNF168 UBIQUITINATES K13-15 ON H2A/H2AX TO DRIVE DNA DAMAGE SIGNALING

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ABSTRACT

Ubiquitin-dependent signaling during the DNA damage response (DDR) to double strand breaks (DSBs) is initiated by two E3 ligases, RNF8 and RNF168, targeting histone H2A and H2AX. RNF8 is the first ligase recruited to the damage site while RNF168 follows RNF8-dependent ubiquitination. This suggested that RNF8 initiates H2A/H2AX ubiquitination with K63-linked ubiquitin chains and RNF168 extends them. Here, we show that RNF8 is inactive towards nucleosomal H2A whereas RNF168 catalyzes the mono-ubiquitination of the histones specifically on K13-15. Structure-based mutagenesis of RNF8 and RNF168 RING domains shows that a charged residue determines whether nucleosomal proteins are recognized. We find that K63 ubiquitin chains are conjugated to RNF168-dependent H2A/H2AX monoubiquitination at K13-15 and not on K118-119. Using a mutant of RNF168 unable to target histones but still catalyzing ubiquitin chains at DSBs, we show that ubiquitin chains *per se* are insufficient for signaling, but RNF168 target ubiquitination is required for DDR.

INTRODUCTION

Ubiquitination of target proteins was discovered by the identification of this mark on histone 2A (H2A) at lysine 119[1-2]. This prevalent modification can be present at up to 10% of cellular H2A in chromatin as a result of ubiquitination by the E3 ligases present in the Polycomb repressive complex 1 (PRC1) during transcriptional repression[3-6]. More recently it was suggested that two RING-type ubiquitin E3 ligases, RNF8 and RNF168, with roles in the DNA damage response (DDR) also modify H2A[7-14].

These ligases participate in the early signaling events of the Double Strand Break (DSB) repair pathway, where their ubiquitination activity is required for proper DDR. RNF8 is the first ligase that binds to damaged sites in an ATM-dependent manner[7-10]. The E3 ligase activity of RNF8, conferred by its C-terminal RING domain, is required for the formation of ubiquitin chains at the site of damage to permit the recruitment of the second E3 ligase, RNF168, by its ubiquitin binding domains[11-13]. The concerted action of these ligases contributes to the ubiquitin-dependent signals necessary for the further recruitment of downstream regulators of the DSB response pathway, such as 53BP1 and the BRCA1 complex[15-20].

Very little is known about the substrate spectrum of the ligase activity of RNF8 and RNF168 during DDR. So far the main targets are histone H2A and its variant H2AX, where the E3s are thought to form K63-linked ubiquitin chains[7-13]. Because of their recruitment order, it has been proposed that RNF8 is the ligase responsible for the initial ubiquitination of the histones, while RNF168 is thought to extend the chains on this target.

More recent data have revealed higher complexity to this pathway, where phosphorylation and sumoylation are signaling in conjunction with the ubiquitination-dependent cascade[21-23]. Additional ubiquitin ligases also participate in the damage response, such as the BRCA1/BARD1 complex that is recruited by the actions of RNF8 and RNF168 and is

responsible for downstream signaling[15-17], the HECT-type E3 HERC2 that is recruited by RNF8 in a phosphorylation dependent manner and appears to have different substrate spectrum than the RING-type ligases RNF8 and RNF168[24], RAD18 and the negative regulator RNF169 whose involvement in DSB repair was recently described[25-26]. Also the polycomb Ring1B/Bmi1 complex is implicated in the DSB response, where its activity towards K119 of histone H2A appears to be important[27-33]. In this complex scenario, the current literature lacks biochemical details to clarify the molecular events that define the DNA damage-dependent H2A/H2AX ubiquitination orchestrated by RNF8 and RNF168.

Here, we study the mechanism of RNF168 and RNF8. Surprisingly we find that the first step of H2A ubiquitination is catalyzed by RNF168, while RNF8 is inactive towards this target. To confirm this finding, we solve the structure of the catalytic RING domain of RNF8 to find the region that is responsible for target specificity for these ligases. Using specific single-point target recognition mutants we validate the importance of this region on RING domains for the function of these E3s in vitro and in vivo.

We find that RNF168-dependent H2A ubiquitination during DDR takes place at a previously unknown site on H2A, on K13 or 15. We also show that K63 ubiquitin chains are specifically formed on this site and not on the Polycomb-modified residue. We find that RNF8 is efficient in catalyzing these chains in vitro on the already ubiquitinated histone. Interestingly we show that it is this target ubiquitination step by RNF168 that is critical for the integrity of the DDR pathway.

RESULTS

RNF168 efficiently modifies H2A in nucleosomes in vitro, while RNF8 is inactive towards this target.

In order to understand the molecular details of the early steps of the DSB repair pathway, we reconstituted in vitro the reactions carried out by RNF8 and RNF168 towards H2A using purified human oligonucleosomes as substrate.

We first assessed the intrinsic activity of these ligases in catalyzing the formation of ubiquitin chains in vitro in absence of the target. Purified full-length human RNF8 or RNF168, as well as their isolated RING domains (Figure 1a) were incubated with E1, Mg^{2+}, ATP, ubiquitin and a set of E2s. The formation of ubiquitin chains was followed by western blot analysis and in these assays both E3 ligases were active with several E2s. RNF8 showed high activity particularly with UbcH5c and the Ubc13/Mms2 complex, depleting the pool of free ubiquitin (Figure 1b and S1a). Although the activity of RNF168 was lower, it still promoted ubiquitin conjugation with these E2 enzymes (Figure 1b and S1b).

Previous studies have shown that both RNF8 and RNF168 are active towards isolated purified histone proteins[9,11,13]. In our hands, both E3 ligases ubiquitinate H2A in its free form, or in presence of the other histones without DNA (Figure 1c). However on purified oligonucleosomes, where the histone octamer is surrounded by DNA only RNF168 maintains its capacity to target H2A, primarily with the E2 UbcH5c. In contrast, full-length RNF8 and its RING domain are extremely inefficient in modifying nucleosomal H2A with any of the E2s (Figure 1c-d and S1c-f). Comparable results were obtained for histone H2AX

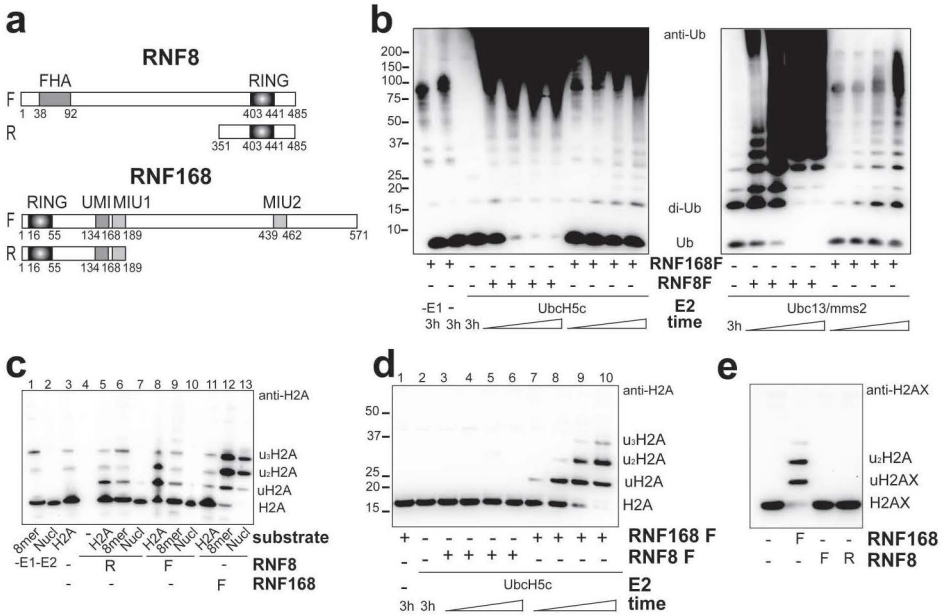

Figure 1 RNF168 efficiently modifies H2A in nucleosomes in vitro, while RNF8 is inactive towards this target. **a)** Domain architecture of human RNF8 and RNF168. Full length (F) and RING (R) domain constructs used. **b)** Time course (10-30-90-180 minutes) ubiquitin chain formation assay of 1μM full-length RNF8 and RNF168 with the E2 enzymes UbcH5c and Ubc13/Mms2 in presence of 15 μM ubiquitin. **c)** Full-length and RING domain of RNF8 can efficiently ubiquitinate isolated H2A or H2A in the context of the octamer (lanes 5-6 and 8-9), whereas they are inefficient in ubiquitinating H2A in the nucleosomal context (lane 7 and 10). RNF168 can efficiently target H2A in each context (lanes 11-13). **d)** H2A ubiquitination in purified oligonucleosomes (time course, as above) with 1μM E3. Concentration series is shown in Figure S1e. **e)** RNF168 can modify H2AX in nucleosomes and RNF8 does not (3 hours). Ring (R), Full-length (F). See also Figure S1.

(Figure 1e). Additionally, from the analysis of the other histone proteins in nucleosomes we see that RNF168 can target H2B, but not H3 and H4, to a lower extent than the H2A-type histones (Figure S1g) while RNF8 is inactive.

The finding that RNF168 targets H2A/H2AX efficiently, whereas RNF8 is inactive, contrasts with the order of recruitment of these ligases to the damage site, and thus challenges the current view on the order of events of H2A ubiquitination by RNF8 and RNF168 in the DDR.

Crystal structure of the RING domain of RNF8

RNF8 is capable of modifying H2A in isolation, but it is inactive when the histone is in its most common form within the nucleosome. We determined the crystal structure of the RING domain of RNF8 to understand the molecular determinants of this target specificity. The structure was solved at 1.9 Å resolution using the anomalous signal of the Zinc ions (Figure 2a), with R/Rfree of 20.0/22.6% and excellent stereochemistry (Table S1). The

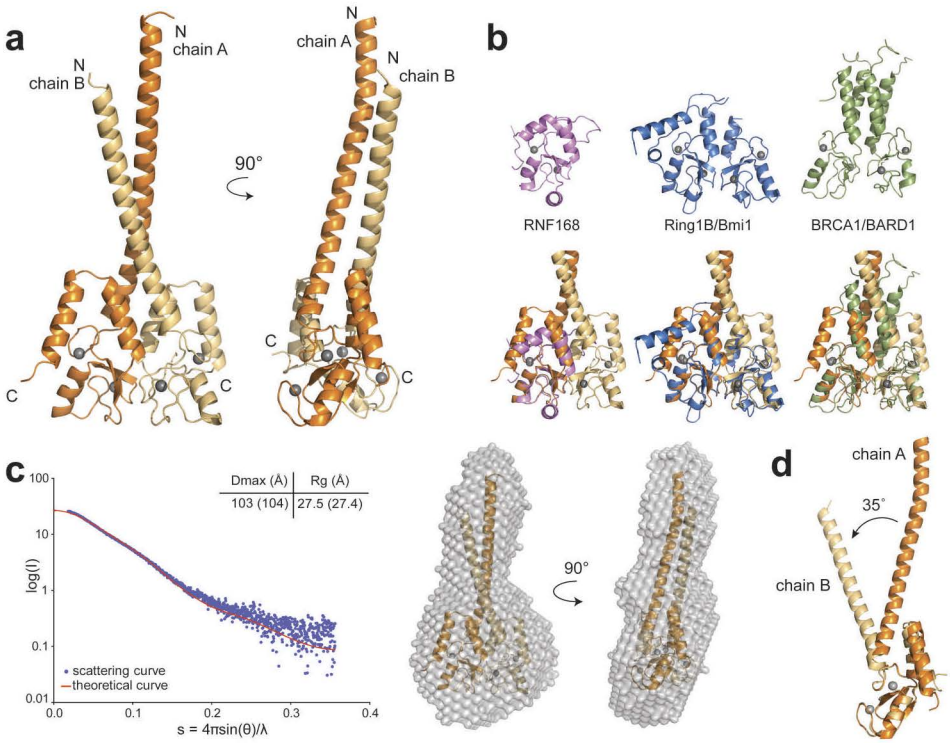

Figure 2 Structure of RNF8 RING homodimer. a) Cartoon representation of the crystal structure of the dimeric RING domain of RNF8 (residues 351-483 (chain A) and 359-485 (chain B) could be built in density, Zn^{2+} grey spheres). Front and 90° rotated view. N- and C-termini are highlighted. **b)** The RNF8 heterodimer resembles other RING E3 ligases: RNF168 monomer (magenta, PDB: 3L11), Ring1B/Bmi1 (blue, PDB: 2CKL), BRCA1/BARD1 (green, PDB: 1JM7). Superpositions with RNF8 shown without the extended N-terminal helices for clarity. **c)** SAXS analysis of the RNF8 RING domain. Plot of the scattering curve used for analysis and theoretical curve calculated from the crystal structure. *Ab initio* model (shown as light gray surface, derived from DAMAVER) based on SAXS measurements superposed to the crystal structure (orange cartoon). Table shows calculated values and expected ones are in brackets based on crystal structure. **d)** Superposition of chain A and B of the RNF8 RING dimer shows asymmetry due to a kink of the N-terminal flanking helices of 35°. Images were prepared using GraphPad Prism and PyMOL. See also Figure S2 and Table S1

Zinc-binding region in the RING (403-441) forms the canonical structure with two Zn^{2+} ions coordinated by the C3HC4 motif. It resembles other RING structures, including the recently determined RNF168 monomer (PDB: 3L11, rmsd 2.0 Å for the monomer) (Figure 2b)[34].

The RNF8 RING domain forms a dimer through interactions of the core RING domain and flanking regions in a manner that resembles other dimeric RING dimers such as Ring1B/Bmi1[5-6], BRCA1/BARD1[35], RAD18[36] and the U-box protein CHIP[37] (Figure 2b), but with an extended buried interface (~2000 Å^2), due to long N-terminal helices that fully contribute to the dimer interface, in accordance with the low resolution structure recently published[34].

We used SAXS (Small Angle X-ray Scattering)[38-39] to study the shape of the RNF8 RING domain and confirmed the extended arrangement of these helices in solution. *Ab initio* modeling based on these SAXS measurements resulted in a molecular shape that fits the crystal structure well (Figure 2c and S2a).

In the crystal structure, these N-terminal helices adopt 35° different orientations in each protomer within the dimer, revealing the possibility of a structural asymmetry that was also observed in full-length CHIP[37] (Figure 2d). Although most likely due to crystal contacts, the point of asymmetry is conserved between RNF8 and CHIP (Figure S2b). In general, structural and functional asymmetry seems to be a conserved feature in this class of E3-ligases, where *e.g.* in Ring1B/Bmi1, BRCA1/BARD1, RAD18 and CHIP only one protomer is active[5,35-37,40-41].

Identification of a target recognition site on RING domains explains the differences in activity towards H2A in nucleosomes

We compared the crystal structure of RNF8, which is inactive against nucleosomal H2A, with crystal structures of two E3 ligases that can catalyze H2A mono-ubiquitination, RNF168 and Ring1B. This allowed us to search for regions involved in substrate recognition in these E3s. When we superimposed the RING domains in these structures (Figure 3a) we noted a significant deviation located just C-terminal to the last cysteine of the RING motif, flanked by a charged residue, that is negative in RNF8 (D443), but positively charged in RNF168 (R57) and Ring1B (K93).

We inverted the charges at this single site on both the RING domain and the full-length proteins to analyze the importance of this charged residue for the activity of the ligases.

Interestingly, a R57D mutation in RNF168, which does not affect the monomeric nature of the protein (Figure S3g), fully abolishes its ability to ubiquitinate H2A in nucleosomes (Figure 3b). Importantly, this single point mutation hardly affects the formation of ubiquitin chains by RNF168 (Figure 3c). It also does not affect the rate of discharging ubiquitin from the E2 enzyme UbcH5c, as shown in single turnover experiments on pre-charged UbcH5c~ubiquitin (Figure 3d and S3a-c). Apparently this site is important for target specificity but not for ubiquitin chain formation.

On the other hand, the reverse change in charge at this site (D443R) in RNF8, confers the ability to the RING domain construct to target H2A in nucleosomes (Figure 3e, time course in Figure S3i). Again, the mutation has almost no effect on the ability to form unanchored ubiquitin chains nor on the rate of discharging the E2 (Figure 3f-g and S3d-f). The gain-of-function, though present, is much less for the full-length RNF8 protein towards the H2A target, suggesting that substrate recognition in RNF8 may be further controlled by regions outside the RING domain.

The structural equivalent mutation in the Ring1B RING domain (K93D) inhibited the E3 ligase function of the RING domain dimer of Ring1B/Bmi1 towards nucleosomal H2A (Figure S3h), in accordance with recent data that identifies this small loop as DNA binding region in Ring1B[42]. In contrast, we could not detect significant binding to DNA by the RING domain of RNF168 (affinity weaker than 100 μM in 150 mM NaCl). Interestingly RNF168 R57D still modifies free H2A, but

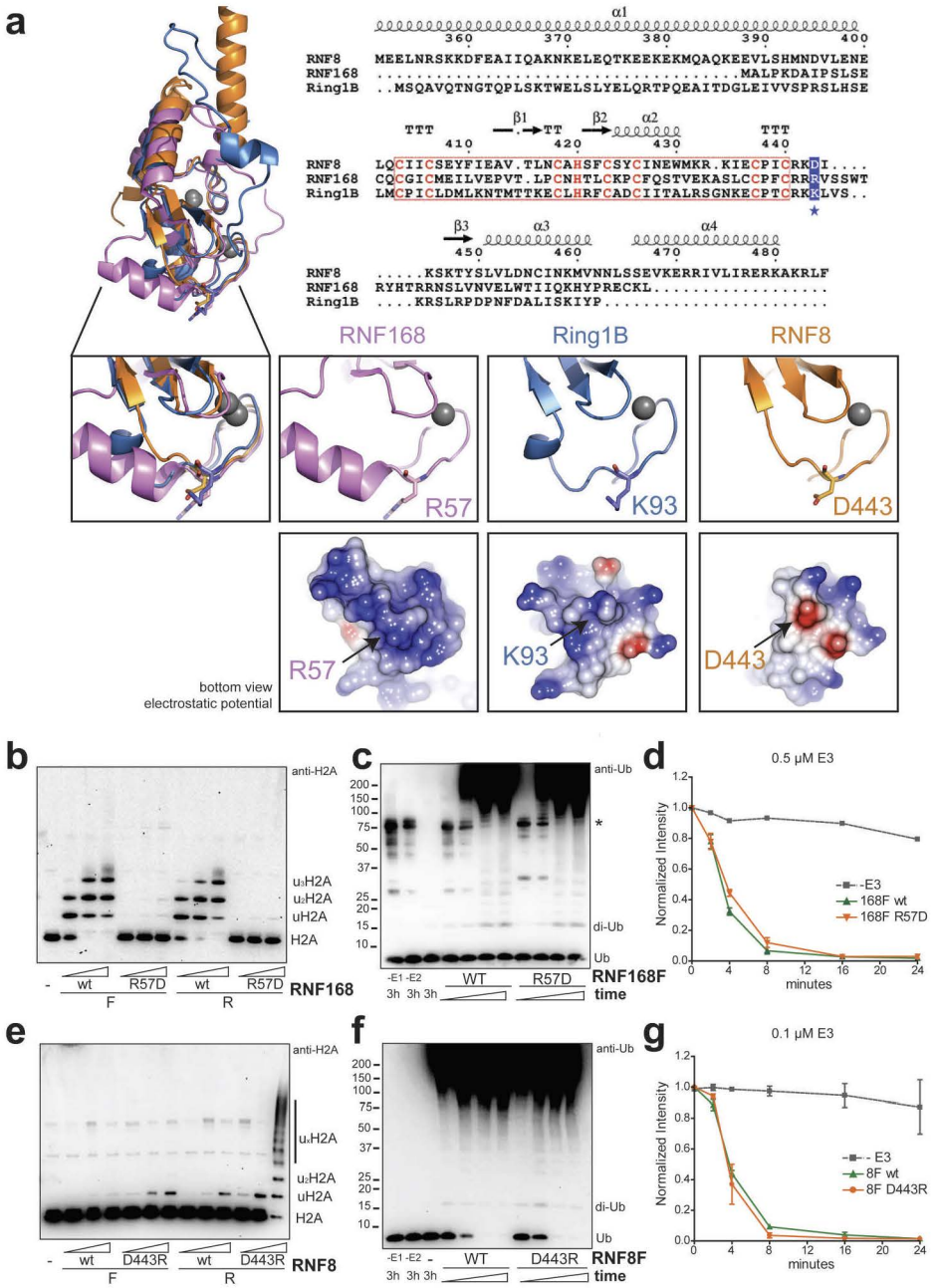

Figure 3 Determination of substrate recognition site in E3 RING domains. a) Superposition of RNF8, Ring1B and RNF168 RING domain structures (shown without RNF8 flanking helices) and structure based sequence alignment of the RING domains (with RNF8 secondary structure and numbering) reveals differences downstream of the core RING domain (red box highlights the core Zn-binding RING domain, Zn coordinating residues in red). The residue that is positively charged ►

it clearly has reduced activity when other histones proteins are present (Figure S3j) indicating that nucleosomal proteins are also important for substrate recognition by this region in RNF168.

Apparently this site near the RING domain can either positively or negatively determine recognition of nucleosomal H2A by these E3 ligases and different aspects of the nucleosome contribute to this recognition process.

RNF168 modifies Lys13 or 15 on H2A and H2AX in vitro and in vivo

Our findings suggest that the E3 initiating H2A/H2AX mono-ubiquitination during DSB signaling is RNF168. Because in vitro RNF168 and UbcH5c are able to attach more than one ubiquitin moiety to H2A/H2AX, we tested if these were chains using a ubiquitin variant without lysines (Ub K0). We still observe multiple bands, showing that RNF168 catalyzes multiple mono-ubiquitination of the histone, rather than forming chains, in these in vitro experiments (Figure 4a).

So far, the known site of mono-ubiquitination on H2A is K119 and in case of a mutation of this lysine, K118. These are targeted by the polycomb PRC1 complex, where the Ring1B/Bmi1 dimer is the main E3 ligase[3]. We wanted to investigate the site of ubiquitination of RNF168 in vitro and in vivo.

Using purified oligonucleosomes that contain mutated FLAG-tagged H2A variants, we show that RNF168 does not target the same site as Ring1B. In vitro we observe a preference for K13-15 (Figure 4b), as shown by its reduced ability to modify the K13-15R nucleosomal H2A variant. The RING domain construct of RNF168 is sufficient to provide specificity for these lysines (Figure S4b) and mutation of the ubiquitin binding domains MIU1 (A179G)[11-13] or UMI (L149A)[43] on this construct do not affect its specificity (Figure S4b).

► in E3 ligases targeting H2A (Ring1B, RNF168) but negative in RNF8, that does not modify H2A in nucleosomes is shown (blue star in alignment, zoom and surface charge (blue positive, red negative), in boxes below). **b)** R57D mutation in full-length RNF168 causes loss-of-function towards H2A in nucleosomes (for anti-ubiquitin blot of these samples, Figure S3a). **c**) Full-length RNF168 R57D is not affected in ubiquitin chain formation capacity, time course experiment (10-30-90-180 minutes) performed with UbcH5c and in absence of oligonucleosomes (RING domain shown in Figure S3b). **d)** Single turnover E2 discharge is not affected by R57D mutation. Graphs showing UbcH5c discharge rates for full-length RNF168 WT and R57D (0.5 μM concentration E3). Mean and Standard Deviation (SD) calculated on two independent experiments, example SDS-PAGE used for analysis in Figure S3c. **e)** Gain-of-function D443R mutation in full-length RNF8 confers ability to ubiquitinate H2A with UbcH5c but to a lesser extent than the RING domain (for anti-ubiquitin blot of these samples, Figure S3d, time course assay Figure S3i). **f)** D443R mutation in full-length RNF8 doesn't affect ubiquitin chain forming capacity, time course experiment (10-30-90-180 minutes) performed with UbcH5c and in absence of oligonucleosomes (Figure S3e shows experiment for the RING domain). **g)** Single turnover E2 discharge is not affected by D443R mutation. Graphs showing UbcH5c discharge rates for full-length RNF8 WT and D443R (0.1 μM concentration E3). Mean and Standard Deviation (SD) calculated on two independent experiments, example SDS-PAGE used for analysis in Figure S3f. Assays shown in panels **c** and **f** are performed with 15 μM of ubiquitin. E3 concentration series in panel **b** and **e** (0.17-0.5-1.5 μM). * indicates background bands. Alignment was done using ESPript. Images were prepared using GraphPad Prism, PyMOL and CCP4mg (electrostatic potential). Ring (R), Full-length (F). See also Figure S3.

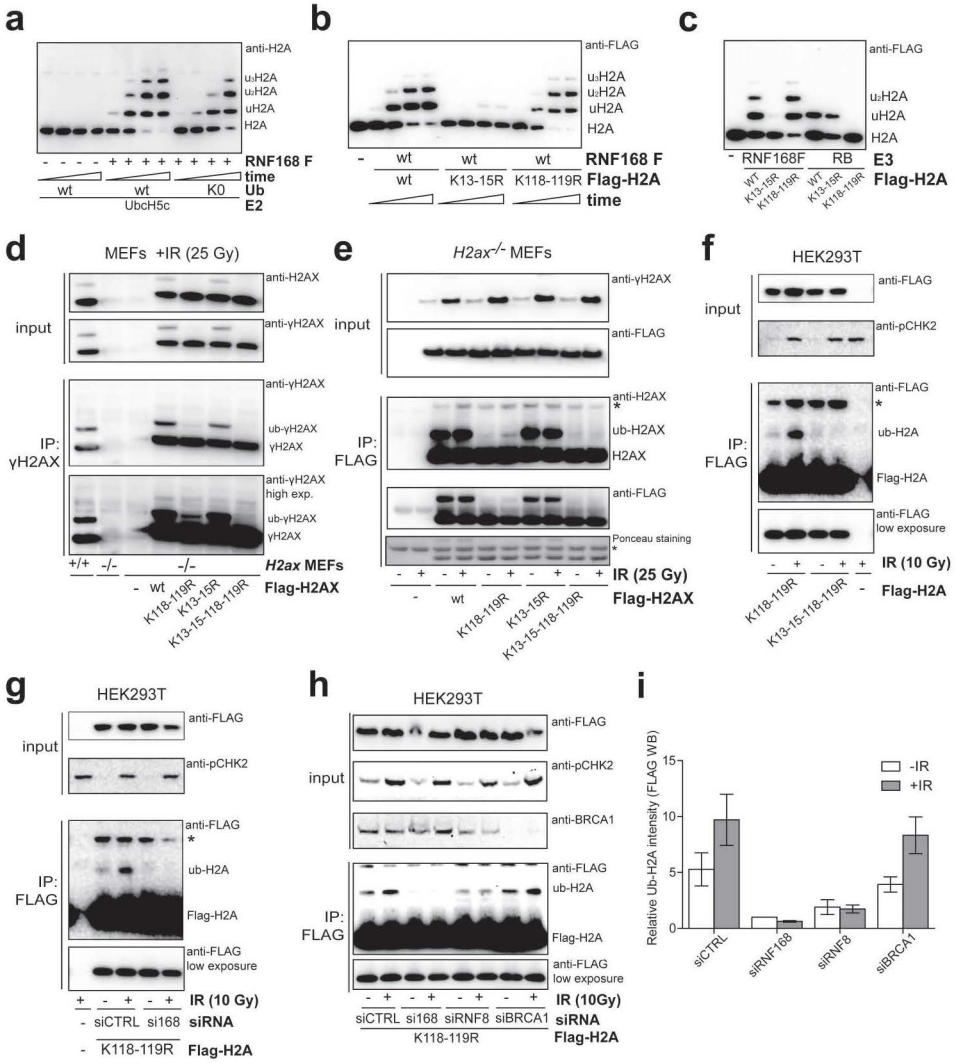

Figure 4 RNF168 modifies K13-15 on H2A and H2AX in vitro and in vivo. a) In vitro time course assay (10-30-90-180 minutes) to compare RNF168 activity with UbcH5c towards nucleosomal H2A with WT or K0 mutant ubiquitin. The Ub K0 reaction is slower because of surface changes. **b)** Time course assay (10-30-90-180 minutes) with oligonucleosomes containing different mutants of FLAG-tagged H2A. **b)** Full-length Ring1B/Bmi1 (RB) and RNF168 have different site specificity on H2A in nucleosomes. 2 hour assay performed with oligonucleosomes containing FLAG-tagged H2A constructs. Ubiquitin was used at 5 μM concentration in panels **b** and **c**. **d)** γH2AX IP from WT MEFs or knock-out for *H2ax* and reconstituted with different FLAG-H2AX constructs shows presence of a ubiquitinated form of H2AX only when K13-15 are present. **e)** FLAG IP from *H2ax*$^{-/-}$ MEFs reconstituted with different FLAG-H2AX constructs to test DNA-damage dependence of K13-15 ubiquitination. **f)** FLAG IP from HEK293T cells transiently transfected with different cDNA of FLAG-H2A mutants shows that ubiquitination on K13-15 is increased after IR. **g,h)** FLAG IP from HEK293T cells transiently transfected with different siRNA and cDNA of FLAG-H2A mutants shows ►

Interestingly the specificity for K13-15 of RNF168 is not present against the isolated H2A (Figure S4c). Like the specificity of RNF168 itself, as reflected by the R57D mutant (Figure S3j), the specificity for the H2A site seems to rely on higher order organization in the nucleosome.

In contrast to RNF168, Ring1B/Bmi1 has high specificity for K118-119, while the K13-15R mutation doesn't significantly affect its ability to target H2A in nucleosomes (Figure 4c).

K13-15 are located on the N-terminal tail of H2A whereas K118-119 are at the C-terminus. As the H2A fold crosses the nucleosome, both tails are at the DNA interface, but diametrically opposite on the nucleosome rim (Figure S4a)[44] . The K118-119 is located between the incoming and leaving strand of the DNA, whereas the K13-15 site is much more exposed. This site is close to the C-terminal tail of histone H2B (Figure S4a). Functionally one could therefore expect different downstream consequences to signals that arise from the RNF168- versus the polycomb-dependent modification.

To validate our results in the cellular context, we used *H2ax*$^{-/-}$ Mouse Embryonic Fibroblasts (MEFs)[45]. We reconstituted them with different mutants of H2AX (Figure S4d) and tested whether we could detect a modification of the histone at this site. We used the K118-119R H2AX mutant to remove the background Polycomb-dependent mono-ubiquitination. When we immunoprecipitated γH2AX after Ionizing Radiation (IR) treatment, we could observe a band corresponding to mono-ubiquitinated H2AX protein. This band was not present when K13-15 were also mutated, confirming that the modification takes place on these N-terminal residues (Figure 4d). This modification was induced by DNA damage as we could see that the ubiquitination at K13-15 was increased upon IR (Figure 4e and S4e).

Comparable results were obtained in human 293T cells where we introduced mutants of FLAG-H2A. Upon immunoprecipitation of the histone we could observe a damage dependent ubiquitination, only when K13-15 were present (Figure 4f and S4e). This specific modification was fully abolished when RNF168 was depleted in these cells, confirming that this ligase is responsible for the mono-ubiquitination of H2A at K13-15 (Figure 4g and S4f).

Two major E3 ligases are also known to take part in DDR after DSBs: RNF8, that acts upstream of RNF168 allowing its recruitment[11-13] and BRCA1, that is thought to act downstream of RNF168[15-16]. When we depleted RNF8 in these experiments, we observed a overall decrease of the signal and the loss of IR-dependent induction, confirming the role of RNF8 in recruiting RNF168 to chromatin in DDR and suggesting

► that RNF168 is responsible for K13-15 modification. BRCA1 knock-down has no significant effect, while depletion of RNF8 affects the total level of the modification and its IR-dependence, confirming a role for RNF8 in recruiting RNF168. Control qRT-PCRs are shown in Figure S4f-g. **i)** Quantification of K13-15 H2A ubiquitination after treatment with different siRNAs. Data is shown relative to the siRNF168 condition in absence of IR. Error bars show SEM (standard error of the mean) calculated from 3 independent experiments used for each condition. In anti-FLAG blots on panels **f-h**, secondary anti-mouse antibodies specific for heavy chain were used. * indicates background bands. See also Figure S4.

that a background K13-15 modification is present in these cells due to RNF168 activity (Figure 4h-i and S4g). Depletion of BRCA1 did not significantly affect the K13-15 ubiquitination mark, supporting the fact that K13-15 modification is an early event in the DSB signaling (Figure 4h-i and S4f-g).

K63-linked ubiquitin chains on H2A/H2AX are formed on K13-15 in vivo.

Previous studies have proposed that K63 linked ubiquitin chains attached to H2A/H2AX and formed by RNF8 and RNF168 are the functional signal for the DSB repair pathway[18-19].

To understand how these two ligases can achieve this modification on the histones, we studied a possible collaboration between these enzymes. As shown in Figure 4A, RNF168 can provide multiple mono-ubiquitination of H2A in nucleosomes incubated with the E2 UbcH5c. We observed that no additional activity was induced when RNF8 is added to this reaction (Figure 5a).

Because the E2 Ubc13 is important for the DSB pathway[7,12,46] and catalyzes K63 chains when complexed with the UEV cognate proteins[47], we included this E2 in our assays. In presence of both E3s and of both E2s, UbcH5c and the complex Ubc13/Mms2, there was a significant increase of chains on H2A, that is dependent on both E2s and both E3 ligases (Figure 5a and S5a). These chains were not formed if Mms2 (UEV2) was omitted (Figure S5b).

To confirm that the RNF8 dependent ubiquitin chains on H2A are K63-linked, we performed a denaturing IP on nucleosomes containing FLAG-tagged H2A upon incubation with the ubiquitin machinery followed by immunoblotting with the specific anti-K63 antibodies[48] (Figure 5b). We observed only minor K63 modification if RNF8 is omitted from the reaction (Figure 5b). Similar results were obtained using a K63R mutant ubiquitin in the reaction (Figure S5c). These results confirm that, although a minor activity is retained by RNF168, K63 chains on histone H2A in nucleosomes are efficiently extended in vitro by RNF8 and Ubc13/Mms2, once the histone is mono-ubiquitinated.

Using a two-step assay, where we prime H2A for ubiquitination with RNF168 and UbcH5c and later add RNF8 and Ubc13/Mms2 we show that the two reactions are uncoupled. The priming activity of RNF168 and UbcH5c is required for chain formation, because the use of a ligase-inactive mutant[49] of RNF168 results in unmodified H2A (Figure 5c). Furthermore, the addition of RNF8 and Ubc13/Mms2 on other mono-ubiquitinated substrates (e.g. H2A ubiquitinated by Ring1B, PCNA[50], or H2B) also leads to K63 chain formation on these substrates (Figure S5d-f), suggesting that the Ubc13/RNF8 E2/E3 pair can efficiently extend ubiquitin chains on previously mono-ubiquitinated proteins.

The formation of K63 chains on H2A in vitro is efficiently achieved with a two-step reaction where two separate E2/E3 pairs are involved: RNF168 and UbcH5c are responsible for the priming of H2A, and RNF8 and Ubc13/Mms2 catalyze the chain extension of the mono-ubiquitinated protein. Such a two-step mechanism, where priming and chain extension are dependent on different ligases, provides tight regulation of target modification as shown for the poly-ubiquitination of the replication factor PCNA[51].

We wanted to investigate K63-linked ubiquitin chain formation in cells during

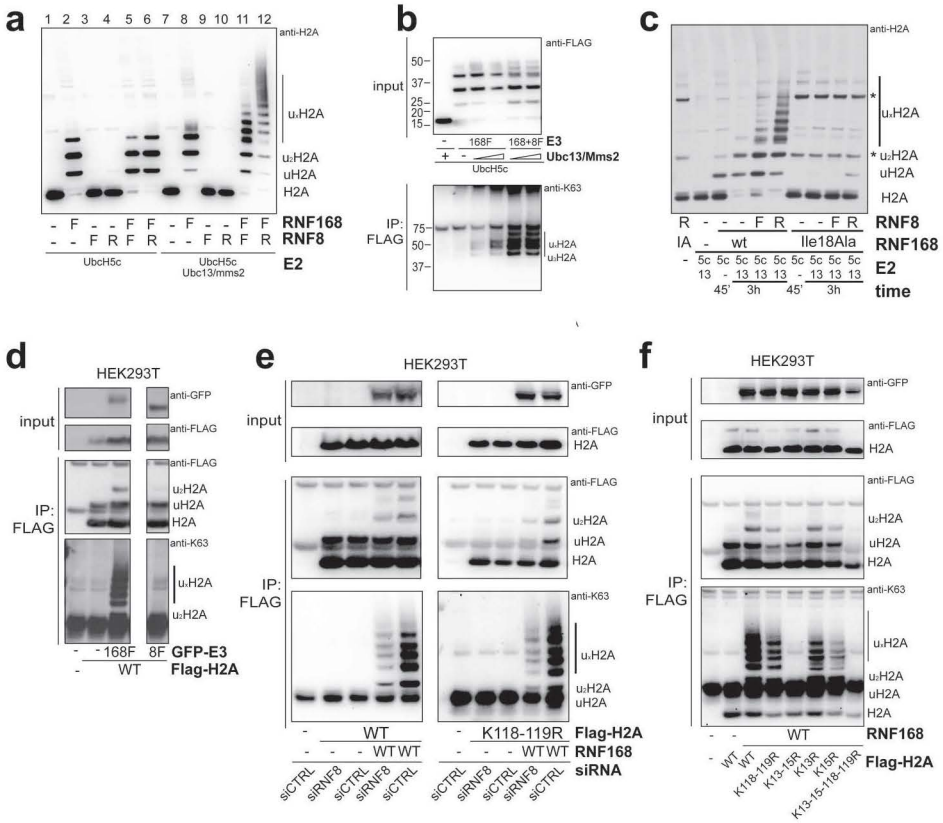

Figure 5 K63 linked ubiquitin chains on H2A and H2AX are specifically conjugated to K13-15. **a)** When RNF8 is added to UbcH5c and RNF168, no additional activity is observed towards H2A (lane 5-6) over RNF168 activity. When Ubc13/Mms2 is added, RNF8 makes chains on ubiquitinated H2A (lane 11-12). **b)** Denaturing FLAG IP was performed after in vitro assay where nucleosomes contained FLAG-H2A. RNF8 efficiently forms K63 ubiquitin chains, while only mild activitity is seen by RNF168. Ubc13/Mms2 was used in equimolar amounts compared to UbcH5c (0.5 μM) and at higher concentration (0.8 μM). **c)** Two-step assay, where nucleosomes were first incubated with RNF168 and UbcH5c to yield mono-ubiquitination and then Ubc13/Mms2 and RNF8 constructs were added to the samples to catalyze K63 chains. RNF8-dependent ubiquitin chain extension is dependent on the catalytic activity of RNF168. GST-tagged RNF168 wt and Ile18Ala (IA) were used. * indicates background bands. **d)** FLAG IP from HEK293T cells transiently transfected with GFP-RNF168 or GFP-RNF8 and FLAG-H2A. No additional ubiquitination of H2A was seen when RNF8 was overexpressed. **e)** Denaturing FLAG IP from HEK293T cells transiently transfected with GFP-RNF168, different siRNAs and FLAG-H2A (WT in left panel, K118-119R in right panel). K63 chains on H2A are dependent on RNF8. Control qRT-PCRs for RNF8 mRNA levels are shown in figure S5h. **f)** K63 chains are specifically formed on K13-15 of H2A. Denaturing FLAG IP from HEK293T cells transiently transfected with GFP-RNF168 and different FLAG-H2A constructs. K63 chains were visualized using the Genentech anti-K63 antibodies. See also Figure S5.

DSB signaling. We initially set out to monitor the chain formation on endogenous histones and therefore immunoprecipitated γH2AX after IR in different cell lines. However we were never able to observe this type of chains (Figure S5g) suggesting that cellular levels of this modification are low or very transient.

To increase the level of mono-ubiquitinated H2A in vivo, we elevated the expression of RNF168. This resulted in robust K63 ubiquitin chains, specifically conjugated to H2A, as observed after denaturating IP of the histone in absence of IR (Figure 5d). Expression of RNF168 is sufficient to drive this poly-ubiquitination, while RNF8 doesn't induce this modification (Figure 5d). Nevertheless, this ubiquitination appears to be RNF8 dependent, as depletion of RNF8 in these cells causes a drastic reduction of this poly-ubiquitin chain consistent with our in vitro data (Figure 5e and S5h). Further unraveling of the relative contributions of RNF8 and RNF168 to H2A ubiquitination is complicated by the tight inter-relation between RNF8 and RNF168 functions during DDR, where activity of RNF168 is dependent on its recruitment by RNF8 and where RNF168-dependent H2A mono-ubiquitination is required for chain extension.

Interestingly, K63 poly-ubiquitin chains are not formed when K13-15 are mutated, whereas the mutation of K118-119 does not significantly affect ubiquitin chain formation on H2A (Figure 5e-f). Thus the K63 chain formation takes place on K13-15 and not on the Polycomb ubiquitination site in vivo.

This mono-ubiquitination by RNF168 in cells is specifically targeted to H2A-type histone, because no increase in H2B ubiquitination was observed upon non-denaturing IP of histone proteins, despite the proximity of the H2B tail to the K13-15 site (10-15 Å) (Figure S5i).

These results demonstrate that RNF168-dependent H2A mono-ubiquitination tightly directs the location of K63 ubiquitin chains during DSB repair and suggest a role for RNF8 in chain extension on this site on H2A.

Target ubiquitination is required for proper DSB signaling

To test whether these findings concerning the specific site of ubiquitination and the order of events that leads to H2A/H2AX modification are relevant during DSB signaling we followed recruitment of factors such as 53BP1 and BRCA1 to DSBs upon DNA damage.

In *H2ax*$^{-/-}$ MEFs reconstituted with lysine mutant variants of H2AX we were unable to observe any significant differences in the recruitment of 53BP1 and BRCA1 to the sites of damage (Figure S6a) indicating that the presence of endogenous wild-type (WT) H2A is sufficient to allow proper signaling. The large number of genes (~16) encoding for histone H2A precludes decreasing the levels of endogenous protein. Therefore we took advantage of the RNF168 target recognition site mutant (R57D) to address whether H2A/H2AX modification is an important step in the ubiquitin-dependent DSB signaling.

First, we tested whether this single point mutation also abolishes H2A/H2AX ubiquitination in cells. Therefore we expressed RNF168 and its loss-of-function mutant R57D in human 293T cells and monitored their ability to target the histone. Wild-type RNF168 strongly enhances H2A ubiquitination and allows K63 chain formation, while the R57D mutant is inactive towards H2A/H2AX (Figure 6a and S6b for H2AX), confirming

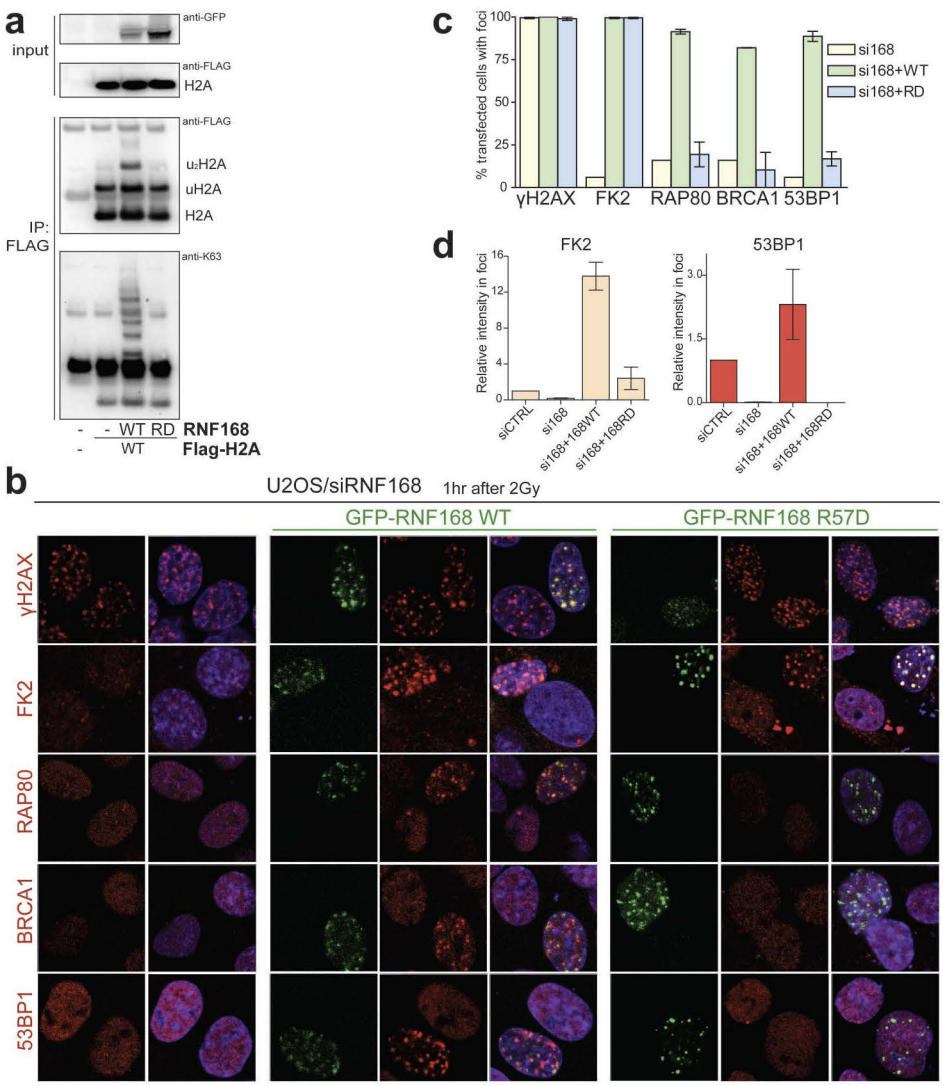

Figure 6 RNF168-dependent target ubiquitination is required for proper DSB signaling. **a)** Loss-of-function mutation R57D in RNF168 inhibits the ligase towards H2A in cells. Denaturing FLAG IP from HEK293T cells transiently transfected with GFP-RNF168 constructs and FLAG-H2A. **b)** Loss-of-function in RNF168 affects recruitment of RAP80, BRCA1 and 53BP1 to DNA damage foci. U2OS cells were transfected with siRNA against RNF168 and with GFP-tagged siRNA-resistant constructs of either empty vector, WT RNF168 or RNF168 R57D, treated with 2 Gy and stained for recruitment of indicated molecules 1 hour after irradiation. The R57D mutant rescues FK2 staining but not recruitment of downstream factors indicating that the H2A modification is critical for damage response. **c)** Quantification of transfected cells with foci for the different markers shown in panel **b**. Error bars show the SEM calculated from 2 independent experiments where 50 cells were counted per condition. **d)** Quantification of the total signal present in foci per cell for FK2 and 53BP1 based on 2 independent experiments similar to the ones shown in panel **b**. Error bars show the SEM. At least 30 cells were counted per condition in each experiment. See also Figure S6.

that this charge reversal affects the target modification also in vivo.

To assess the integrity of the DSB signaling pathway in presence of this RNF168 mutant, we depleted U2OS cells of endogenous RNF168. As shown by others, this renders the cells unable to accumulate ubiquitin chains at the site of damage, which in turn impairs recruitment of RAP80, BRCA1 and 53BP1 (Figure 6b-c).

We then reconstituted these cells with siRNA-resistant constructs of either RNF168 WT or R57D mutant. As expected the WT protein fully restored the pathway, allowing accrual of downstream DSB factors (Figure 6b-c). In contrast the mutant RNF168 R57D was not able to drive the recruitment of RAP80, BRCA1 and 53BP1 (Figure 6b-c).

However, in contrast to RNF168 knockdown, complementation with this mutant resulted in significant formation of ubiquitin chains at the site of damage, as seen by FK2 staining (Figure 6b-c). This finding reflects the in vitro results, where the R57D mutation does not affect chain formation by RNF168. Notably, these ubiquitin chains stain positive for K63, but not K48 chains (Figure S6c-d), in line with published data showing that K48 chains occur at earlier time points after damage as a result of RNF8 activity and are independent of RNF168[52-54].

It is unclear what the docking site of these RNF168-dependent ubiquitin chains is, although autoubiquitination of the ligase could play a role. The total FK2 signal in foci per cell expressing RNF168 R57D mutant is lower than for cells complemented with WT protein (Figure 6d), consistent with the lack of chains on H2A/H2AX. Although this signal is comparable to the endogenous FK2 signal in control cells (siCTRL), the RNF168 R57D mutant does not rescue 53BP1 recruitment (Figure 6d) showing that ubiquitin chains themselves are not sufficient to signal, but that H2A/H2AX ubiquitination is the crucial signal that drives DSB signaling.

Overall these results show that proper modification of H2A is necessary for recruitment of downstream effector proteins in the DDR, while the presence of K63 ubiquitin chains *per se* is not sufficient for signaling.

DISCUSSION

Our study sheds light on the molecular details of the ubiquitination of H2A/H2AX during DSB signaling. Based on the order of their recruitment to the site of damage, RNF8 was previously described as the first ligase to target these histones for ubiquitination, while RNF168 was thought to be involved in the extension of such modification. We show that the previously described order of recruitment for these ligases does not predict the order in which they target H2A/H2AX (Figure 7).

We show that histone ubiquitination during the DSB pathway is initiated by RNF168 on H2A and H2AX, whereas RNF8 is inactive toward them (Figure 1). This finding is further explained by our structure-based mutagenesis, where we identify a single residue that is responsible for target recognition in the RING domain of these ligases (Figure 2-3). Mutation at this site affects H2A/H2AX modification but does not alter the ubiquitin chain forming capacity of these ligases, in vitro or in vivo (Figure 3).

We identify an H2A/H2AX ubiquitination site for the activity of RNF168, K13-15 (Figure

4). We show that during DDR, K63 chains are formed specifically on this site, which distinguishes the DNA damage induced modification from polycomb-mediated K119 mono-ubiquitination (Figure 5). Importantly, we show that histone modification at the damage site is required for proper DSB signaling, because the mere formation of ubiquitin chains is not sufficient to drive the response pathway (Figure 6).

Revised model for RNF8 function in H2A/H2AX ubiquitination during DSB signaling

Our findings challenge the current model for H2A/H2AX ubiquitination during DSB signaling. Once RNF8 accumulates at the lesions, its catalytic activity is required to form ubiquitin chains that will recruit RNF168[11-13]. In this study we provide evidence that RNF168 is the priming ligase for histones, therefore the chains that are responsible for the recruitment of RNF168 must be conjugated to a different substrate (Figure 7). RNF8 was recently suggested to be involved in the ubiquitination of other proteins localized at the site of damage[52-53,55-56], These and possibly more yet to be discovered ubiquitination products of RNF8 might be the docking site for the recruitment of RNF168. Additionally, we show that RNF8 is highly active in making ubiquitin chains (Figure 1B), suggesting that RNF8 could catalyze free chains in proximity of the site of damage similarly to what happens with K63 ubiquitin chains during the NF-kB signaling[57].

Once RNF168 is recruited to the DSB, it will initiate H2A/H2AX ubiquitination on K13-15, where K63 chains will then be extended (Figure 7). We show that in vitro RNF8 efficiently catalyzes formation of this type of chains on the ubiquitinated histones, suggesting that collaboration between the two ligases can take place on H2A/H2AX, with RNF168 catalyzing the priming reaction and RNF8 efficiently extending the K63 chains.

Distinct mono-ubiquitination sites on H2A/H2AX may signal differently

Our study shows that RNF168 is responsible for a ubiquitination on histone H2A/H2AX on K13-15, a different site than the known polycomb site K118-119. The nucleosomal H2A targeting site on the E3 ligases does not account for this H2A lysine specificity, since Ring1B and RNF168 both have a positive charged targeting site, but differ in lysine choice.

We provide evidence that K63 chains are specifically formed on the DDR dependent K13-15 ubiquitination site, suggesting that during DSB signaling the priming reaction of RNF168 is important to tightly control the pathway and to maintain distinct ubiquitin signals on the same target, H2A/H2AX.

Recent studies show involvement of the polycomb E3 ligase proteins Ring1B/Bmi1 in the DSB repair pathway[28-33,58]. Apparently both modifications on H2A are important during the DDR, but since they are located on opposite sides of the nucleosome (Figure S4A) they could provide independent signals in the DDR. On the one hand mono-ubiquitination of K119 could be important for the transcriptional silencing of the regions around the damage, as previously suggested[30,59]. On the other hand the K13-15 poly-ubiquitination could represent a signal that not only allows the recruitment of downstream proteins of the DSB cascade through the K63 chain (e.g. RAP80 binding), but also might induce nucleosomal rearrangements that are important during the assembly of

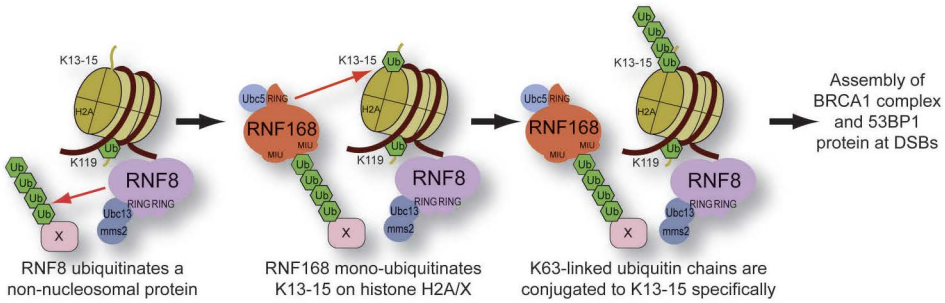

Figure 7 Proposed model for RNF8- and RNF168-dependent activity at DSBs. RNF168 is recruited to DSBs by the activity of RNF8 towards a non-nucleosomal target. RNF168 mono-ubiquitinates H2A-type histones on K13-15 and this leads to K63 Ub chain formation on these lysines, which is required for proper DSB signaling.

the repair machinery through the actual location of this ubiquitination. In fact, K13-15 of H2A are located in proximity to K120 of H2B, which is also a target for mono-ubiquitination in late DSB signaling and in actively transcribed regions where it favors the opening of chromatin[60-62] . This could suggest that RNF168-dependent ubiquitination at K13-15 might induce similar open conformations of the chromatin around the site of damage. It is an interesting question whether the site of attachment of these ubiquitin chains might act as a more complex regulator of the chromatin organization around the damage rather than merely a recruitment station.

EXPERIMENTAL PROCEDURES

Details of experiments are presented in the Supplemental Experimental Procedures.

Cell culture and generation of DSBs

Mouse Embryonic Fibroblasts (MEFs) were received from A. Nussenzweig. All cell lines were cultured in DMEM containing 10% Fetal Bovine Serum (FBS). *H2ax*$^{-/-}$ and wild-type MEFs were cultured at 3% oxygen concentration. Retroviruses carrying FLAG-tagged H2AX constructs were used to generate reconstituted stable cell lines. IR was delivered using a ^{137}Cs irradiation unit with a dose rate of 1 Gy/min.

Protein preparations and crystallography

Full-length RNF8 and Ring1B/Bmi1 were expressed in insect cells, RNF168 and the RING domain constructs were made in E. coli. Oligonucleosomes were purified from human cells as described[5]. Crystals were grown of the RNF8 RING domain (351-485). Crystallographic data collection and refinement statistics are shown in Supplemental Table S1.

Small Angle X-ray Scattering (SAXS)

Samples for the SAXS experiments were prepared in five different concentrations (from 0.4 to 7 mg/ml) in gel-filtration buffer. Data were collected at EMBL Hamburg (beamline X33). Data were analyzed using the ATSAS software package[63].

In vitro ubiquitination assays

Purified human Uba1 at 0.2-0.6 μM was mixed with E2s (0.5 μM), the E3 ligases (0.5-2 μM), ubiquitin (100 μM in all assays

unless otherwise stated), ATP (3mM) and 10 μM of H2A in oligonucleosomes. The reactions were incubated at 32 °C for 3 hours (unless otherwise stated) in buffer 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 μM $ZnCl_2$, 1 mM TCEP. Specificity for K13-15 on H2A for RNF168 is achieved by incubation with lower excess of free ubiquitin (below 10 μM).

E2 discharge assays

UbcH5c was loaded with ubiquitin in presence of E1, Mg^{2+} and ATP. After stopping the reaction with EDTA, this mixture was incubated with the E3s or buffer as control. Samples were analyzed by SDS-PAGE and quantified.

Immunoprecipitations (IP)

For FLAG IPs, transiently transfected 293T or stable MEF cell lines were harvested directly after irradiation. After a PBS wash, cells were lysed and sonicated in E1A buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Tween-20) in presence of phosphatase, protease inhibitors. In denaturing IPs, 0.5% SDS was added to the lysates after sonication. The samples were then diluted 10 times and incubated with beads to avoid denaturation of the antibodies.

For γH2AX IPs, cells were irradiated and were directly harvested. Acidic extraction of histone proteins was performed from isolated nuclei. After neutralization, histones were incubated O/N with anti-γH2AX antibodies (Millipore) and pulled-down with Protein G beads (GE Healthcare).

Immunofluorescence studies (IF)

U2OS cells were first transfected with siRNAs, followed by DNA transfections 24 hours later. Cells were damaged with ionizing radiation (2 Gy) 48 hours after siRNA transfection and fixed in 2% paraformaldehyde in presence of 0.1% Triton X-100 1 hour after damage. Coverslips were washed and incubated at room temperature with primary antibody for 1 hour. After incubation for 1 hour with secondary antibody, samples were embedded in DAPI containing Vectashield mounting medium (Vector). Quantifications were done using confocal images, analyzed by the Fiji Software.

ACCESSION NUMBER

Coordinate and structure factor were deposited in the Protein Data Bank under identification code 4AYC.

SUPPLEMENTAL DATA

The Supplemental Data includes Supplemental Experimental Procedures and 6 supplemental figures (39 panels).

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F.M. designed and performed all experiments and wrote the manuscript. J.H.A.V. contributed to cell-based experiments and discussions. J.A.M. + W.V. + E.C. supervised immunofluorescence experiments and imaging. J.A.M. performed quantifications of immunofluorescence data. W.J.vD. assisted in purification and western blots. P.I. cloned and purified the RNF8 RING domain. T.K.S. designed and supervised experiments and wrote the manuscript. All authors critically read the paper.

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids and Cloning

cDNAs for human full-length RNF8, RNF168, HA-RNF8, GFP-RNF168, were received from J. Lukas[3-4], for Ube2w and Ube2e2 from R. Klevit. pET expression plasmids for *Xenopus laevis* histones were a gift of K. Luger. Expression vectors for the RING domains of Ring1B and Bmi1 were described[5], for full-length RNF8 a modified version of pFastBac1 with a GST-tag with 3c cleavage site for insect cell expression was used, for the RNF8 RING (351-485) domain a pGEX6p1 vector, for the RNF168 RING domain (1-189) a pETNKI-His-SUMO2-LIC-kan vector for E. coli expression was used[6]. FLAG-H2A and H2AX (from L. Penengo) were cloned in pCDNA3.1 and in pMSCVpuro. Mutations were inserted using the QuikChange Mutagenesis Kit from Agilent.

Antibodies, oligos and enzymes

Antibodies used for Western blot analysis: anti-Ub (P4D1 from Santa Cruz, 1:1000), anti-H2A (Millipore, 1:1000 dilution), anti-H2AX (Millipore; 1:1000), anti-H2B (Millipore, 1:1000 dilution), anti-UbH2B (Medimabs, 1:1000 dilution), anti-H3 (Millipore, 1:500), anti-H4 (Millipore, 1:1000), anti-PCNA (Santa Cruz, 1:2000 dilution), anti-CDK4 (Santa Cruz, 1:1000), anti-pCHK2 (Cell Signaling, 1:500), anti-BRCA1 (Cell Signaling, 1:1000 dilution) anti-mouse HRP conjugated (BioRad, 1:10000 dilution), anti-rabbit HRP conjugated (BioRad, 1:20000 dilution), anti-mouse (heavy chain specific) HRP-conjugated (Jackson ImmunoResearch, 1:10000). Antibodies used for immunofluorescence (IF) experiments are: anti-53BP1 (Santa Cruz, 1:500 dilution), anti-BRCA1 (Santa Cruz, 1:100 dilution), anti-RAP80 (a gift from J. Chen), anti-γH2AX (Millipore, 1:1000 dilution), Conjugated ubiquitin (FK2, BIOMOL International 1:1000-5000), anti-FLAG (Sigma, 1:1000 dilution), anti-K63 anti-K48 (Genentech[2], 1-0.3 μg/ml) in combination with the corresponding secondary antibodies labeled with Alexa Fluor 488 or 568 or 594 (Invitrogen). Purified K0 ubiquitin was purchased from Boston Biochem. siRNA targeting RNF168 (code J-007152-05: ACACUUUCUCCACAGAUA), RNF8 (5'GGACAAUUAUGGACAACAA) and BRCA1 (pool of D-003461-06 and D-003461-07) were purchased from Thermo scientific. Enzymes used for cloning were purchased from ROCHE and NEB. Chromatography columns and beads material is from GE Healthcare.

Protein purification

E1 and E2s, ubiquitin and the oligonucleosomes were purified as described previously[5,7]. Recombinant histones purification and histone octamer refolding was performed as previously shown[8]. GST-tagged RNF168 was expressed in E.coli and purified using GSH beads in buffer containing 30 mM HEPES 8.0, 250 mM NaCl, 10% glycerol, 1 μM $ZnCl_2$, 1 mM TCEP, in presence of COMPLETE EDTA-free, DnaseI and 1 mM $MgCl_2$, the tag was cleaved by 3C protease and the protein was further purified by Heparin affinity chromatography. Recombinant full-length human RNF8 was expressed in Sf-21 cells and purified on glutathione beads in buffer 50 mM HEPES pH 8, 500 mM NaCl, 10% glycerol, 1 μM $ZnCl_2$, 1 mM TCEP. After

elution with 50 mM Glutathione, the protein was cleaved in solution with 3c protease. Protein was diluted to 50 mM NaCl and purified by Heparin affinity chromatography and gel-filtration. The RING domain of human RNF8 was expressed in E.coli and purified over glutathione beads in buffer 50 mM HEPES pH 8, 50 mM NaCl, 10% glycerol, 1 μM $ZnCl_2$, 1 mM TCEP. After elution with 50 mM Glutathione, the protein was cleaved in solution using 3c protease and purified on an S75 Superdex column in tandem with a GSH column, followed by ion exchange chromatography on a ResourceQ column. The RING domain of human RNF168, was expressed in E.coli and purified over Nickel Sepharose beads in buffer 50 mM HEPES pH 8, 500 mM NaCl, 10% glycerol, 10 mM imidazol, 1 μM $ZnCl_2$, 1 mM TCEP. The protein was eluted in 400 mM imidazol and the imidazol was then removed using a desalting column. The protein was concentrated and cleaved with SENP2 for 4 hours or O/N. The cleaved sample was reloaded on Nickel beads and the unbound sample was injected on a Superdex 75 column for final purification in 50 mM HEPES pH 8, 250 mM NaCl, 10% glycerol, 1 μM $ZnCl_2$, 1 mM TCEP. Full-length and the RING domain constructs of Ring1B/Bmi1 was purified as previously shown[5,9]. Proteins were concentrated to 1-10 mg/ml and stored at -80°C in their gel-filtration buffer. Mutants were purified as wild-type proteins.

Multi-Angle Laser Light Scattering (MALLS)

MALLS experiments were performed at 4°C on a Mini-Dawn light scattering detector (Wyatt Technology) online with a S75 10/300GL in 50 mM HEPES pH 8, 250 mM NaCl, 10% glycerol, 1 μM $ZnCl_2$, 1 mM TCEP. The RING domain construct of RNF168 WT and R57D mutant had comparable elution profiles from the gel-filtration. The retention volume was lower than expected from the molecular weight measured by the MALLS, suggesting an elongated shape of the proteins as expected from the helical arrangement of the UMI-MIU1 region at the C-terminal part of these constructs (Figure 1a). Data were analyzed using ASTRA Software and GraphPad Prism was used to prepare the figure.

Crystallization and structure determination

Crystals of RNF8 RING domain (351-485) protein (~10 mg/ml) were grown at room temperature in 0.1 M Na Cacodylate pH 6.6-6.9, 2.4-2.5 M Ammonium Sulphate and 0.8 mM of N,N'-Bis(3-D-gluconamidopropyl)deoxycholamide) (Deoxy Big CHAP), by vapor diffusion in 150 nl drops with 1:1 precipitant to protein ratio. The crystals appeared within 24-48 hours, were transferred to mother liquor containing an additional 25% glycerol and vitrified in liquid nitrogen. Diffraction data were collected at SLS, beamline X06SA to a resolution of 1.9 Å. Intensities were processed with XDS[10] and Scala[11-12]. The structure was determined using the Single Anomalous Dispersion (SAD), from the anomalous signal of the zinc ions at 1.282Å wavelength. The four zinc sites were located by SHELXD[13] and were used to calculate the phase probability distributions in Phaser[14]. Following density modification by DM[15], ARP/wARP 7.0[16] built most of the structure automatically (131 residues for chain A, 122 for chain B out of 135 total residues per chain), using the SAD function in REFMAC5[17] for refinement. The model was completed in iterative cycles of model building using

COOT[18] and refined using REFMAC5[17], and autoBUSTER[19]. Optimized weighting terms for B-factor restrains and X-ray weight were derived with PDB_REDO[20] and used for final refinement. Structure images were generated with PYMOL[21] and CCP4mg[22]. The final model comprises residues 351-483 in chain A, residues 359-485 in chain B. Structure validation was done with MolProbity[23] (98th percentile), with no outliers in the Ramachandran plot. Data collection and refinement statistics are presented in Supplemental Table S1.

Small Angle X-ray Scattering (SAXS)

The samples were thawed and centrifuged at high speed for 1 minute just before measurement. Samples were exposed in a measuring cell cooled to 10 °C for 15 s per frame with 8 frames per sample, after which the frames that did not indicate radiation damage were averaged. Data was analyzed using the ATSAS software package[24]. PRIMUS was used for the buffer subtraction and data reduction. No difference in scattering was observed between the curves for the different concentrations. Data quality was then assessed using Guinier plot for low-angle data and signal-to-noise in PRIMUS for wide-angle data and the curve measured at 3.5 mg/ml of protein was chosen for analysis. GNOM generated the particle distance distribution function P(r). DAMMIF generated a set of 10 *ab initio* models, which were all compatible with the elongated shape of the crystal structure (NSD values between 0.6 and 0.74). DAMAVER averaged the models and generated the envelope that was used for the final superposition to the crystal structure, done with SUPCOMB. CRYSOL generated the theoretical scattering curve for our crystal structure for the comparison to the experimental data.

In vitro ubiquitination assays

The PCNA ubiquitination assays were carried out at the same temperature, same buffer and time, with 150 nM of E1, 2 μM of E2, 1 μM of E3, 500 nM of PCNA and 45 μM of ubiquitin. Mono-ubiquitinated H2B enriched Drosophila oligonucleosomes were a gift from the P. Verrijzer lab[25]. To widely screen conditions for RNF8 activity towards H2A in nucleosomes, we tested ranges of 0.1-0.6 μM E1 (Uba1), 0.5 μM E2, 0.5-8 μM E3 and 10-20 μM of H2A in oligonucleosomes, 0.5-100 μM ubiquitin, A very low activity for full-length was detected only when ubiquitin was present in saturating amount compared to the nucleosomes and after more than 1 hour reaction.

E2 discharge assays

To load the E2, E1 at 0.2 μM was mixed with 20 μM UbcH5c in presence of ubiquitin (20 μM) Mg^{2+} and ATP (5 mM) in buffer 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 μM $ZnCl_2$, 1 mM TCEP. Sample was incubated 15 minutes at 37°C. Reaction was stopped with 50 mM EDTA final concentration. This mixture was then incubated at 37°C with the E3s or buffer as control. Samples were stopped at given times and were analyzed by SDS-PAGE. Quantifications were done using Image Lab software (Bio-Rad). GraphPad Prism Software was used for statistics.

Western blotting

SDS Loading buffer was added to samples from in vitro reactions or from immunoprecipitations. Samples were boiled and loaded on 4-12% NuPAGE gels in MES buffer (Invitrogen), except for panels 4 f-h where 12% NuPAGE gels were used in MOPS buffer. Detection

was performed by Western Blot using the corresponding antibodies.

Generation of stable cell lines

Retrovirus carrying empty vector, wild-type or mutants of H2AX were harvested and used to infect *H2ax*$^{-/-}$ MEFs cells overnight. The next day, puromycin was added to the cells to select the infected population.

FLAG Immunoprecipitation (IP)

Plasmids and siRNAs were transfected into HEK293T cells using Calcium Phosphate. Cells were irradiated ~45 hours after transfection and were directly harvested. WT and reconstituted MEFs were proliferating for 3 days before irradiation. After a PBS wash, cells were lysed in E1A buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Tween-20) in presence of phosphatase, protease inhibitors and iodoacetamide. Lysates were sonicated and spun down, the supernatant was used as input sample. ANTI-FLAG® M2 Affinity Gel from SIGMA was used for the IP. Beads were incubated 4-16 hours, washed and then loaded on gel for Western blot analysis. In denaturing IPs, 0.5% SDS was added to the lysates after sonication. After centrifugation the supernatant was diluted 10 times and incubated with beads to avoid denaturation of the antibodies. For immunoprecipitation of FLAG-tagged histones after in vitro reactions, 0.5% SDS was added to the samples and after 10 fold dilution they were incubated with ANTI-FLAG® M2 Affinity Gel (SIGMA) for 4-16 hours.

γH2AX immunoprecipitation (IP)

Cells were irradiated and were directly harvested. Hypotonic buffer (10 mM HEPES pH 7.4, 10mM KCl, 0.05% NP-40 and protease inhibitors) was used to isolate the nuclei. Acidic extraction of histone proteins was performed in 0.2 M HCl and after neutralization with 0.4 M Tris HCl pH 8.0, 200 mM NaCl, 10 mM $MgCl_2$, phosphatase and protease inhibitors, histones were incubated O/N with anti-γH2AX antibodies (Millipore). Protein G beads (GE Healthcare) were added to the samples and after 3 hours incubation, beads were spun down, washed and samples were prepared for western blot analysis.

Immunofluorescence studies

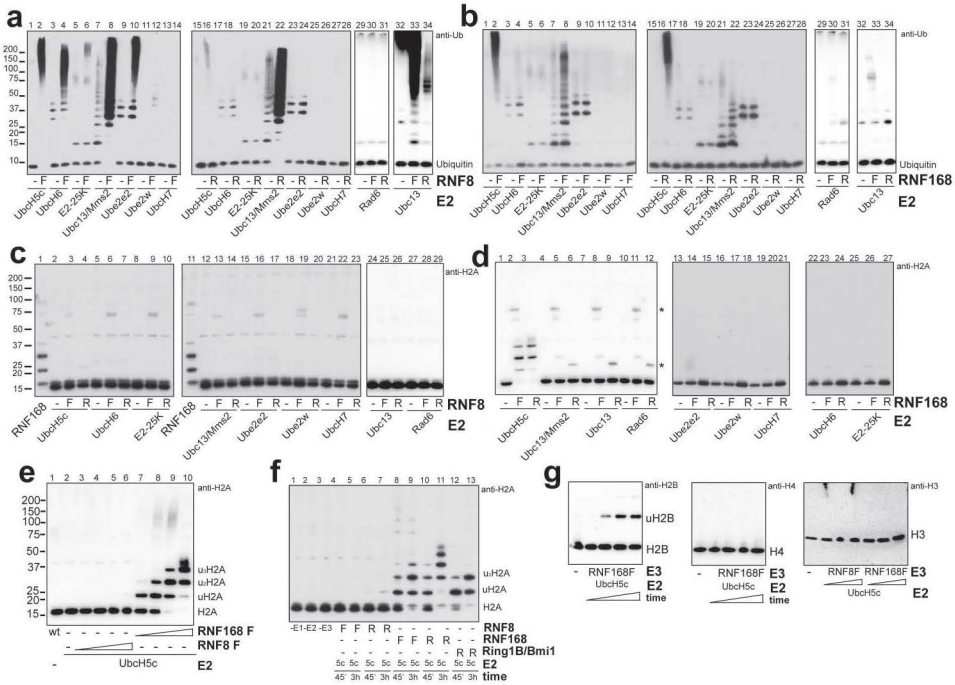
DNA transfections in U2OS cells were carried out using FUGENE6 (ROCHE), siRNA transfections were done using LIPOFECTAMINE RNA iMAX (Invitrogen). The cells were first transfected with siRNAs, 24 hours later the DNA transfection was performed. Cells were damaged with 2 Gy, 40 hours after siRNA transfection and fixed in 2% paraformaldehyde in presence of 0.1% Triton X-100 1 hour after damage. Coverslips were washed 5 times with PBS containing 0.1% Triton X-100 and subsequently washed with PBS^{+} (PBS containing 0.15% glycine and 0.5% bovine serum albumin). Cells were incubated at room temperature with primary antibody for 1 hour, then washed 5 times with PBS-Triton X-100 and PBS^{+}. After incubation for 1 hour with secondary antibody and wash in PBS-Triton X-100, samples were embedded in DAPI containing Vectashield mounting medium (Vector). Immunofluorescent images were obtained using confocal microscope (LSM 510 META; Carl Zeiss, Inc.) equipped with a 63× 1.4 NA Plan Apochromat oil immersion lens (Carl Zeiss, Inc.) or LEICA AOBS confocal microscope with ×40 (NA 1.25) oil and ×63 (NA 1.4) oil objectives (Leica). LSM image browser acquisition software (version 4.0; Carl Zeiss, Inc.) or Leica confocal Software was used.

Quantification of FK2 and 53BP1 signal was based on two independent experiments where 30-70 cells were counted per condition in each experiment. Confocal images taken from these samples were used for the quantification using the Fiji software. The DAPI channel was used to select the nuclei of the cells in the field, the green channel was used to select the transfected cells and quantify the protein level of GFP-RNF168. Foci in the red channel were defined as particles bigger than 0.2 μm^2 with an intensity higher than 80 (on a 1-255 scale). The total signal in foci per cell is calculated as the product of the number of foci, foci mean intensity and focus area. Cells expressing high levels of RNF168 (cutoff 80 on a 1-255 scale) were excluded from the quantification. Three different siCTRL and si168 samples were used per experiment and the average intensity was used for quantification. Data is presented as normalized to the siCTRL samples. Excel (Microsoft Office) and GraphPad Prism were used for the analysis.

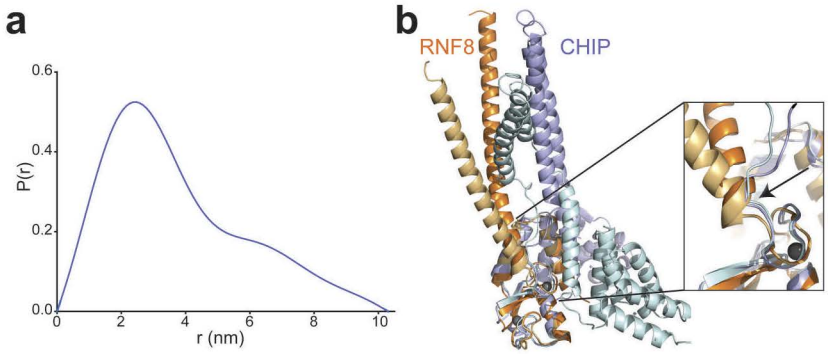
qRT-PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen) and cDNA was prepared using Superscript II RT and oligod$(T)_n$ primers (Invitrogen). qRT-PCR was performed on a StepOne Plus Realtime PCR system using SYBR Green PCR mastermix (Applied Biosystems). The amount of target, normalized to an endogenous reference (HPRT) was calculated by: $2^{-\Delta\Delta C}$T. The primer sequences were as follows: RNF8fwd 5'-ttacagtccc agctgtgtgc-3'; RNF8rev 5'-ccttgggctatctcc aaacc-3'; RNF168fwd 5'-caacgtggaactgtgg acgat-3'; RNF168rev 5'-tactgagcagacgaact ggctg-3'; HPRTfwd 5'-cggctccgttatggcg-3'; HPRTrev 5'-ggtcataacctggttcatcatcac-3'.

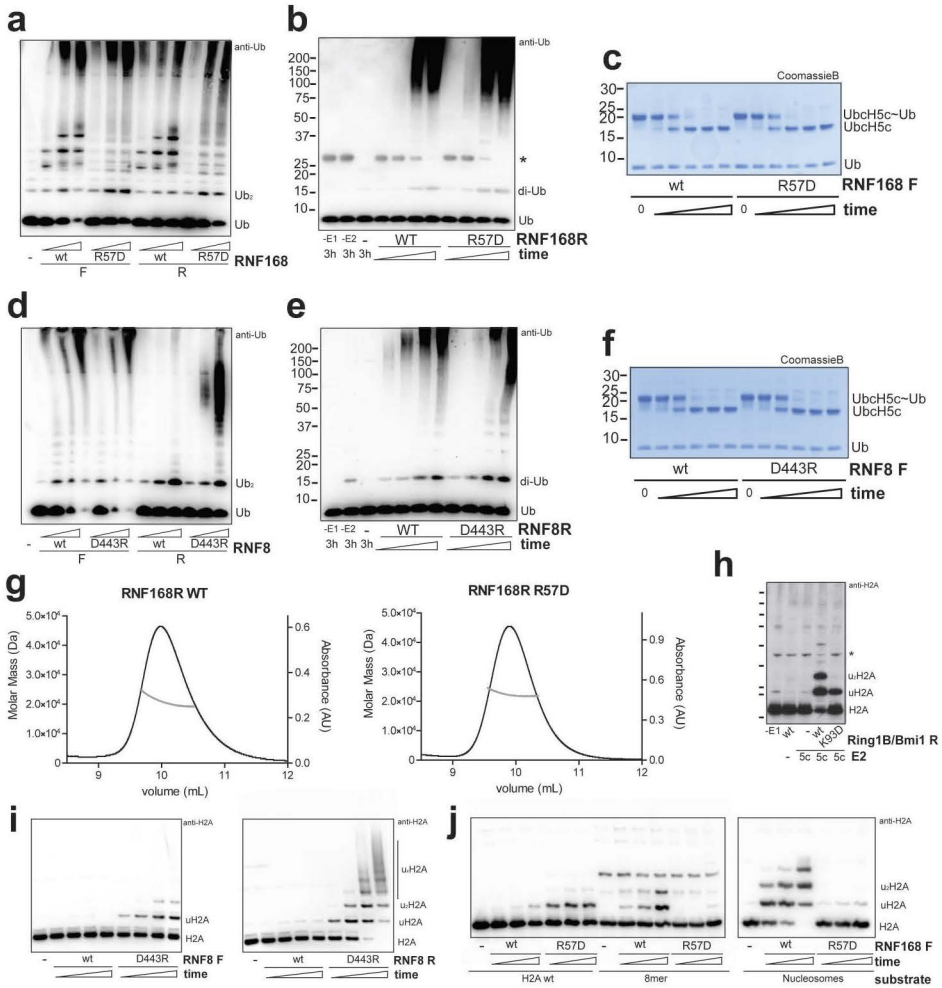
SUPPLEMENTAL MATERIAL



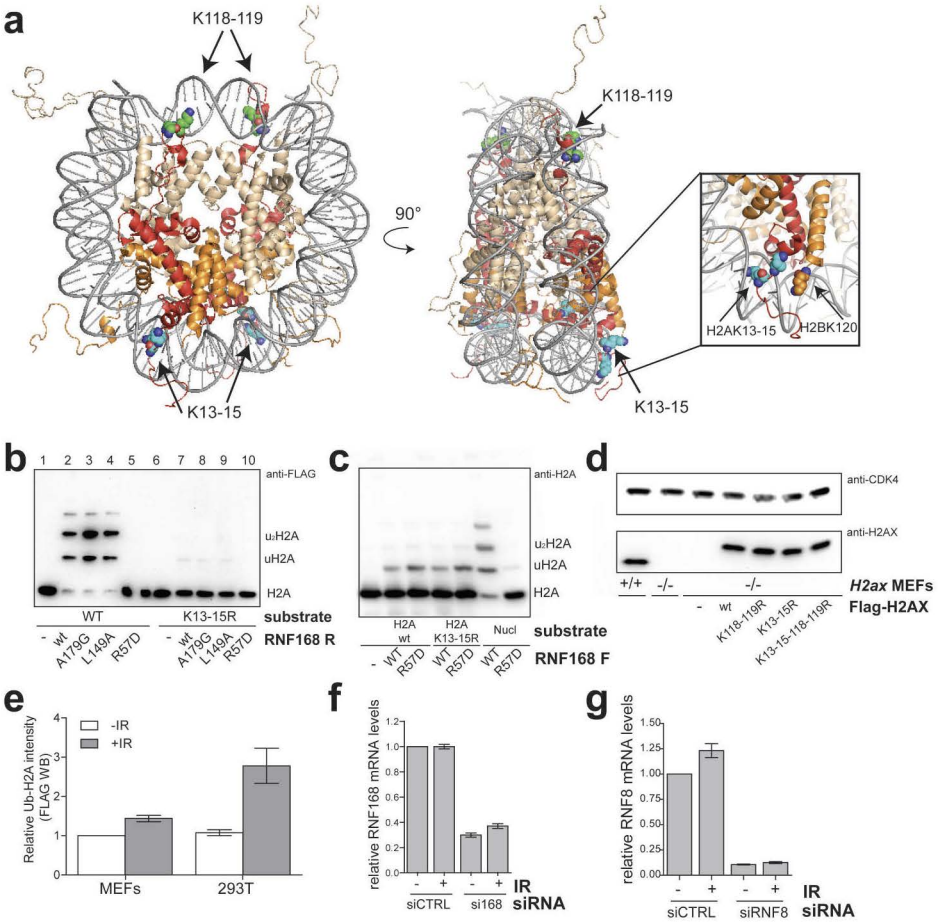
Supplemental Figure S1. Related to Figure 1. Ubiquitin chain formation and nucleosome-directed activity of RNF8 and RNF168 **a,b)** Ubiquitin chain formation activity of the full-length and RING domain of RNF8 (**a**) and RNF168 (**b**) with different E2s. Ubiquitin was used at 15 μM concentration. **c)** Full-length RNF8 and its RING domain are unable to modify H2A in nucleosomes with any E2. **d)** Full-length RNF168 and its RING domain can modify nucleosomal H2A primarily with UbcH5c. * indicates background bands. **e)** Concentration series (0.05- 015-0.45-1.35 μM E3) assay in presence of purified oligonucleosomes. Full-length RNF8 has virtually no activity against H2A in nucleosomes, whereas full-length RNF168 is active in presence of UbcH5c. **f)** Full-length RNF8 (F, lanes 4 and 5) and its RING domain (R, lane 6 and 7) have no activity (F) or almost no activity (R) against H2A in nucleosomes, whereas full-length RNF168 (lanes 8 and 9), its RING domain (lanes 10 and 11) and the RING domain of Ring1B/Bmi1 (lanes 12 and 13) are active. **g)** Time course experiments to show full-length RNF168 activity towards H2B, H3 and H4 with UbcH5c. Concentration series for full-length E3s activity towards H3 (0.5-1-2 μM).



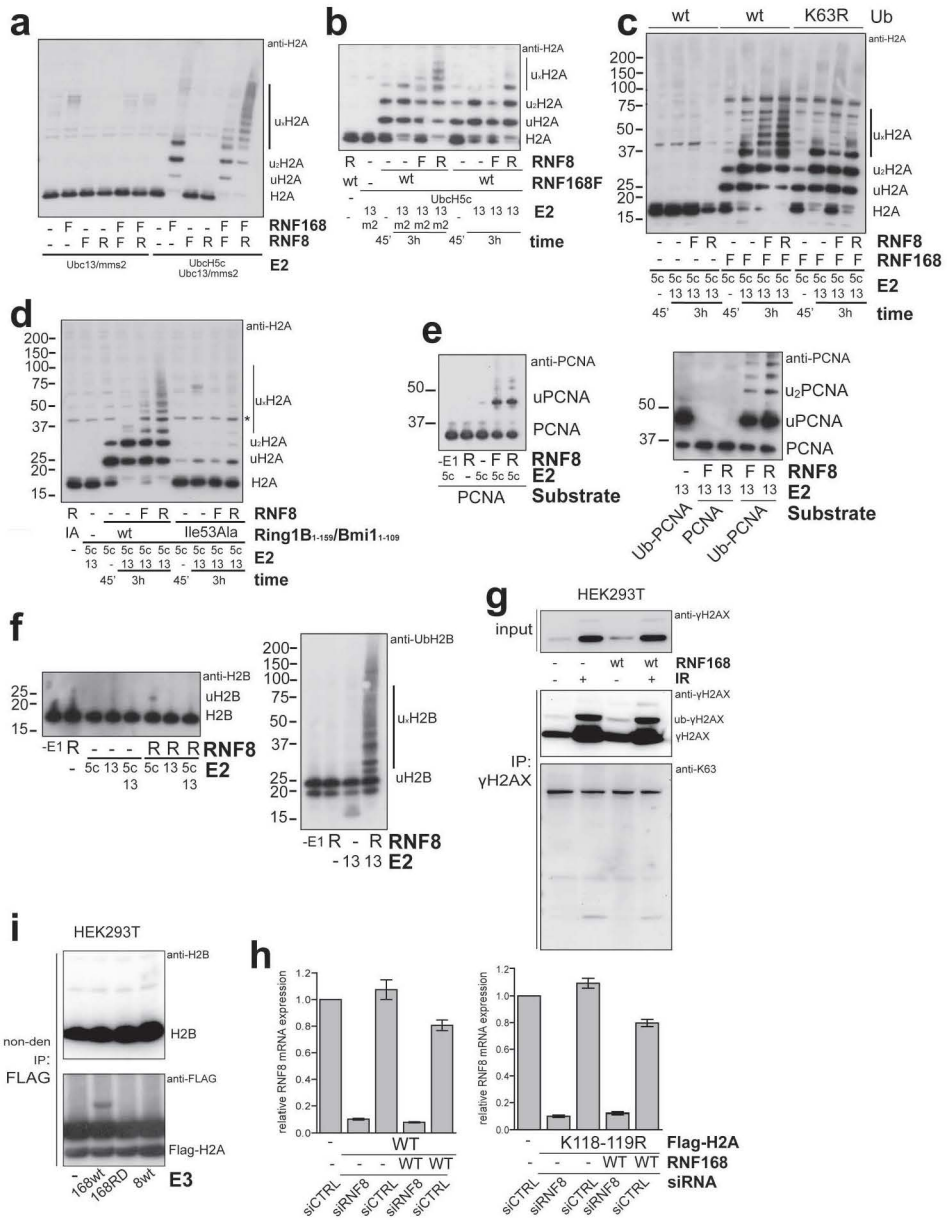
Supplemental Figure S2. Related to Figure 2. **a)** The symmetrical interatomic distance probability distribution (P(r)) for the atoms of RNF8 RING domain calculated with GNOM using the SAXS experimental data. **b)** Superposition of the monomers of the RNF8 RING domain dimer in orange and asymmetric monomers of the crystal structure of full-length CHIP in blue (PDB code: 2C2L) shows that the location of the point of divergence between the protomers is conserved. Arrow in the close up indicates the point of divergence between protomers. Images were prepared using GraphPad Prism and PyMOL.



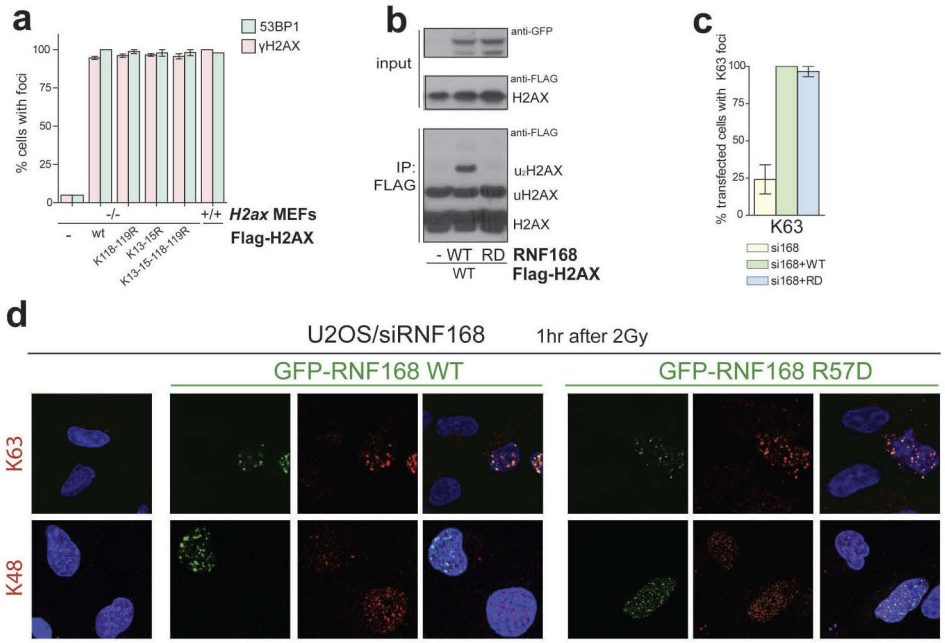
Supplemental Figure S3. Related to Figure 3. Analysis of target recognition mutations in RING E3 ligases. **a, d)** Anti-ubiquitin blots of the samples shown in Figures 3b for RNF168 (**a**) and 3d for RNF8 (**d**). **b,e)** Time course (10-30-90-180 minutes) ubiquitin chain formation assay in the presence of UbcH5c and 1μM E3 to compare wild-type and mutant activity of RING domains of RNF168 (**b**) and RNF8 (**e**) reveals that chain formation abilities are not affected by charge reversal. Ubiquitin was used at 15 μM concentration. * indicates aspecific bands. **c,f)** Basis for quantification shown in figures 3d,g. SDS-PAGE gel showing discharge rate of UbcH5c by full-length RNF168 WT and R57D mutant (**c**) and full-length RNF8 WT and D443R (**f**). Time course: 2-4-8-16-24 minutes, E2 concentration: 20 μM. E3 concentrations: 0.5 μM for RNF168 and 0.1 μM for RNF8. **g)** Multi-Angle Laser Light scattering (MALLS) and gel filtration profiles of the RING construct of RNF168 WT (left panel) and R57D (right panel). Left Y-axis shows scale for measured molecular mass (gray line), Right Y-axis shows UV absorbance values (black line). Calculated molecular weight of the RING domain construct is 22.1 kDa. **h)** The equivalent charge reversal mutation the Ring1B RING domain (K93D) also reduces ligase activity towards nucleosomal H2A. **i)** Time course (10-30-90-180 minutes) assay to compare WT and D443R RNF8 constructs towards nucleosomal H2A. **j)** Time course assay (30-60-180 minutes) to assess the activity of full-length RNF168 WT and R57D towards H2A in free form, in presence of the other histones or in oligonucleosomes. Ubiquitin concentration was set to 5 μM.



Supplemental Figure S4. Related to Figure 4. **a)** Crystal structure of the nucleosome (PDB code: 1KX5[1]). In red is H2A, in orange H2B, in wheat H3 and H4. DNA is in gray. K118-119, the polycomb target lysines are shown as spheres in green. K13-15, the RNF168 target residues are shown in cyan spheres. Close up showing the proximity between the N-terminal tail of H2A (K13-15 shown in cyan spheres) and the C-terminal part of H2B (K120 shown in orange spheres). Images prepared using PyMOL. **b)** The RING construct of RNF168 retains specificity for K13-15 on nucleosomal H2A. MIU1 (A179G) or UMI (L149A) mutations do not affect its activity or the site specificity. The R57D mutant as used as control. **c)**. Full-length RNF168 doesn't retain specificity for K13-15 when the histone is in free form; oligonucleosomes were used as control. Ubiquitin concentration was set to 5 μM and the samples were incubated for 2 hours at 32°C in assays shown in panels **b** and **c**. **d)** Expression levels of exogenous H2AX constructs in reconstituted *H2ax*$^{-/-}$ MEFs is comparable to WT MEFs. CDK4 was used as loading control. **e)** Quantification of the relative induction of H2A ubiquitination on K13-15 for MEFs and HEK293T cells. 3 different experiments were used per cell line. Image Lab (BioRad software) was used for the quantification. **f)** Graph showing relative amount of RNF168 mRNA transcript in samples shown in Figure 4g, measured by qRT-PCR and normalized to HRPT levels. **g)** Graph showing relative amount of RNF8 mRNA transcript in siRNF8 samples shown in Figure 4h, normalized to HRPT levels. Error bars show the SEM (standard error of the mean).



Supplemental Figure S5. Related to Figure 5. K63-linked ubiquitin chains in vitro and in vivo. **a)** In absence of UbcH5c there is no modification of H2A in nucleosomes. Samples were treated as the ones shown in Figure 5a. **b)** In absence of Mms2 chains on H2A are not formed. In vitro two-step assay as in Figure 5c. 13-m2 stands for the complex Ubc13/Mms2, while 13 stands for Ubc13 alone. In all the other assays the complex Ubc13/Mms2 was used. **c)** K63R ubiquitin mutant abolishes chain extension on H2A by RNF8 and Ubc13/Mms2. **d)** RNF8 can catalyze K63 chains on H2A mono-ubiquitinated by Ring1B/Bmi1. RNF8-dependent chain extension capacity is dependent on the catalytic activity of Ring1B. IA stands for the mutant protein I53A. * indicates background bands. **e,f)** RNF8 and ►



Supplemental Figure S6. Related to Figure 6. Importance of RNF168-dependent H2A/H2AX mono-ubiquitination during DDR. **a)** Quantification of γH2AX and 53BP1 foci in WT and *H2ax*$^{-/-}$ MEFs reconstituted with different FLAG-H2AX constructs. 50 cells were counted for 2 independent experiments. **b)** Non-denaturing FLAG-IP from 293T cells expressing FLAG-H2AX and GFP-RNF168 construct. R57D mutation abrogates the activity of RNF168 towards H2AX in cells. **c,d)** Additional information to Figure 6b. Immunofluorescence staining using anti-K63 and anti-K48 antibodies for cells depleted of endogenous RNF168 and then expressing WT or R57D RNF168 constructs. Both variants of RNF168 can catalyze K63 ubiquitin chains at the site of damage. Quantification of transfected cells with K63 foci are shown in panel c. 30 cells where counted per condition, from two independent experiments. Antibodies were obtained from Genentech[2]. Error bars show the SEM.

► Ubc13/Mms2 efficiently recognize and poly-ubiquitinate already ubiquitinated PCNA (e) and H2B (f), but they are inactive towards the unmodified proteins. **g)** γH2AX IP to test K63 chain formation on endogenous histones did not yield to the visualization of any K63 chains. **h)** Control qRT-PCR showing mRNA levels for RNF8 in samples used for figure 5e. Values were normalized to HPRT levels. Error bars show the SEM. **i)** Non-denaturing FLAG-IP for histone H2A shows activity of RNF168 WT towards H2A but not towards histone H2B.

Supplemental table S1. Related to Figure 2. Data collection, phasing and refinement statistics (SAD) for the structure of the RING domain of RNF8.

Data collection	
Space group	$P2_12_12$
a, *b*, *c* (Å)	47.37, 213.72, 34.10
Resolution (Å)	47.37-1.9 (2.00-1.9)
R_{merge}	0.041(0.602)
I/s*I*	25.8 (2.7)
Completeness (%)	99.1 (95.2)
Redundancy	6.9 (5.9)
Refinement	
Resolution (Å)	1.9
No. reflections	28115
$R_{work/}$ R_{free} (%)	20.0 / 22.6
No. atoms	
Protein	2206
Ligand/ion	86
Water	58
B-factors	
Protein	48.3
Ligand/ion	76.4
Water	41.0
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.198

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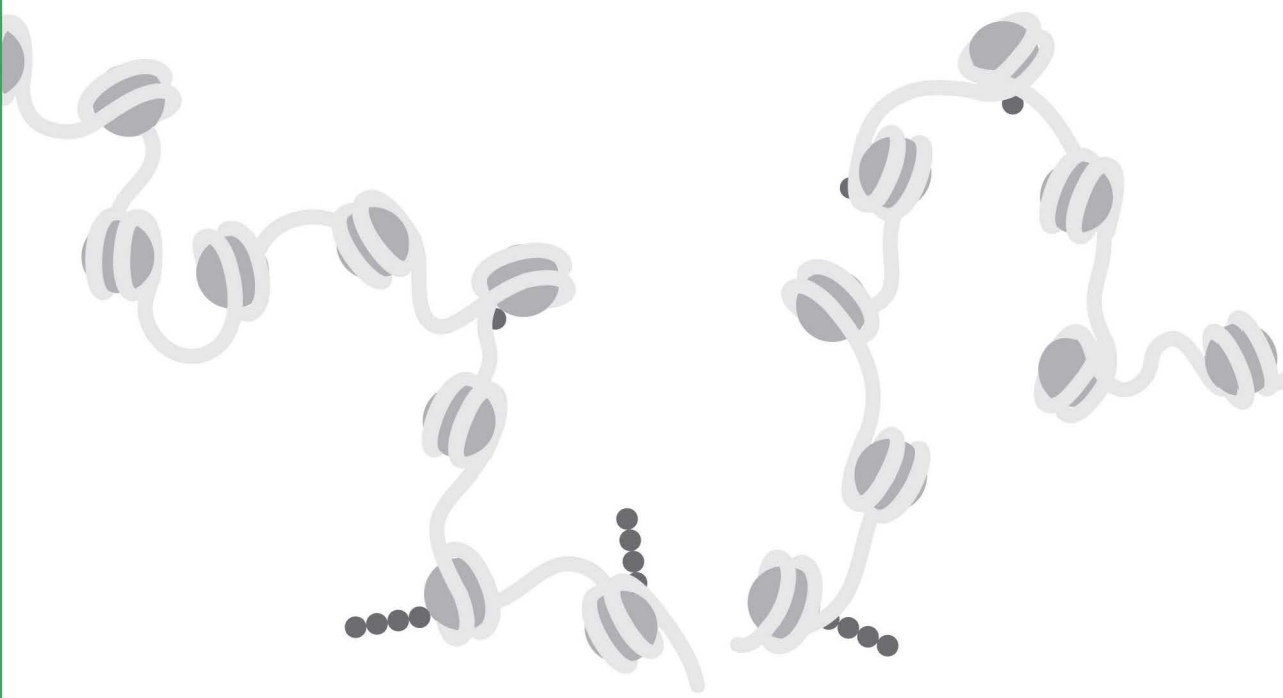

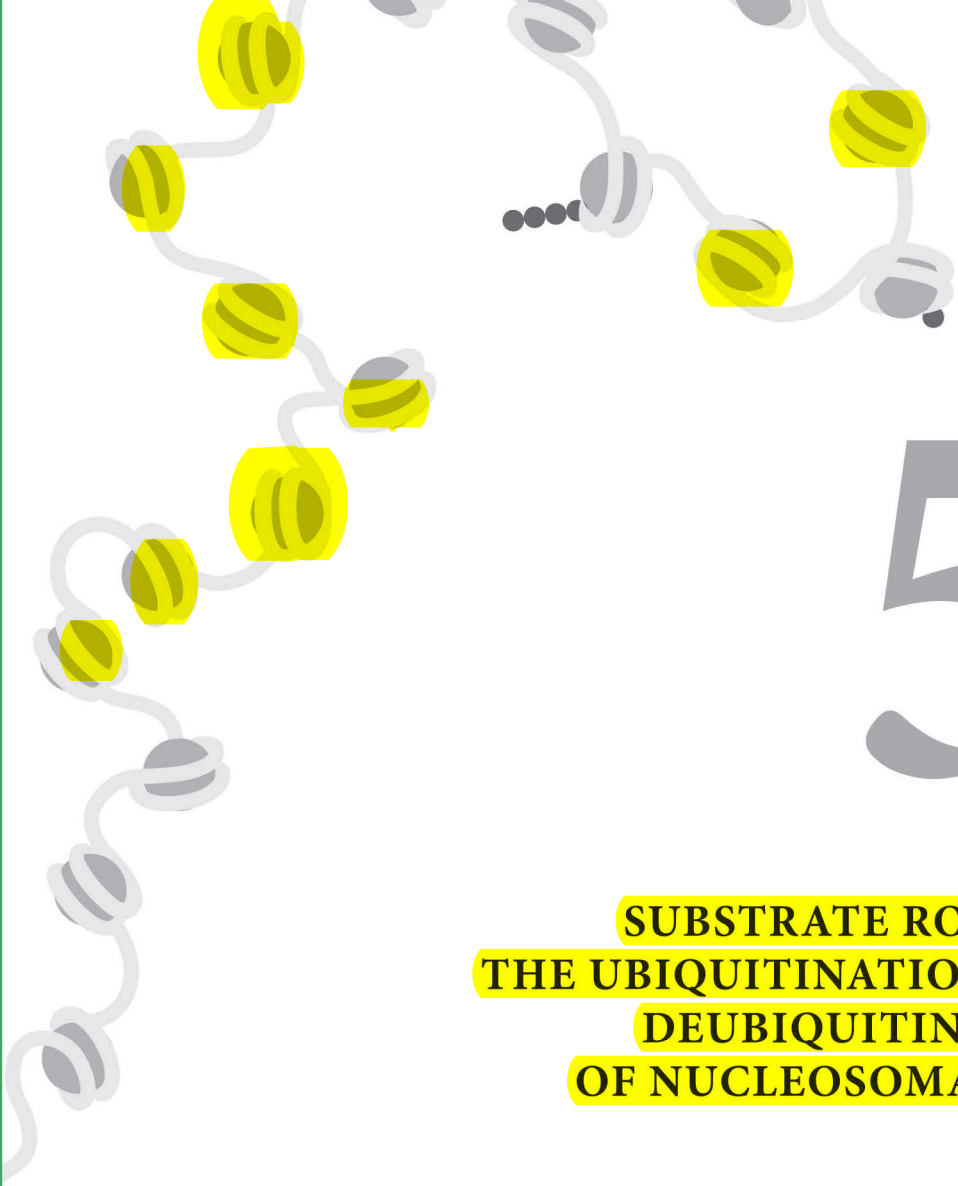

5

SUBSTRATE ROLES IN THE UBIQUITINATION AND DEUBIQUITINATION OF NUCLEOSOMAL H2A

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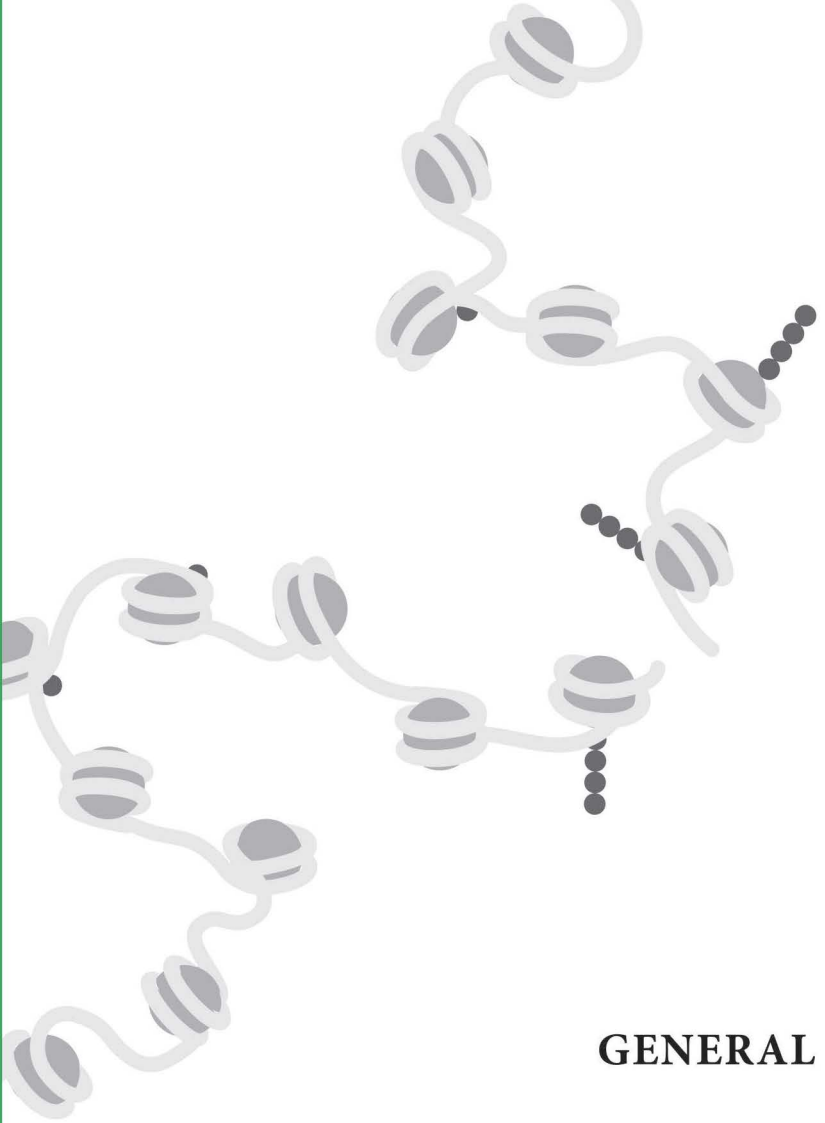

6

GENERAL DISCUSSION

Ubiquitination is a post-translational modification widely used by the cell to regulate function, fate and localization of proteins. The conjugation of ubiquitin takes place on a lysine residue on the target protein and it requires a three step cascade of E1, E2 and E3 enzymes. To balance this modification, a variety of deubiquitinating enzymes (DUBs) are present in the cell to de-conjugate the ubiquitin moiety.

These processes lead to diverse types of modification (monoubiquitination or polyubiquitination) on the substrate that will have diverse effects. While for some modifications the cellular and molecular function are known, in other cases the details of the signaling remain obscure. Understanding the structural and biochemical details of these processes is key to the possibility of use them in targeted therapy. This thesis presents new molecular insights into the mechanisms of histone H2A ubiquitination. Modification of H2A is crucial in the epigenetic transcriptional silencing by Polycomb proteins as well as in the DNA damage response pathway. The work presented contributes to dissect the differences and similarities of these pathways with particular focus to the E3 ligases reaction. Here I'll discuss the main findings and will point out possible future directions.

Dimeric RING E3 ligases

We began in chapter 2 with an overview of the structural aspects of multi-domain RING E3 ligases involved in DNA repair[1]. Among other aspects, we focused our attention on the way dimerization occurs in this class of enzymes. Interestingly, a large number of these E3s either heterodimerizes or homodimerizes in an asymmetrical fashion[2-6]. In these instances it is plausible that only one of the two RING domains within the dimer is capable to bind the charged E2 enzyme to allow catalysis. This is confirmed for PRC1 by our biochemical analysis of the full-length E3 ligases presented in chapter 3 where only the E2 interaction interface of the Ring1B subunit is important for activity. Moreover, the crystal structure of the dimeric RING domain of RNF8 presented in chapter 4 shows an asymmetrical arrangement that although it may be induced by crystal contacts, it could represent a structural and functional feature conserved also in the context of the full-length protein[5].

So, if only one RING domain is required for catalysis, why are RING E3s mostly dimeric? While in the case of heterodimers a putative role in target selection could be assigned to the partner RING protein, in cases of homodimers the answer to this question is at the present moment still a mystery. It is conceivable that the presence of two charged E2s on one dimer is not compatible with selective catalysis on a specific lysine, nevertheless it could provide a highly efficient reaction in cases of multiple ubiquitination events. Further studies addressing these questions and comparing the enzymatic mechanism of monomeric RING ligases, such as RNF168[7], with the dimeric ones are of real interest for the understanding of the activity of these enzymes.

Modulation of PRC1 E3 ligase activity

In chapter 3 we present a biochemical analysis of the different E3 ligase complexes present in PRC1. Interestingly we observed that the two full-length paralogs Bmi1 and Mel18 differentially stimulate the E3 ligase activity of Ring1B and Ring1A. This finding is interesting, particularly in the light that, when using the isolated RING domains

of these proteins, these differences are not present. Investigation of the aspects important for the cross-talk between the two components of the dimer will aid to the understanding of the role of Bmi1 and Mel18 for the E3 ligase function in PRC1. These differences may reflect the different functions that are proposed in vivo for PRC1 complexes containing the different paralogs[8-11]. Several studies have begun to unravel possible roles for the C-terminal domains of these PRC1 proteins[12-16]. The C-terminal part of Ring1B and Ring1A, as well as of the PCGF subunits contains a ubiquitin-like domain (RAWUL domain)[13]. This domain in Ring1B is important for binding to other PRC1 proteins (e.g. CBX7 and RYBP)[14-16], but it has also been proposed that this region is involved in homodimerization[12,16]. No study has yet addressed the consequences of such dimerization for the E3 ligase activity of the complex, and particularly the possibility of heterodimerization between the RAWUL domains of RING and PCGF subunits. It would be interesting to investigate if these domains have a role in regulating E3 activity of the PRC1 ligases.

On a different note, it has been shown that some Ring1B target genes are not enriched in ubiquitinated H2A[17]. This raises the question: how is the E3 ligase activity regulated in those regions? Post-translational modifications as well as PRC1 complex composition may well have a role. In vitro analysis of the influence of such modifications and of PRC1 subunits can now be performed using the tools described in chapter 3.

Order of events in DNA double-strand break repair pathways

Previous to the studies presented in chapter 4, the order of activity of RNF8 and RNF168 towards H2A was believed to be synchronous to their order of recruitment to the site of damage, namely RNF8 first to initiate ubiquitination[18-21] and RNF168 next to extend the polyubiquitination[22-24]. Our work shows that on H2A the order of recruitment is not predictive of the order of their activity. Although RNF8 is recruited first, only RNF168 is capable of initiating H2A ubiquitination and RNF8 may be involved in the formation of the K63 polyubiquitin chain on the ubiquitinated histone[5].

This finding implies that recruitment of RNF168 must occur via ubiquitin chains formed on a yet unknown substrate. Identification of this substrate is crucial for our understanding of the molecular steps required during DSB repair signaling. The molecular response to DSBs has proven to be extremely complex. The coexistence of simultaneous signaling cascades in this response has become evident by the work of several groups. In fact, in parallel to ubiquitination, sumoylation and phosphorylation are employed by the cell for signaling[25-28]. Being able to uncouple the different signals and focus on one ligase reaction in cells proves to be difficult to date. Nevertheless mass spectrometry analysis to identify and dissect ubiquitin-related processes has proven successful in other pathways[29-33], it is then conceivable that proteomic approaches may aid to the unraveling of these enzymatic step in the DSB signaling.

Novel H2A ubiquitination site

In chapter 4, in parallel to a study from a different group[34], we identify a novel

target site on H2A, K13-15, specific for the DNA damage dependent modification by RNF168[5]. Because K119 ubiquitination is so abundant and long-known, previous studies have assumed that during DSB signaling H2A ubiquitination would take place on the same site. The finding of a novel site greatly widens our understanding of the role of H2A ubiquitination during DDR. We discuss aspects of this in chapters 4 and 5, here we would like to expand on different points.

First, this finding uncouples DDR ubiquitination from the Polycomb ubiquitination, indicating that regulation of H2A ubiquitination at this site is likely to be distinct and differentially controlled, as we began to unravel in chapter 5. Nonetheless, it is clear that Polycomb E3 ligases can accumulate at the site of DSBs. These observations bring up the question: why do both modifications exist at the lesion and how can they differentially signal? As multiple pathways are working in parallel during DDR, it is conceivable that their functional distinction may well reflect a selected organization of the location of their target on chromatin regions around the damage. More explicitly, we can envision that Polycomb proteins may target only the nucleosomes in the chromatin regions that require transcriptional control in proximity of the damage, e.g. coding regions. Instead, RNF168-dependent modification may represent the local signal for damage and its deposition may only be dependent on the location of lesion and independent on the chromatin organization. A possibility could be that the presence of specific histone modifications cross-talks to the ubiquitination machineries to direct the deposition of the ubiquitin mark at specific nucleosomes, in particular for Polycomb E3s.

The structural separation of the two ubiquitination sites on nucleosomes suggests that the two modifications could even coexist on the same nucleosome particle. This would allow a specific DDR signaling in cases of damage in regions were the Polycomb mark is present and it raises structural and functional consequences for the binding partners of the two modifications. Can they coexist on the same nucleosomes? But more importantly, can both of them properly function when they are simultaneously present on the same nucleosome? Our understanding of the readers of the two ubiquitination sites is very limited at this moment, but these questions are of great relevance in the understanding the signalings arising from them. A recent study[35] has investigated the co-existence of epigenetic histone marks on single nucleosomes, these aspects of H2A ubiquitination could be addressed in a similar fashion.

Moreover, the N-terminal ubiquitination site K13-15 is modified with K63 ubiquitin chains as we show in chapter 4, while to our knowledge the Polycomb site K119 is modified by monoubiquitination only (chapter 3). In addition to the diverse signals that the two types of modification will stimulate, an interesting question arises: how is the K13-15 initial ubiquitination specifically selected for polyubiquitin extension? This could just be a topological issue in the context of the nucleosome: the extension E3 ligase may recognize the nucleosome in a specific way targeting only the K13-15 side. Our work proposes that RNF8 is the ligase involved in this polyubiquitination step, we also show that RNF8 activity in vitro is highly promiscuous and it can target K119 as well as K13-15 monoubiquitination[5]. This would argue in disfavor of this model, unless the binding of RNF8 to the phosphorylated MDC1 protein

has roles in its local orientation at chromatin. Alternatively, the choice for K13-15 extension versus K119, could involve binding proteins to H2AK119ub: it is plausible that interacting proteins may have roles in masking the ubiquitin moiety from further modification. So far only ZRF1 and Ring1B itself have been suggested as binding partners of H2AK119ub[36], future work should expand on additional binders and on their potential role in preventing K63 chain formation. A combination of *in vitro* and *in vivo* studies is required to this end, with the work described in this thesis we have set up a variety of assays that may assist to investigate these points.

Finally, a variety of DUBs targeting H2A have been proposed prior to the identification of the K13-15 ubiquitination site. This discovery urges us to investigate the specificity of these enzymes for either of the H2A modification sites, in chapter 5 we show a first analysis in this direction. It's clear that the modulation of the signals rising from H2A ubiquitination at these two sites also depends on the activity of these DUBs. In a first place, identification of the target lysine on H2A for each DUBs is crucial to assign roles to these DUBs in each signaling cascade. Subsequently, investigation of the timing of action of the DUBs is key to the understanding of the duration of the presence of these ubiquitination marks in cells. Lastly, dissecting the mechanisms driving the dynamics of ubiquitin conjugation and de-conjugation on nucleosomal H2A represents an important goal to understand the cellular and molecular functions of these modifications.

Participation of the nucleosome in substrate-assisted catalysis

The work presented in chapter 5 suggests that the acidic patch on the nucleosome is involved in substrate-assisted catalysis by the RING domain of RNF168, and preliminary data support a similar role of this patch for Ring1B.

This finding represents a similarity between Ring1B- and RNF168-driven reactions on nucleosomes and it is somewhat surprising. In this context the diverse nature of the RING domains of these E3s may have a role. RNF168 is monomeric, while Ring1B heterodimerizes with Bmi1 in these experiments and they seem to bind the nucleosome in a different fashion. These observations suggest that while RNF168 likely sits on the acidic patch during catalysis, for Ring1B/Bmi1 RING domains the Bmi1 subunit may be in contact to the nucleosomal patch, while Ring1B contacts DNA. These speculations are supported by our structural knowledge of these proteins. Nevertheless, they suggest a novel interesting aspect where Bmi1 binding to the nucleosome induces an additional activation of the Ring1B-mediated catalysis. This needs to be tested and can be addressed with *in vitro* reconstitution experiments.

Moreover, this shared surface on the nucleosome required for activity of both E3s may be part of a different level of regulation of the two modifications. It will prevent the two ubiquitination reactions to happen simultaneously on the same nucleosome and it may function to establish order for the modifications which may be important to cross-talk to other molecular events during signaling.

More interestingly, the concept of the substrate actively participating to the last step of the ubiquitination reaction by RING domains is novel and fascinating. It was already known that RING domains

are allosteric activators of the release of ubiquitin from the charged E2 enzyme[37-39], our finding would suggest that the substrate molecule can potentially further activate this release. This mechanism could provide an additional level of regulation developed by the cell in optimizing selectivity and efficiency of E3 reactions. The complexity of this mechanism allows specific ubiquitination only when the E3/E2~ub comes in contact with the substrate in the right orientation, where the lysine is available in proximity to the E2-ubiquitin thioesther and the RING domain is contact with a distal region involved in the activation (the acidic patch in the case of H2A). This may explain the low affinity interactions that are observed between RING E3s and their substrates: binding might not be the only mechanism for target recognition, but allosteric activation at the moment of binding is insuring an efficient and specific reaction. A similar role for substrate-assisted catalysis has been proposed for residues on ubiquitin itself during E2-dependent ubiquitin chain formation[40-41] and these mechanisms may well apply to other targets during E3-directed ubiquitination.

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SUMMARY

Ubiquitination refers to the attachment of a small protein named ubiquitin to a target protein. This modification can change the function, the localization or the fate of the target in the cell. Ubiquitination is a three-step enzymatic process, where E1, E2 and E3 enzymes are involved. Target proteins are recognized by the E3 ligase which allows the final ubiquitin conjugation on a lysine (K) residue. This class of enzymes is vast and includes different sub-groups. In **chapter 2** we present an overview of the structural aspects of one of those sub-groups, the RING/Ubox E3 ligases with focus on their role in DNA repair pathways. Collecting structural and mechanistic observations, we discuss diverse aspects of their activity, in particular roles of dimerization, interaction with the E2s and chain-type specificity.

In the following chapters of this thesis, we present structural and biochemical studies on the mechanisms that yield the ubiquitination of the histone protein H2A. H2A is one of the structural components of the nucleosome, the minimal units responsible for DNA compaction in the cell nucleus. Modifications on histone proteins, including ubiquitination, are known to be important for a variety of cellular processes. In particular, ubiquitination of H2A occurs in cells during two cellular signaling pathways: the transcriptional regulation by Polycomb proteins and the DNA damage response to DNA double-strand breaks (DSBs). This thesis aims at the definition of the structural and biochemical requirements for functional H2A ubiquitination in both these pathways.

In **chapter 3**, we focus on the biochemical analysis of the different E3 ligases present in the Polycomb PRC1 complex. We describe methods for *in vitro* purification of these ligase dimers. We find that their activity towards nucleosomal H2A is comparable in the context of the isolated RING domains, but for the full-length proteins we observe differences. We investigate some aspects of the modulation of the activity of the full-length Ring1B/Bmi1 complex. We confirm that in this complex Ring1B is the active RING ligase, by using mutagenesis of residues important for E2 interaction. Moreover, focusing on the role of phosphorylation of the complex, we find that AKT can stimulate the activity of Ring1B/Bmi1 *in vitro*. In order to study the influence of other PRC1 components to the E3 function, we set up insect cell expression systems to obtain three- and four-component complexes.

In **chapter 4**, we move to the analysis of the H2A ubiquitination by the DNA damage ligases RNF168 and RNF8. With this work, we define a novel order of events that lead to the polyubiquitination of H2A, challenging previous models. We identify a novel ubiquitination site on H2A, K13-15 that is targeted in response to DSBs by RNF168. We show in vitro and in cells that this E3 targets this new site and initiate H2A ubiquitination, while we propose that RNF8 functions in the extension of K63 polyubiquitin chains on the ubiquitinated histone. We identify a target recognition site on the RING domain of these ligases that determines their ability to target H2A. By mutational analysis of this site in RNF168 in cells we show that ubiquitin chain formation at DSB sites *per se* is not sufficient for the integrity of the signaling, while modification of H2A during is required for the response pathway.

In **chapter 5**, in consequence to the identification of a novel target site on H2A, we investigate the determinants for the specificity of the E3 ligase reactions. We find that RNF168 and Ring1B differentially recognize H2A within the nucleosome. We determine that the minimal substrate required for specific K13-15 modification is the H2A/H2B dimer. In contrast to Ring1B that requires the assembly of the whole nucleosome. We also identify a novel role for the acidic patch present on the nucleosome in participating to the catalysis of both E3 ligases.

In **chapter 6**, we discuss the main findings of the work presented in this thesis and we speculate on their future implications.

Overall, this thesis presents evidences of the functional separation between Polycomb- and DNA damage-related H2A ubiquitination. This has broadened our way of thinking about these two pathways and it elucidates how, even on one small protein like H2A, parallel and distinct ubiquitin signals can coexist to sustain and regulate the healthy survival of the cell.

SAMENVATTING

Ubiquitinering is het proces van vastmaken van een klein ubiquitine-eiwit aan een substraateiwit. Deze modificatie kan de functie, lokalisatie en de bestemming van een substraateiwit veranderen. Ubiquitinatie is een enzymatisch proces dat wordt verzorgd door een cascade van E1, E2 en E3 enzymen. Hierbij worden substraateiwitten herkend door E3 ligases, die de uiteindelijke ubiquitine conjugatie aan een lysine residu (K) bewerkstelligen. Deze E3 ligases vormen een uitgebreide groep enzymen die bestaat uit meerdere subgroepen. In **hoofdstuk 2** geven we een overzicht van de verschillende aspecten van een van deze subgroepen weer, de RING/Ubox E3 ligases. Hierbij richten we ons op de rol die deze enzymen spelen in DNA reparatie mechanismen. In een combinatie van structurele en mechanistische observaties, bespreken we diverse aspecten van de activiteit van E3 ligases, met name de rol van dimerisatie, interacties met E2's en de specificiteit van ubiquitine keten formatie.

In de daarop volgende hoofdstukken van dit proefschrift laten wij structurele en biochemische studies zien van de mechanismen die leiden tot de ubiquitinering van het histon eiwit H2A. H2A is één van de structurele componenten van de nucleosoom, de minimale bouwsteen die verantwoordelijk is voor compactie van het DNA in de celkern. Modificaties van histon eiwitten, zoals ubiquitinering, is belangrijk voor een groot scala aan cellulaire processen. Ubiquitinering van H2A vindt in het bijzonder plaats in twee verschillende signaalcascades in cellen: de transcriptionele regulering van Polycomb eiwitten en de reactie op DNA schade bij DNA dubbel-strengs breuken (DSB's). In dit proefschrift definiëren we de structurele en biochemische vereisten voor functionele H2A ubiquitinatie binnen deze signaleringsroutes.

In **hoofdstuk 3** concentreren wij ons op de biochemische analyse van de verschillende E3 ligases die aanwezig zijn in het Polycomb PRC1 complex. Wij beschrijven hierin methoden voor de opzuivering van deze ligase dimeren *in vitro*. Hierbij vinden wij dat de activiteit van de geïsoleerde RING domeinen van deze eiwitten op nucleosomaal H2A vergelijkbaar is, maar we zien verschillen in de activiteit van de complete eiwitten op dit substraat. We onderzoeken verschillende aspecten van de modulatie van de activiteit van het Ring1B/Bmi1 complex en bevestigen dat Ring1B de actieve RING ligase is in dit complex door mutagenese van residuen die belangrijk zijn voor E2 interacties. Door de rol van fosforylering van het complex te analyseren vinden we bovendien dat AKT de activiteit van Ring1B/Bmi1 *in vitro* kan stimuleren. Om de invloed van de andere PRC1 componenten op de E3 functie te kunnen bestuderen, hebben we een expressie systeem in insectencellen opgezet om drie- en vier-componenten complexen te verkrijgen.

In **hoofdstuk 4** verleggen wij de analyse naar de H2A ubiquitinatie ten gevolge van DNA schade die door RNF168 en RNF8 wordt uitgevoerd. Hier definiëren wij een nieuwe volgorde van de gebeurtenissen die leiden tot de polyubiquitinering van H2A, waarmee bestaande modellen worden aangevochten. We vinden dat K13-15 op H2A is een nieuwe site die door RNF168 geubiquitineerd wordt ten gevolge van DSBs. We laten *in vitro* en in cellen zien dat deze residuen als doelwit dienen voor RNF168 ter initiatie van

H2A ubiquitinering, en stellen dat RNF8 de initiële ubiquitinatie van de histonen verlengt met K63 polyubiquitine ketens. Ook identificeren we residuen op het RING domein van deze ligases die de mogelijkheid om H2A als substraateiwit te gebruiken bepalen. Vervolgens laten we in cellen zien met mutant RNF168, waarin deze site gemuteerd is, dat ubiquitine keten formatie bij DSBs niet voldoende is voor de integriteit van de DSB signalering, maar dat modificering van H2A vereist is voor de signaalcascade.

In **hoofdstuk 5**, bouwen we voort op de identificatie van K13-15 als een nieuwe ubiquitinatie-site op H2A. We bestuderen we de factoren die nodig zijn om de specificiteit van de E3 ligases te bepalen en ontdekken dat RNF168 H2A binnen nucleosomen op een andere manier herkent dan Ring1B. We vinden dat de H2A/H2B dimeer het minimale substraat is voor de K13-15 modificatie door RNF168, terwijl Ring1B het hele nucleosoom nodig heeft voor herkenning. Ook identificeren we een nieuwe rol voor het negatief geladen gebied dat aanwezig is op het nucleosoom voor de katalyse van de activiteit van beide E3 ligases.

In **hoofdstuk 6** bespreken we de bevindingen die staan beschreven in dit proefschrift en speculeren we over de toekomstige implicaties hiervan.

Dit proefschrift laat verschillen zien tussen de functionele verdeling tussen Polycomb- en DNA schade-gerelateerde H2A ubiquitinering. Dit heeft onze manier van denken over deze twee signaalcascades verbreed en licht toe hoe, zelfs op een klein eiwit als H2A, parallelle en verschillende ubiquitine signalen kunnen samenkomen ter ondersteuning en regulatie van een gezonde overleving van de cel.

SOMMARIO

Nelle nostre cellule alcune proteine vengono modificate mediante l'attacco di una piccola proteina chiamata "ubiquitina". Questo processo prende il nome di ubiquitinazione. Questa modificazione può cambiare la funzione, il destino o la localizzazione della proteina substrato. L'ubiquitinazione avviene attraverso tre reazioni enzimatiche che coinvolgono gli enzimi E1, E2 ed E3. Le ligasi E3 hanno il compito di riconoscere il substrato e sono in grado di catalizzare la coniugazione dell'ubiquitina su residui di lisina (K) del substrato. Questa classe di enzimi è vasta ed è formata da diversi sottogruppi. Nel **capitolo 2** rivisitiamo alcuni aspetti di biologia strutturale del funzionamento di uno di questi sottogruppi, le ligasi E3 che contengono il dominio RING/Ubox e che sono implicate in processi di riparazione dei danni del DNA. Utilizzando informazioni sul loro meccanismo d'azione discutiamo vari aspetti delle loro funzioni, in particolare la loro capacità di dimerizzare, le interazioni con l'E2 e la specificità nella formazione di certi tipi di catene di ubiquitina.

Nei capitoli successivi, presentiamo studi strutturali e biochimici sui meccanismi utilizzati da diverse ligasi E3 nel catalizzare l'ubiquitinazione dell'istone 2A (H2A). Questo istone è una delle proteine strutturali che compongono i nucleosomi. I nucleosomi sono le unità che permettono il compattamento del DNA dentro al nucleo delle nostre cellule. Gli istoni vengono modificati in molti modi e queste modificazioni sono importanti per la regolazione di diversi processi cellulari. In particolare, l'ubiquitinazione di H2A avviene in risposta a due segnali cellulari: la regolazione trascrizionale dettata dalle proteine Polycomb e il processo cellulare stimolato dalla presenza di danni alla doppia elica del DNA. Questa tesi ha l'obiettivo di definire i dettagli strutturali e biochimici delle reazioni che permettono la corretta ubiquitinazione di H2A in entrambi questi processi.

Nel **capitolo 3**, ci focalizziamo sull'analisi biochimica delle diverse ligasi presenti nel complesso Polycomb PRC1. Descriviamo nuovi metodi usati per la purificazione *in vitro* dei diversi dimeri di E3 presenti in questo complesso. Mostriamo che usando i construtti contenenti solo i domini RING di queste ligasi, la loro attività verso H2A nei nucleosomi è comparabile, mentre quando usiamo le proteine full-length osserviamo differenze nell'attività verso questo substrato. In questo capitolo, studiamo come l'attività di una di queste ligasi, Ring1B/Bmi1 viene modulata. Mediante un analisi mutazionale dei residui importanti per l'interazione con l'E2, mostriamo che in questo dimero Ring1B è la componente attiva della ligasi. Inoltre, analizziamo il ruolo della fosforilazione del complesso e troviamo che la chinasi AKT è in grado di stimolare l'attività di Ring1B/Bmi1 *in vitro*. Con l'intento di studiare l'influenza di altri componenti di PRC1 sull'attività delle E3, abbiamo sviluppato e testato un sistema di espressione in cellule d'insetto per la produzione di complessi contenenti tre o quattro proteine.

Nel **capitolo 4**, analizziamo invece l'ubiquitinazione di H2A da parte delle ligasi RNF168 e RNF8 nei processi cellulari in risposta a danni del DNA. Qui definiamo un nuovo ordine di eventi che porta alla poliubiquitinazione di H2A, in contrasto con i modelli proposti in precedenza. Identifichiamo un nuovo sito

di ubiquitinazione su H2A, K13-15 che è specificamente modificato da RNF168 in conseguenza a rotture della doppia elica del DNA (DSBs). Mostriamo sia *in vitro* che in cellule che questa ligasi modifica le lisine K13-15 iniziando l'ubiquitinazione di H2A e proponiamo che RNF8 agisce nel processo di estensione delle catene K63 sull'istone già ubiquitinato. Inoltre, identifichiamo un residuo sul dominio RING di queste ligasi che è conivolto nella fase di riconoscimento del substrato e che determina la loro capacità di ubiquitinare H2A. Mediante esperimenti di mutagenesi di questo sito su RNF168 in cellule, mostriamo che la formazione di catene di ubiquitina in prossimità della lesione al DNA non è sufficiente *per se* per l'integrità del segnale cellulare, mentre l'ubiquitinazione di H2A è necessaria per questo processo.

Nel **capitolo 5**, in conseguenza all'identificazione del nuovo sito di ubiquitinazione su H2A, investighiamo i fattori che determinano la specificità della reazione di ligazione delle E3. Mostriamo che RNF168 e Ring1B riconoscono in maniera diversa H2A nel contesto del nucleosoma. Qui troviamo che il dimero di H2A/H2B è il substrato minimo necessario per l'ubiquitinazione specifica delle K13-15 da parte di RNF168. Invece, Ring1B necessita dell'assemblamento completo del nucleosoma per poter ubiquitinare l'istone. Infine, mostriamo che la regione acidica del nucleosoma ha un nuovo inaspettato ruolo nel processo di catalisi di entrambe le ligasi.

Nel **capitolo 6**, riassumiamo i contenuti principali del lavoro presentato in questa tesi e discutiamo le loro potenziali implicazioni per lavori futuri.

In conclusione, questa tesi descrive le distinzioni funzionali dell'ubiquitinazione di H2A da parte delle proteine Polycomb e di RNF168 e RNF8. I nostri dati permettono una più ampia comprensione di questi due meccanismi cellulari. Inoltre, i nostri risultati descrivono come due segnali paralleli e distinti possono coesistere anche su una piccola proteina come H2A per consentire una corretta omeostasi cellulare.

CURRICULUM VITAE

Francesca Mattiroli was born on March 28th, 1982 in Tradate (Italy). After obtaining her Scientific Lyceum diploma in 2001 she began her studies in Biotechnology at the University of Pavia (Italy), where she obtained her Bachelor degree in October 2004. The same year, Francesca joined the Master Program in Industrial Biotechnology and performed a research internship in the laboratory of Prof. Andrea Mattevi where she learnt structural biology approaches to study protein function. Francesca obtained her Master degree in June 2006. In August 2006 she started her doctoral research in the laboratory of Prof. Titia K. Sixma at the Netherlands Cancer Institute in Amsterdam (the Netherlands), where she combined structural biology, biochemistry and cell-based assays to study the mechanisms of histone H2A ubiquitination. The results of this work are presented in this thesis. After her promotion, Francesca will join the laboratory of Prof. Karolin Luger in Fort Collins, Colorado where she will focus on structural aspects of chromatin biology.

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