UNIVERSITÀ DEGLI STUDI DEL PIEMONTE ORIENTALE

"AMEDEO AVOGADRO"

Dipartimento di Scienze del Farmaco

Dottorato di Ricerca in Biotecnologie Farmaceutiche ed Alimentari

XXVIII ciclo a.a. 2012-2015

INSIGHTS INTO THE RNF168-DEPENDENT UBIQUITIN SIGNALLING

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"*Non aetate verum ingenio apiscitur sapientia*"
Trinummus - Plauto

CONTENT

CHAPTER 1

INTRODUCTION

CHAPTER 2

CHAPTER 3

PAPER

CHAPTER 4

UNPUBLISHED RESULTS

CHAPTER 5

1. *The genome integrity network*

The survival of the organism is the meaning of life, which is thoroughly preserved inside the smallest biological unit: the cell.

What survival demands is genetic stability that is essential for the inheritance of traits to offspring, indeed it requires not only an extremely accurate mechanism for replicating DNA, but also systems for repairing the many accidental lesions that continually occur on DNA. To counteract the genotoxic threat induced by endogenous cellular events and exogenous environmental agents, cells developed a complex network of surveillance processes, which is mainly constituted by three evolutionarily conserved cellular pathways: the chromosome replication pathway, which governs the accurate and unhindered replication of DNA[1]. The chromosome segregation pathway, that preserves the correct number of chromosomes during cell division[2] and the DNA damage response (DDR), which ensures efficient repair of all types of damage[3]. Due to the existing crosstalk between these pathways, the disruption of one of them leads to the engagement of the others to protect genome integrity while maintaining cell homeostasis.

As the in common genome integrity network-protagonist, chromatin is subjected to regulatory mechanisms, including covalent histone modifications able to function as a docking site for the recruitment of non-histone proteins. In addition, ATP-dependent chromatin remodelling enzymes prompt changes in nucleosome position permitting gene transcription as well as the eviction or the incorporation of histone variants. This eviction or incorporation provides several biophysical properties to the chromatin fibre regulating the different post-translational modifications (PTMs) options and the binding of chromatin-related proteins. All of these chromatin-based regulations are implicated in the maintenance of genome stability participating to the coordination of the correct signal spread.

2. *DNA damage response*

DDR is an intricate cellular network able to detect DNA lesions, to signal their presence and to promote their repair by several proteins that act in concert as sensors, transducers and effectors[4, 5] ***(Fig.1)***.

The sensors directly recognize the aberrant DNA structures, induced by DNA damage or DNA replication stress, and activate the most upstream DDR kinases that are ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-Related), and DNA-PKcs (DNA-dependent protein kinase). These serine/threonine kinases are members of the phosphatidylinositol-3-kinase-like kinase family (PIKKs) and provide to amplify the signal by inducing the phosphorylation of a large number of proteins, which includes the checkpoint kinases (Chk) Chk1 and Chk2[6]. Both the upstream kinases and the two Chk are the transducers of the pathway. Below them lie the effectors that execute the function of the DDR (DNA repair, transcription regulation and cell-cycle control) and, depending on the context, have multiple functions in the different pathways of the network.

The hierarchical recruitment of these different proteins create a time window that allows the removal of the threat triggering a specific DNA repair pathway, inducing cell cycle arrest[7,8] and activating the apoptotic machinery[4]. The biological importance of a functional DDR network is illustrated by the severe consequences, for the human health, of inherited defects in the factors involved in DNA Damage Response, which result in various diseases, including neurological degeneration, premature aging, immunodeficiency and cancer susceptibility[9, 10].

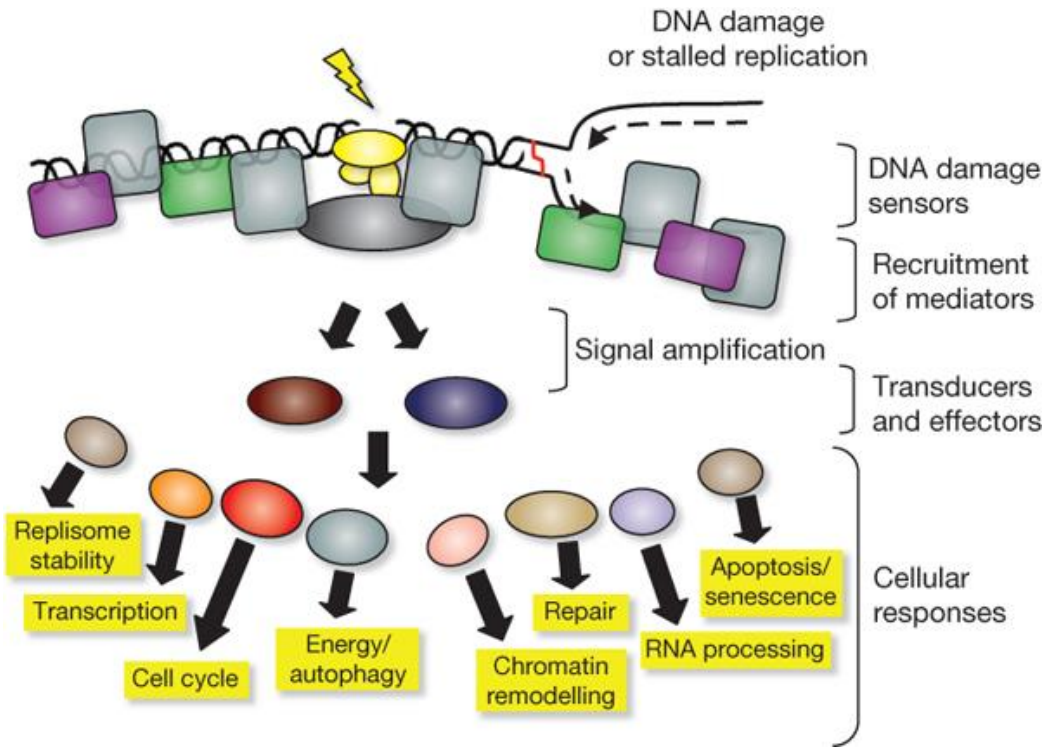

FIGURE 1: MODEL FOR DDR. The presence of a lesion in the DNA is recognized by various sensor proteins. These sensors initiate signalling pathways that have an impact on a wide variety of cellular processes[11].

2.1 *Insights into the molecular mechanisms of DNA repair*

The inheritance of an organism lies in the genetic information carefully kept into DNA, which is a dynamic chemical entity subject to several injuries. Any of these injuries can result in a damage that, if not repaired, will lead to mutation and possibly disease[11].

The types of lesion that can affect DNA are diverse and include the single strand breaks (SSBs) and the double strand breaks (DSBs) which are the most detrimental lesions since they do not leave an intact complementary strand to be used as a template for DNA repair and can lead to chromosome breaks and translocations[11].

The intricate network of DNA damage response coordinates DNA repair and determines the fate of the cell after DNA damage. DDR is mainly composed of distinct evolutionarily selected DNA-repair mechanisms, which are induced by the wide diversity of DNA damage and divided into five categories ***(Fig.2)***: direct repair, base excision repair (BER), nucleotide excision repair (NER), double-strand break repair and repair of interstrand cross-links[12].

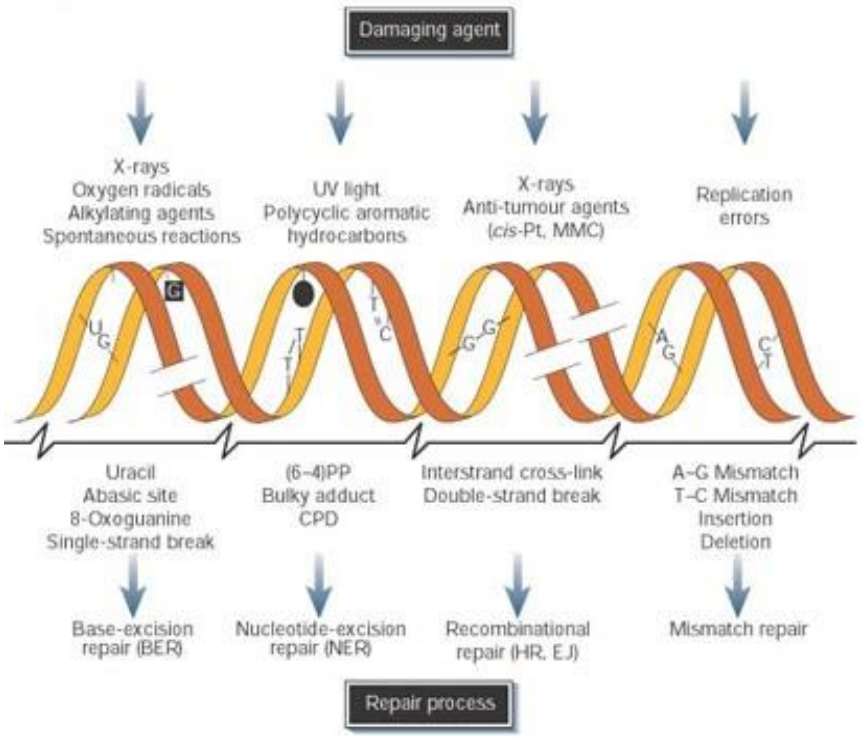

FIGURE 2: DAMAGING AGENTS, DNA DAMAGES AND REPAIR MECHANISMS. Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle) and the DNA repair mechanisms responsible for the removal of the lesions (bottom)[9].

While direct repair is a protein-mediated reversal, such as the methylguanine DNA methyltransferase that provides the removal of the O6-methyl group from O6-methylguanine (O6MeGua), the other repairing mechanisms are based on a sequence of catalytic events mediated by multiple proteins. Among these

mechanisms, the repair of DSBs has been extensively studied, due to its deleterious effects and to its relevance in the physiological process of V(D)J recombination and immunoglobulin class-switching process. DSBs are produced by exogenous or endogenous sources, such as reactive oxygen species or replication fork arrest and collapse, and are repaired by either homologous recombination (HR) or non-homologous end-joining (NHEJ) mechanisms[13] ***(Fig.3)***.

HR is an error-free pathway and is based on the preferential use of a sister chromatid as template, which is present in late S- or G2-phase of the cell cycle, to repair the damaged DNA[14]. It also has a prominent role in the high-fidelity duplication of the genome by providing critical support for DNA replication and telomere maintenance[14]. In the HR signalling the DNA end-resection is recognized by the Mre11-Rad50-Nbn (MRN) complex, which recruits ATM that in turn triggers the pathway phosphorylating the histone H2A.X (ɣ-H2A.X) and other proteins involved in reparation and checkpoints signalling[15]. The core steps of HR are homology search and DNA strand invasion by the Rad51 coupled to the single-strand DNA (ssDNA) filament complex, positioning the invading 3'-end on a template duplex DNA. Initially, the ssDNA is recognized by the eukaryotic ssDNA-binding factor, replication protein A (RPA), which displays higher affinity for both ssDNA and Rad51[16, 17]. Then RPA is replaced by Rad51 with the help of the mediator proteins of the pathway. Rad51 is the eukaryotic RecA homolog that, once loaded onto ssDNA, promotes homology search and DNA strand invasion in a reaction called synapsis[18, 19, 20]. During synapsis, Rad51 facilitates the formation of a physical connection between the invading DNA substrate and homologous duplex DNA template, leading to the generation of heteroduplex DNA (D-loop). Finally, when DNA is synthesized using the invading 3'-end as a primer, Rad51 dissociates from the double stranded DNA (dsDNA) to expose the 3'-OH required for DNA synthesis[21].

By contrast to HR, NHEJ occurs in all phases of the cell cycle and does not require a homologous template. It is an error-prone repair process promoting the fusion of the broken DNA ends together[22]. NHEJ is also essential for V(D)J recombination where DSBs are intentionally generated during T- and B-cell lymphocyte development[23]. The repairing process prompted by NHEJ is divided into four sequential steps: (I) recognition of the damage and assembly of the protein complexes at the DSBs; (II) bridging of the DNA ends and promotion of end stability; (III) DNA end processing; (IV) ligation of the broken ends and dissolution of the NHEJ complex[24]. The first step is the recognition and binding of the Ku heterodimer to the break, which is composed of the Ku70 and Ku80 subunits[25, 26]. Ku is massively expressed inside cells and has a high affinity for DNA ends interacting with DSBs in a sequence independent manner[27]. Once bounded to the DSBs, the Ku heterodimer functions both as a stabilizer, by binding the DNA ends in order to maintain their stability by protecting them from non-specific processing, and as a scaffold to recruit the other NHEJ factors, including DNA-PKcs, to the damaged site. DNA-PKcs activates, through phosphorylation, a large number of substrates and prompts the formation of a synaptic complex that holds the two ends of the broken DNA molecule together. Among the proteins activated by DNA-PKcs there is ARTEMIS, which, thanks to its 5' endonuclease activity, processes the DNA ends to create ligatable ends. The final step is the ligation of the broken ends, catalyzed by the DNA Lig IV in complex with XRCC4 and XLF, and the dissolution of the NHEJ complex[24].

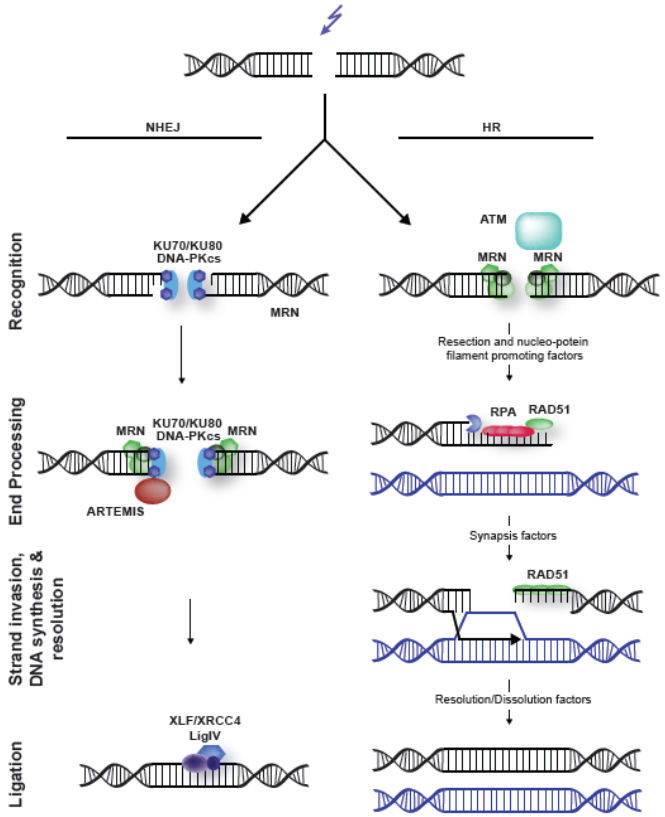

FIGURE 3: DOUBLE-STRAND BREAKS REPAIR. DSBs are predominantly repaired by either NHEJ or HR. In NHEJ the broken DNA ends are bound by the Ku70/Ku80 heterodimer, which recruits DNA-PKcs. The DNA ends are joined by the activity of polymerases and a ligase complex consisting of XRCC4, XLF and LigIV. Instead, in HR the MRN complex recognizes the DNA end-resection and recruits ATM, which phosphorylates the histone H2A.X triggering the pathway. ssDNA generated by DNA end-resection are bound by RAD51, which promotes the invasion of the ssDNA to a homologous dsDNA template, leading to synapsis, novel DNA synthesis, strand dissolution, and repair[28].

2.1.1 *Formation of DDR* foci

When a damage occurs on DNA, DDR proteins accumulate at the sites of lesion forming detectable supramolecular structures, which encompass megabase lengths of DNA adjacent to the sites of breaks, called DDR *foci* or IRIF (ionizing radiation-induced *foci*)[29] ***(Fig.4)***.

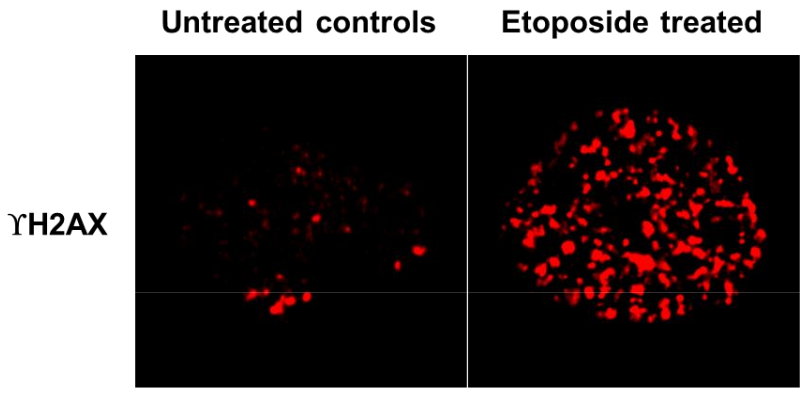

***FIGURE 4: DDR* FOCI.** The figure shows a typical staining of phosphorylated histone H2A.X (named ϒ-H2A.X) in U2Os cells before and after etoposide treatment, which induces DSBs by inhibiting Topoisomerase II.

The first event occurring upon DNA damage is the phosphorylation on Ser139 of the histone variant H2A.X in the proximity of the lesion, induced by ATM, ATR and DNA-PKcs[30, 31, 15]. The resulting phospho-H2A.X, also referred as ϒ-H2A.X, acts as a docking station for a number of downstream DDR factors. The creation of these high concentrations of proteins increases the efficiency of the DNA damage response and repair[32]. Once formed, *foci* function as dynamic structures due to the exchange of proteins from the damaged chromatin and the freely diffusing proteins[29, 33]. While the phosphorylation triggers the formation of the *foci*, these structures are also finely regulated by other PTMs, including ubiquitylation, sumoylation, methylation, acetylation, and PARylation[34, 35].

2.2 *The role of post-translational modifications in DDR*

The importance of the maintenance of genome integrity is evident from the large investment that cells make in the number of proteins and mechanisms involved in DDR. Indeed, the assembly of the protein complexes at DNA breaks is tightly

organized by an intricate pathway of PTMs, prompted by several enzymes[34, 35] ***(Fig.5)***. PTMs are reversible modifications that have a key role in DDR in virtue of their ability to alter the activity of an existing protein without the necessity of *de novo* protein synthesis, in order to avoid transcription of damaged DNA. In addition, these modifications generate docking sites on target proteins at the damaged site, contributing to the formation of DDR *foci*[34, 35].

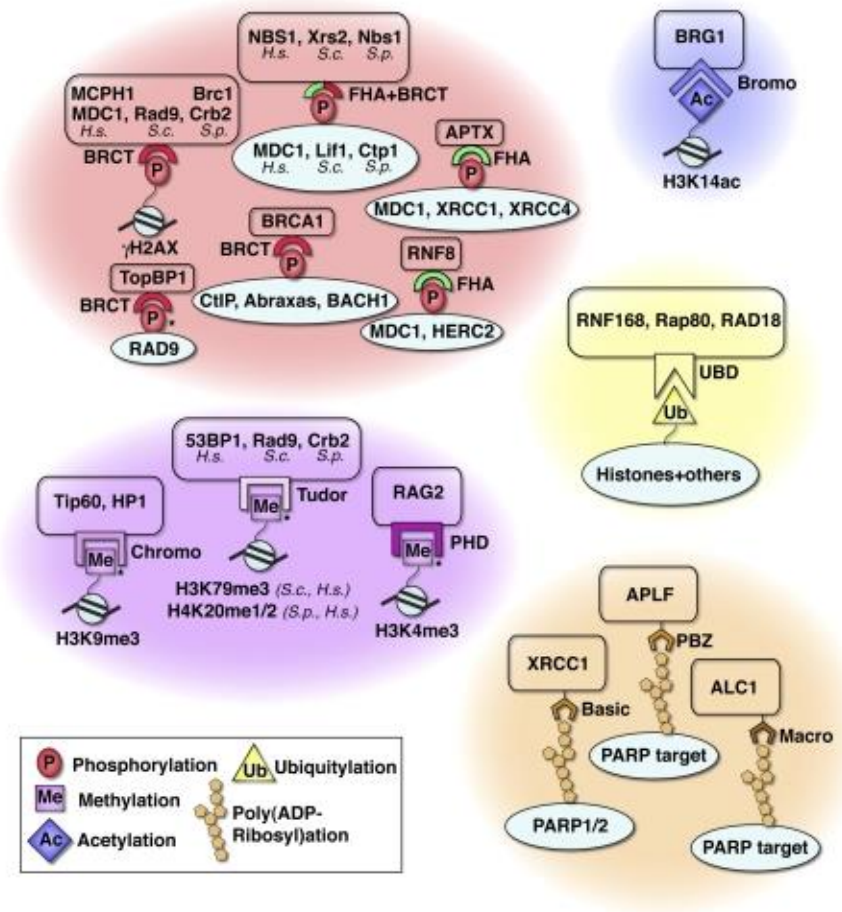

FIGURE 5: THE ORGANIZED AND INTRICATE PATHWAY OF PTMS IN DDR. The recruitment of DDR proteins to sites of DNA breaks is mediated by specific interactions between the PTMs and a dedicated binding module. BRCT and FHA domains, which are represented by red and green semicircles, bind phosphorylated serine or threonine residues; Tudor domains,

chromodomains, and PDH finger domains bind methylated histones; bromodomains (Bromo) bind acetylated histones; and UBDs bind ubiquitylated proteins. The PAR-binding domain can take the form of a basic stretch of aminoacids (Basic), a PAR-binding zinc finger (PBZ), or a macrodomain (Macro). The species of the proteins are indicated, unless only human proteins are listed. (*H.s.*) *H. sapiens*; (*S.c.*) *S. cerevisiae*; (*S.p.*) *S. pombe*[36].

As described above, phosphorylation is the first signalling device able to trigger the pathway inducing the formation of the DDR *foci*[15]. Due to its central role, hundreds of phosphorylated targets have been already identified by mass spectrometry-based screens[37, 38, 39].

DDR proteins frequently display phospho-binding motifs such as BRCT (breast cancer C-terminal) or FHA (Forkhead-associated) domains that mediate the phospho-dependent assembly of DDR protein complexes[40, 41, 42, 43]. Due to this, γ-H2A.X, being the prime substrate of the DDR kinases, directs the assembly of downstream DDR components, including checkpoint mediators such as MDC1 and MCPH1 (Microcephalin; also named BRIT1)[44, 45]. In addition, it recruits chromatin-modifying complexes such as p400, a component of the NuA4 histone acetyltransferase complex[46, 47]. Once bound to γ-H2A.X, MDC1 acts as a loading platform for other DDR components, which in turn recognize the phosphorylation on the mediator; the ATM-dependent phosphorylation of MDC1 on Thr-Gln-X-Phe (TQXF) motifs creates binding sites for the FHA domain of the ubiquitin ligase RNF8 (Ring Finger protein 8), which in turn promotes the focal accumulation of 53BP1 and BRCA1 at DSBs sites[33, 48, 49].

In virtue of this role in the fine orchestration of the assembly of DDR proteins, ubiquitination stands up as a critical PTM in DNA repair[50, 51]. It was demonstrated that several ubiquitin ligases, such as BRCA1[56], RNF8[33, 48, 49], RNF168[54, 55], RAD18[58], HERC2[57] and Polycomb-repressive complex 1 (PRC1)[63] accumulate at the sites of DNA breaks, where they amplify the signal by recruiting the DDR transducers and

effectors[52, 53, 59]. Similarly to phosphorylation, ubiquitination can be recognized by specific domains: termed ubiquitin-binding domains (UBDs). It was demonstrated that ubiquitin is necessary to recruit the DDR proteins to the site of damage. For instance, when a damage occurs during DNA replication, the FA core complex mono-ubiquitinates the FA proteins FANCD2 and FANCI in a manner that promotes their recruitment to the HR *foci*[60, 61].

Other PTMs playing a role at DNA breaks are: SUMOylation, which consists of the addition of SUMO (small-ubiquitin-like modifier) to a substrate in order to increase the residence time of the target protein at damage *foci*[62, 63, 64]; PARylation, an early DDR process catalysed by PARP enzymes which promote the addition of ADP-ribose polymers to a substrate. These polymers contribute to the recruitment of the MRN complex as well as the Polycomb complex[34, 65, 66, 67]. Moreover, several studies suggested a role for histone acetylation in the regulation of the dynamics of DDR factors[68, 69, 70, 71, 72]. Indeed, the acetylation status of histone proteins in the proximity of a DNA break can regulate the assembly of the proteins involved in DDR both indirectly by modulating chromatin compaction, and directly by creating binding sites. Finally, a necessary modification for the focal recruitment of DDR proteins in the site of damage is methylation[73, 74]. For instance, the recruitment of 53BP1 to the breaks is mediated by the recognition, through its tandem Tudor domains, of the methylated histone residues[75].

Once the DNA has been repaired, the DDR machinery must be switched off by the disassembling of the *foci* and this process appears to occur mainly by reversing PTMs. Indeed γ-H2A.X dephosphorylation by several phosphatases plays an important role in terminating checkpoint signalling[76, 77, 78] and in recruiting of histone acetyltransferase as Tip60, which promotes the acetylation-dependent eviction of H2A.X from chromatin[79]. Acting similarly to phosphatases,

deubiquitinating enzymes (DUBs) have also been implicated in the terminating process of DDR by antagonizing or suppressing the ubiquitin ligase-mediated ubiquitination. An example of this mechanism is given by OTUB1 (OUT domain ubiquitin aldehyde binding 1) which is a DUB that suppresses the RNF168-mediated ubiquitination through direct inhibition of the E2 conjugating enzyme Ubc13[80].

3. *Ubiquitination*

Ubiquitination, as suggested by the name, is a ubiquitous catalytic reaction that affects a plethora of cellular processes, such as cell growth, endocytosis, apoptosis, innate immune response, neuron degeneration and cellular trafficking. Moreover, several studies in the last years highlight the key role of ubiquitination as a modification that influences almost all aspects of DDR[81, 82]. The process of ubiquitination promotes the addition of a ubiquitin (ub) monomer to a target protein and occurs through three sequential steps catalysed by different enzymes: an (E1) ub-activating ezymes, an (E2) ub-conjugating enzymes and an (E3) ub-ligases[83]. The first step is represented by the activation of a ub moiety in an ATP-dependent two-step reaction prompted by an E1: initially a ub-adenylate intermediate is produced, followed by the reaction of this intermediate with an E1 cysteine residue to form an E1-ub thioester. The following second step requires that the activated ub monomer is transferred from the E1 to an E2 in order to form an E2-thioester. At last, ub is added to a substrate through the formation of an isopeptide bond with the ub carboxyl-terminus and the ε-amino group of a lysine residue within the sequence of the target protein. In this step the E3, which binds both the E2 and the substrate, is crucial ***(Fig6)***.

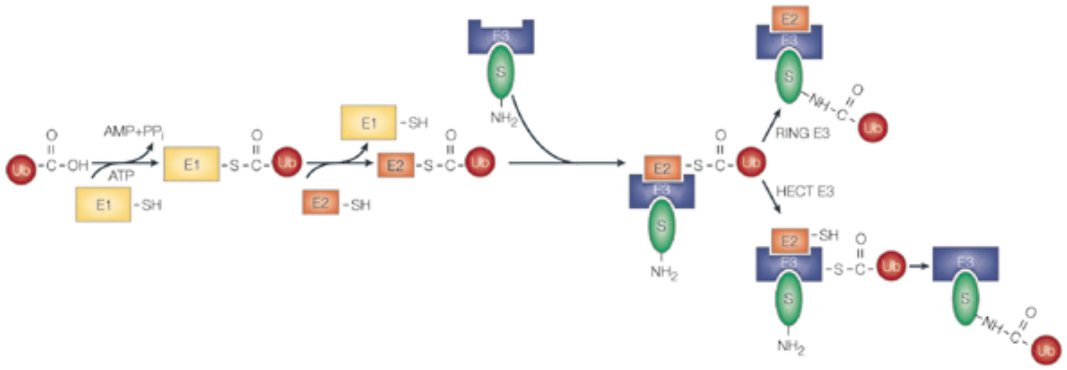

FIGURE 6: THE UBIQUITINATION REACTION. Free ubiquitin (ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ub. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s. For HECT domain E3s, ubiquitin is next transferred to the active-site cysteine of the HECT domain followed by transfer to substrate (S) (as shown) or to a substrate-bound multi-ubiquitin chain. For RING E3s, current evidence indicates that ubiquitin might be transferred directly from the E2 to the substrate[84].

The first clue of the complexity of ubiquitination is well represented by the high number of ubiquitinating enzymes. Indeed, the first step of the reaction can be catalyzed by two E1s, which can transfer the ub moiety to at least 40 different E2s, which then may serve more than 600 E3s. Moreover, the E3 ligases can be classified into three major groups depending on their catalytic domain: the HECT domain (Homologous to E6-associated protein C-terminus), the RING finger (Really Interesting New Gene) and the U-box E3s. Due to the different nature of the catalytic site, the E3s function differently: the HECT-type presents a catalytic cysteine residue, which accepts an ub moiety from the E2 before transferring it to the substrates. Conversely, both the RING-finger and U-box types function as scaffolds between the E2 charged with ub and the substrates in order to facilitate the direct transfer of the ubiquitin from the E2 to the target protein.

The interesting pattern behind the E3s is their ability to be active in different cellular pathways suggesting a different mechanism of action. In DDR several RING finger E3, which possess a RING-finger domain with the following primary sequence: Cys-X2-Cys-X[9-39]-Cys-X[1-3]-His-X[2-3]-Cys-X2-Cys-X[4-48]-Cys-X2-Cys, where "Cys" or "His" are respectively a conserved cysteine or histidine residue involved in zinc coordination and X is any aminoacid[85], are well characterized. The three dimensional structure of this domain reveals that its conserved cysteine and histidine residues are buried within the core of the domain maintaining the overall structure ***(Fig.7)***.

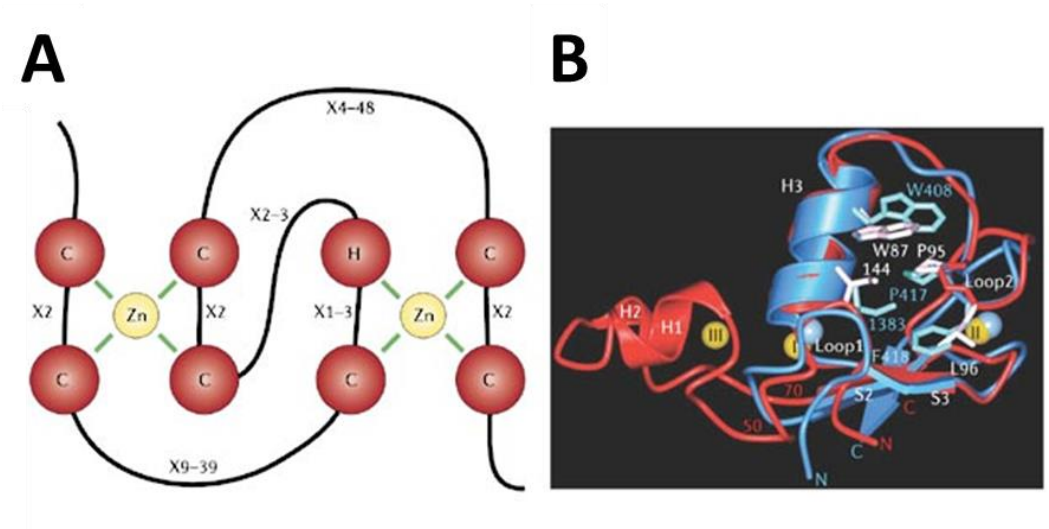

FIGURE 7: THE RING-FINGER DOMAIN. **(A)** Schematic representation of a C3HC4 RING finger. Most RING fingers contain two zinc atoms (yellow) coordinated with cysteine or cysteine/histidine-rich clusters (red). The general consensus sequence is: C-X2-C-X9–39-C-X1–3-H-X2–3-C-X2-C-X4–48-C-X2-C, although some variations exist. **(B)** Overlay of crystal structure of the RING-finger domains found in c-CBL (blue) and RBX1 (red) reveals a significant degree of structural similarity in their E2-binding components. RBX, RING-box protein[86].

3.1 *Complexity of the ub system*

Ubiquitin is a 76-aminoacids long globular protein highly conserved throughout the eukaryotes. The secondary structure of this little protein is composed by five-stranded β-turns and an α- helix, which confers a relative rigidity to the protein. Moreover, ub has an hydrophobic patch, centered on Ile44 and located in the middle of the domain formed by the α/β structure, which is an important interaction site[87, 88] ***(Fig.8)***.

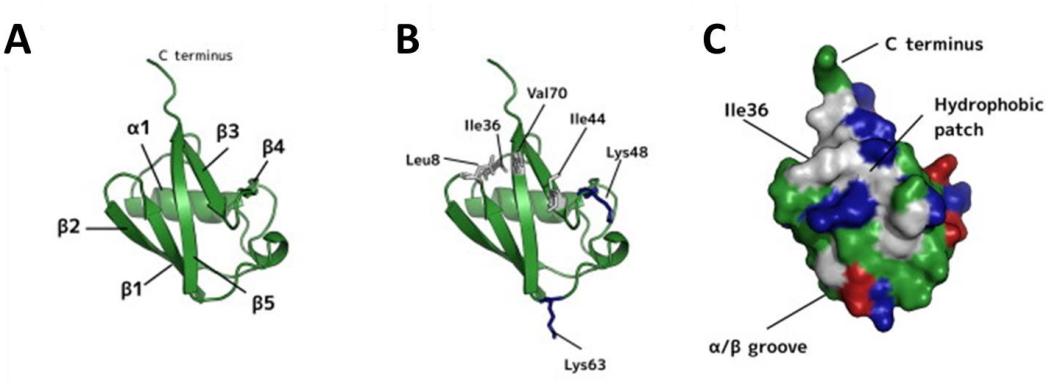

FIGURE 8: THE UB STRUCTURE. **(A)** Backbone fold of ubiquitin, with secondary structure elements labelled. **(B)** Selected residues on ubiquitin are rendered as sticks and labelled. **(C)** The surface of ubiquitin, coloured by residue type. The colour scheme is gray for nonpolar, green for polar (uncharged), red for acidic, and blue for basic[88].

In spite of its little dimensions, ub has an incredible signalling power: it can form different polymeric chains through the addition of at least one ubiquitin molecule on any of the eight amino groups (the N terminus Methionine (M1) and the seven Lysines: K6, K11, K27, K29, K33, K48, and K63) present in the first monomer added to the target protein[89, 90]. This means that a substrate could be ubiquitinated on a single residue (mono-ubiquitination) or on multiple residues (multi-mono-ubiquitination). Moreover, the substrate could be poly-ubiquitinated on a single

residue, forming different linkages that increase the complexity of the ub system, giving rise to ub chains with distinct topology, providing structural flexibility that results in a multitude of functional outcomes able to considerably affect cell life[91, 92] ***(Fig.9)***.

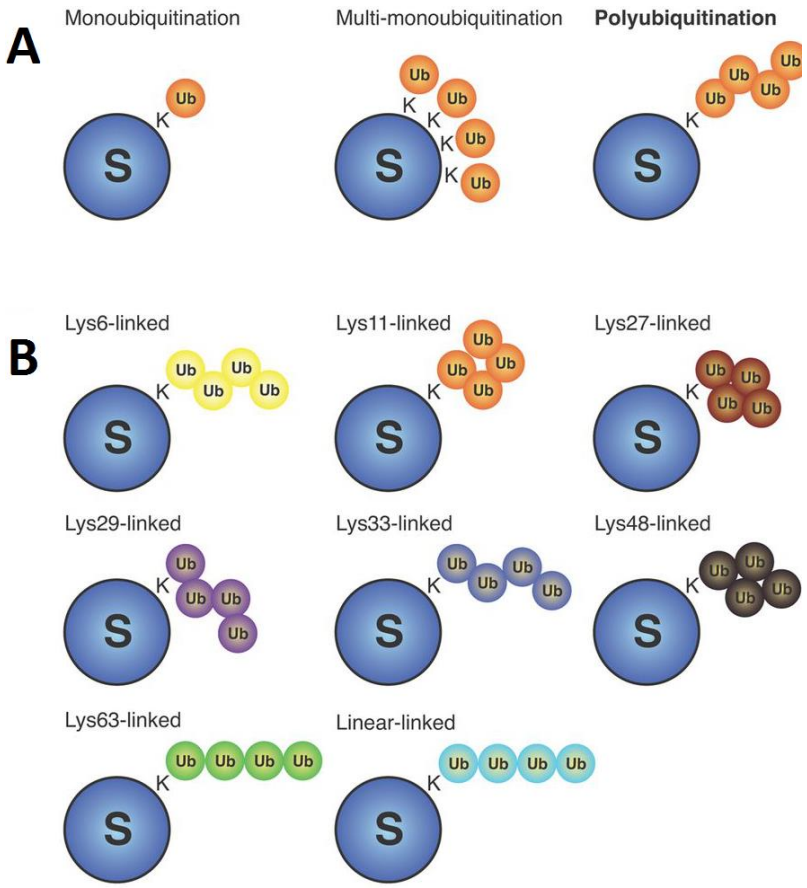

FIGURE 9: THE UB SYSTEM. **(A)** The ubiquitin modification has three general layouts: mono-ubiquitination, multi-mono-ubiquitination and poly-ubiquitination. **(B)** Forms of homotypic poly-ubiquitination, where each ubiquitin chain contains a single linkage type. Individual linkages may lead to distinct ubiquitin chain structure[93].

For instance, the structural studies of the well characterized K48- and the K63-linked ubiquitin chains highlight the different spatial conformation between the

two chain types, which is read by the other proteins in different ways. K48- poly-ubiquitination is predominantly a mark to target proteins for 26S proteasomal degradation, the K63- linked ubiquitination instead mediates a variety of non-degradative pathways, including DNA repair, transcriptional regulation, endocytosis and activation of protein kinases[94, 95, 96, 97] ***(Fig.10)***.

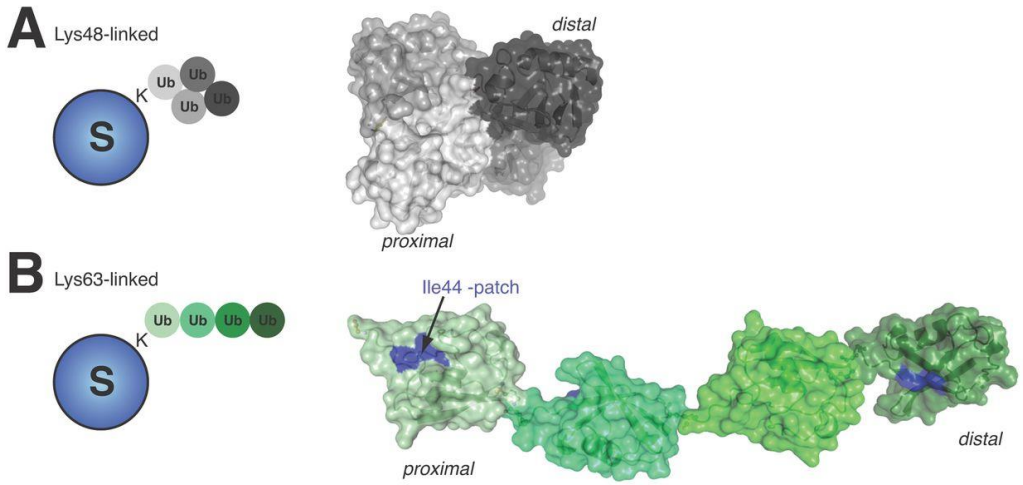

FIGURE 10: STRUCTURES OF K48- AND K63-LINKED UBIQUITIN CHAINS. **(A)** Structure of K48-linked tetra-ubiquitin. Proximal (white) and distal (black) molecules are labelled. Proximal/distal describes the position relative to the substrate, see cartoon on the left. In K48-linked chains, all ubiquitin molecules interact with each other, and the Ile44 patches are not exposed. **(B)** K63-linked ubiquitin chains display an open conformation, both in the crystal structure and in solution. The Ile44 patches (shown as blue surface on the molecules) are exposed, and can adopt different relative positions due to the flexibility in the ub chain[93].

A further level of complexity lies in the formation of the branched poly-ubiquitin chains, which are mixed-linkage ubiquitin chains that form bifurcations [98].

The E3 ligases as well as the E2 conjugating enzymes control the formation of a specific ub chain on the substrate. The diverse combination of E3s with several E2s induces a specific signalling through the addition of a peculiar modification on a broad number of substrates, including the ubiquitin ligases itself. For instance,

the E3 ligase BRCA1 (Breast Cancer Susceptibility Gene 1) promotes auto mono-ubiquitination by interacting with the E2s UbcH6, Ube2e2, UbcM2 and Ube2w, whereas it promotes K63- or K48-linked auto-poly-ubiquitination by interacting with Ubc13-Mms2 and Ube2k, respectively[99]. These diverse and potent signals, which intrinsically lie in the ub molecule, result in an actual intriguing ubiquitin "code".

4. *Inside the double strand breaks: the RNF8/RNF168-dependent signalling*

DSBs arise on DNA in response to radiomimetic drugs or ionizing radiation (IR) and also after treatment with Topoisomerase II inhibitors that prevent relegation of DNA strands broken by Topoisomerase II activity. They are naturally occurring, not only in lymphocytes as mentioned before, but also at chromosome ends, where they are associated with human cell aging[11, 100].

Over the last years, it has become clear that, even if the initial trigger of the DSBs repair pathway lies heavily on phosphorylation, a key step in the activation of the DDR machinery relies on histone ubiquitination[101]. Indeed, the early steps of the pathway induces the quick recruitment of RNF8, which is the first ubiquitin ligase recruited on the site of damage. The RNF8-dependent ubiquitination is assisted by HERC2 (HECT domain E3 ligase) which promotes the interaction between the RING finger ligase and the E2 conjugating enzyme Ubc13 that prompts the formation of K63-linked ubiquitin chains on chromatin at the damaged site[57]. Although the critical substrates of RNF8 and K63-linked ubiquitination remain elusive, very recently Thorslund et colleagues demonstrated that the concerted action of RNF8 together with Ubc13 predominantly targets the H1-type linker histones, expanding the concept of "histone code"[102]. The local RNF8/Ubc13-mediated

histone ubiquitination renders the DSBs-flanking chromatin susceptible to assemble additional regulators, including RNF168 which through its UBDs recognizes the RNF8-dependent ubiquitination and uses it as docking site to localize itself in the site of damage[33, 48, 49, 53, 54, 103].

The ubiquitination prompted by RNF168 targets the histones H2A and H2A.X on the bidentate K13/15 site at the N-terminal tail[104, 105]. The RNF168-dependent ubiquitination, in turn, generates docking sites for the recruitment of the effectors of the pathway, such as BRCA1 and 53BP1 (p53 binding protein). Both proteins are able to recognize ub signals. BRCA1 accumulation depends on Rap80, which possesses two adjacent UBDs named UIM1 and UIM2 or tandem UIMs[103, 106], instead 53BP1 localization at DSBs is due to its UDR (ubiquitination-dependent recruitment) motif which, together with the Tudor domain, reads a bivalent ubiquitination-methylation signal at damage sites[107] ***(Fig11)***.

The interplay between BRCA1 and 53BP1 determines the effective DSBs repair by one of the two major DSBs repair pathways. Indeed BRCA1 promotes HR while 53BP1 commits to NHEJ[108, 109, 110].

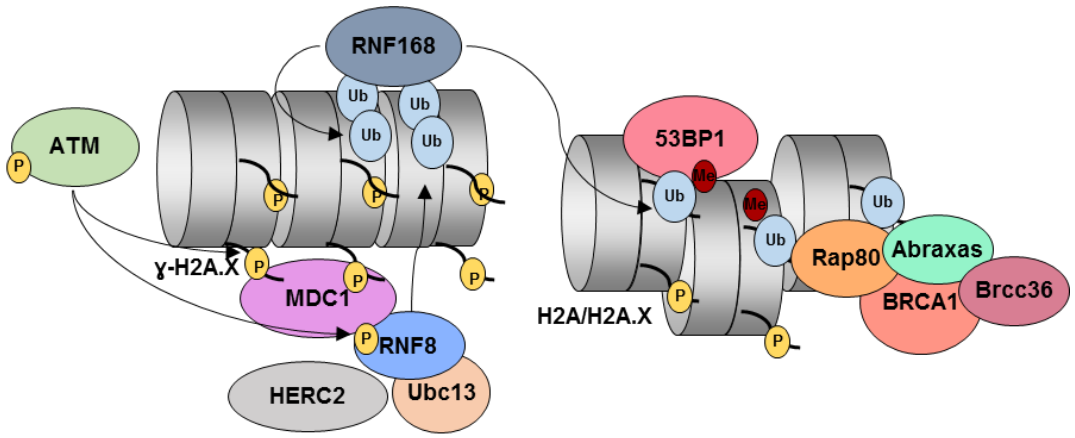

FIGURE 11: THE RNF8/RNF168-DEPENDENT SIGNALLING. Schematic representation of the ATM-mediated DDR pathway with a focus on RNF8/RNF168. In response to a DSB, ATM

phosphorylates the histone H2A.X, which is recognized by MDC1 that in turn recruits RNF8. Simultaneously, ATM phosphorylates HERC2, which stimulates the interaction between RNF8 and Ubc13 promoting the RNF8/Ubc13-mediated K63-linked ubiquitin chains on chromatin. This ubiquitination is fundamental for the recruitment of RNF168 that recognizes the ubiquitin through its UBDs and using it as a docking site in order to stay in the site of damage amplifying the signal by ubiquitinating the bidentate K13/15 site at the N-terminal tail of the histones H2A/H2A.X. In turn, this ubiquitination allows the recruitment of 53BP1 and BRCA1.

4.1 *The ub-mediated fine-tuning of the DSBs reparation*

The ubiquitin-dependent signalling during DDR is multifaceted, inasmuch being read by different proteins, through their UBDs, determining distinct outcomes in the pathway[111]. For instance, the PcG transcriptional repressor complex Ring1b/Bmi1, which is a part of the polycomb complex, induces the silencing of the transcription in response to DSBs by mono-ubiquitinating the Lys119 of both histones H2A and H2A.X (H2A/H2A.XK119ub)[112, 113, 114, 115].

It was recently found that both Ring1b/Bmi1- and RNF168-dependent ubiquitination on histones H2A/H2A.X depend on an intact nucleosome acidic patch which could function as a scaffold to integrate differential signals on H2A[116].

Also the ubiquitination of H2B on Lys120 (H2BK120ub) by RNF20 is involved in gene transcription after damage; unlike the Ring1b/Bmi1-dependent ubiquitination, H2BK120ub is linked to gene activation, in part through chromatin decompaction at transcribed regions[117, 118]. In addition, the physical proximity between K120 on H2B and the bidentate K13/15 site of H2A/H2A.X ubiquitinated by RNF168 within the nucleosome suggests a possible cooperation of these marks during DNA repair[97, 105, 117].

An important consideration is that the E3 ligases function not only as positive regulators of the pathway but also as modulators of the signal magnitude in order to contribute to the proper ub spread on chromatin at the damaged sites. Among

these E3s there is RNF169, a paralogous to RNF168, which negatively regulates the RNF8/RNF168-dependent signalling. Due to its UBDs, RNF169 is able to compete with 53BP1 and Rap80 for the binding to the RNF168-mediated chromatin ubiquitination at the sites of damage[119]. Intriguingly, this ability of RNF169 to function in a non-catalytic manner provides the first example of an E3 ligase that, independently from its catalytic activity, is able to regulate the protein accumulation at DSBs-flanking chromatin.

Other examples of magnitude regulators of the signal are the two HECT domain E3 ligases: TRIP12 and UBR5. These two enzymes are able to control the accumulation of RNF168 at the site of damage by preventing its excessive spread and therefore an abnormal chromatin ubiquitination that could have deleterious consequences, including unscheduled transcriptional silencing or sequestering cellular pools of limiting genome caretakers[120].

Since one of the consequences of the RNF168-dependent ubiquitination is the conspicuous increase in chromatin retention of 53BP1, the NHEJ is preferred under these conditions[108, 109, 121]. This suggests a possible regulatory role for the two HECT domain E3s so to funnel the signal towards one of the two mechanisms of DSBs repair.

Another important, yet not well-understood, aspect of the ubiquitin-dependent signalling in DDR is how the control of the spatio-temporal events is achieved. Particularly, it remains to be clarified in detail how the disassembly of the repair complexes occurs after the damage is solved, which enzymes are involved and how this impacts on termination of the DNA damage checkpoint and ultimately on genomic stability and survival. An example of such mechanisms could include the DUBs activity that seems functionally connected with the DSBs response[97, 122].

5. *Deubiquitinating enzymes at work*

Ubiquitination is a reversible process prompted by the deubiquitinating enzymes (DUBs, also known as deubiquitinases or deubiquitylating enzymes). They can be divided into five families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs), Josephins and JAB1/MPN/MOV34 metalloenzymes (JAMMs; also known as MPN (+) or JAMM/MPN (+)).

In the last years the importance of the DUBs in the modulation of the ub-based DDR was highlighted by several studies on different deubiquitinases. These DUBs were found actively involved in the regulation of the recruitment of RNF168-responsive factors, in checkpoint recovery and DSBs-induced transcriptional silencing[53, 54, 103, 123, 124, 125] ***(Fig.12)***. Since then, an increasing body of evidence supports the reversal of DNA damage-induced chromatin ubiquitination by DUBs as a key aspect of the DSBs response[97, 122]; in particular, a great part of these enzymes has been implicated as regulatory components of the RNF8/RNF168-mediated ubiquitination at DSBs.

Different DUBs can have a positive or a negative effect on signalling. The negative regulators usually limit the RNF8/RNF168 pathway by reversing the ubiquitination catalysed by these ligases. Examples are given by USP3 and USP44, two chromatin-associated proteins able to prevent the recruitment of 53BP1 to the IRIFs by deubiquitinating histone H2A[54, 123]. BRCC36 (BRCC3), a JAMMs DUB, regulates negatively by acting on K63-linked ub chains at DSBs. BRCC36 is part of the BRCA1 complex and, together with Rap80, inhibits HR early upon DSBs induction[126, 127]. Instead, DUBs such as OTUB1 are able to attenuate ub-based DDR through non catalytic inhibition of RNF168[80].

Another DUB that conversely acts as a positive regulator of DDR, through an indirect mechanism, is USP34 which removes degradative ub chains on RNF168, thereby stabilizing the E3 and promoting DDR signalling[128]. Similarly USP7 also promotes ubiquitin-dependent DNA damage signalling by stabilising RNF168 [129].

These findings suggest the importance of the regulation of the RNF8/RNF168 signal magnitude that can be obtained by the tight balance between the positive and the negative feedback on the pathway. Moreover, for the fine orchestration of the DDR machinery, the DUB selectivity is critical in order to give the necessary diversity to the ubiquitin signals at DSBs.

Hereto, there are DUBs not directly involved in the RNF8/RNF168-dependent ubiquitination but still involved in the regulation of the pathway. For instance, USP16 (also known as Ubp-M) counteracts PcG-mediated gene silencing through deubiquitination of H2AK119ub[130, 131, 132]. Local transcription inhibition is dependent on Ring1b/Bmi1 and is partially regulated through RNF8/RNF168-dependent ubiquitination[112, 113, 114, 115, 125]. These findings place USP16 at the interplay between the DNA repair machinery and the transcriptional regulation. Also BRCA1-associated protein 1 (BAP1; also known as UCHL2) displays DUB activity towards H2AK119ub[133]. Until now, the functional characterization of this deubiquitination is not yet fully understood. Considering that BAP1 is recruited to DSBs together with the evolutionary conserved PcG repressive complex PR-DUB (formed by BAP1 and additional sex combs-like 1 (ASXL1)), it is thought that it is related to the regulation of the PcG-mediated gene silencing[112, 114, 115, 125].

At last it behoves us to mention the characteristic role of the POH1/PSMD14-dependent deubiquitination. POH1/PSMD14 is a JAMMs DUB and is also the intrinsic DUB of the 19S proteasome lid[134], which has a role in the restriction of the ub conjugates at sites of DNA damage, leading to a restrained accumulation of 53BP1 and promoting Rad51 loading. These results suggest a cross-talk between

proteasome and DNA repair, where the proteasome has the capacity to fine-tune DNA repair by balancing the activity of the ubiquitin ligases.

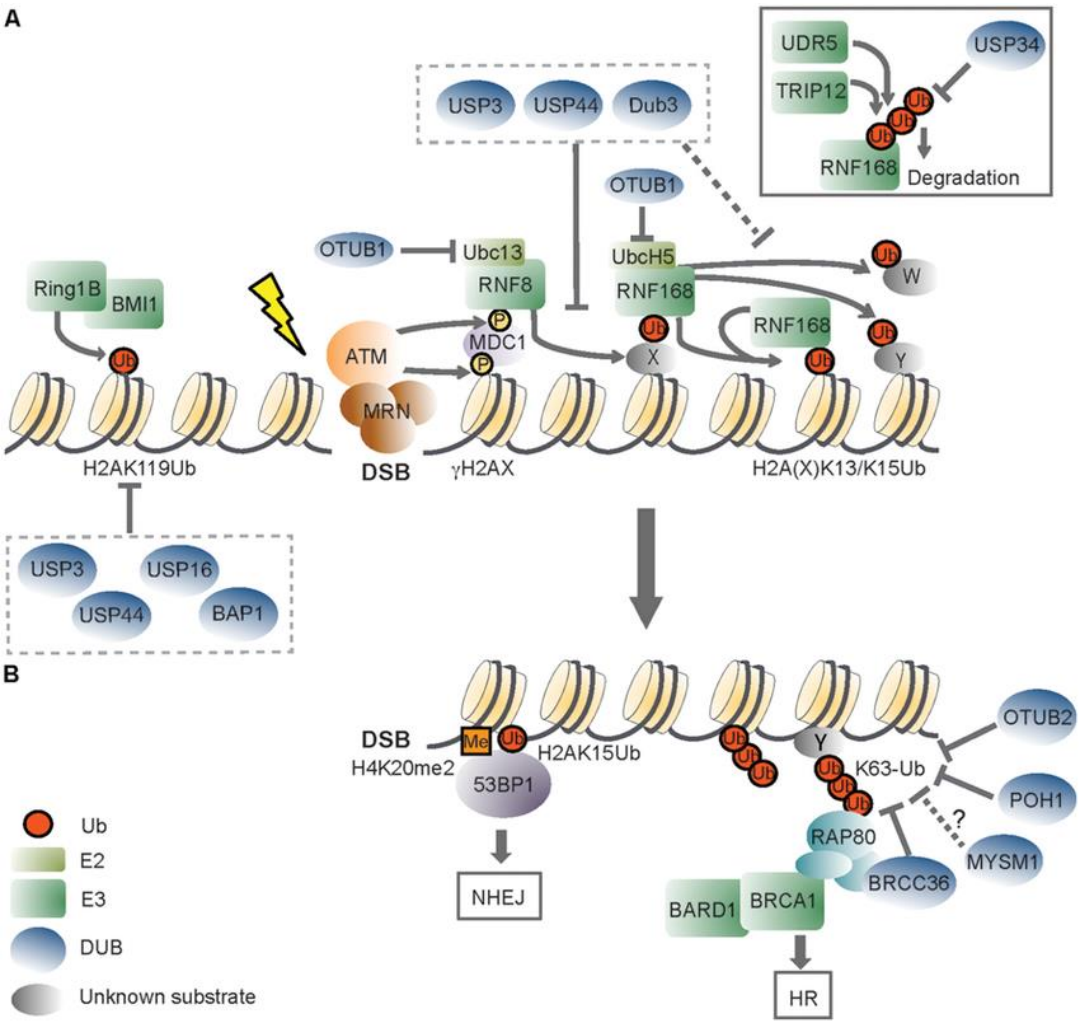

FIGURE 12: THE ROLE OF DEUBIQUITINATING ENZYMES IN THE CHROMATIN-BASED RESPONSE TO DNA DSBS. **(A)** OTUB1 opposes RNF168 activity by binding to the E2 ubiquitin-conjugating enzymes UBC13 and UbcH5. USP3, USP44, and Dub3 DUB activities impair RNF168 recruitment, suggesting that they can target RNF8 substrates. These DUBs may also cleave RNF168-mediated ubiquitinated H2A/H2AX. Excessive RNF168-dependent chromatin ubiquitination is limited by the TRIP12 and UBR5 E3 ligases, which target RNF168 for proteasomal degradation. USP34, instead, counteracts DSBs-induced RNF168 ubiquitination. DSBs also trigger the recruitment of the Polycomb group E3 RING1B/Bmi1 which mono-ubiquitinates H2A on Lys119 (H2AK119Ub) to locally repress transcription.

USP16 and BAP1 target the H2AK119Ub mark, and USP16 activity is required for re-activation of DSB-induced transcriptional silencing. USP3 and USP44 oppose to steady-state mono-ubiquitinated H2A, which is primarily constituted by H2AK119Ub. **(B)** The DUBs BRCC36, POH1, and OTUB2 regulate DDR signalling by hydrolyzing DSB-induced K63-ub chains. Whether MYSM1, which also possesses K63-ub cleavage activity, participates in this step of DDR is an open question. Dashed lines indicate proposed protein–protein interactions. X indicates an unknown RNF8 substrate(s), and Y and W indicate unknown RNF168 substrates[135].

6. *The DSBs-associated DUBs in cancer biology*

De-regulation of DDR mechanisms can contribute to cancer but may also promote functional decline of the stem cells with consequential deterioration in tissue function and aging[11, 136, 137]. Consistent with this and considering the importance of some DDR proteins in human cancer (e.g. BRCA1), in the last years several studies were addressed to the relationship between the DNA repair-dependent ubiquitination and cancer biology.

Although much attention has been paid to the role of ubiquitin ligases in tumorigenesis, only recently it has been started to shed light on the mechanisms behind cancer prompted by the enzymes that reverse ubiquitination: the DUBs.

By direct investigation of the consequences of inactivation of some DUBs in mouse models, a first step was done towards the identification of candidates which could be considered as future possible pharmaceutical targets. Notably, the screening highlights the ability of the engineered depleted mouse to develop neoplasia: Usp3-depleted mice develop a broad spectrum of tumour types with a latency of one year of age[138]. Further, MYSM1-deficent mice are tumour prone, developing thymic lymphoma with a latency of 4-6 months[139]. Moreover, USP44 knock-out mice are prone to develop spontaneous tumours, displaying in particular an approximately nine-fold increase in adenomas of the lung compared to wild type upon aging[140]. More importantly, USP44 was also frequently found

down-regulated in human bronchial adenocarcinomas and patients with low USP44 expression had significantly shorter overall survival, underscoring a tumour suppressive function in human cancer[140]. Zhang and colleagues also reported in their study of 2012 that mouse cells lacking USP44 not only show a defect in silencing the mitotic checkpoint, but they mis-segregate their chromosomes and exhibit whole chromosome aneuploidy. These findings suggest that one mechanism by which USP44 suppresses tumorigenesis is by preventing aneuploidy, a feature commonly associated with human cancer[140].

But not all the DUBs act as tumour suppressors: for instance, BRCC36 is overexpressed in primary human breast tumours, suggesting that its excessive activity may predispose to tumorigenesis. In support of this hypothesis, a correlation between high levels of BRCC36 and some cancer type (e.g. bladder, ovary, prostate, breast cancer) was reported in the Oncomine database[141].

On the other hand USP10 is a rather controversial DUB which is stabilized upon DNA damage and acts as regulator of p53 degradation and activity by reversing Mdm-2-mediated K48-linked ubiquitination[142]. In cells expressing wild-type p53, USP10 acts as oncosuppressor by abolishing tumour cell proliferation. Therefore, cancer has selected a mechanism in order to induce a downregulation of USP10 expression in cells expressing wild-type p53 or an upregulation of the deubiquitinase expression in p53-mutated cells[142, 143, 144].

Considering all this, it is clear that DUBs are involved in cancer-related pathways and that the current studies are only beginning to comprehend the molecular mechanisms behind the impact on organism physiology and human diseases. Gaining insight into the functional role of the DUBs could provide an important rationale to develop dedicated drugs for more specific therapeutic approaches.

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OUTLINE OF THE THESIS

DNA is damaged all the time by a plethora of injuries; in spite of this, only a few changes accumulate as mutations in the DNA sequence. This preservation of genome integrity is due to a large investment that cells make in the action of a complex network called DNA damage response (DDR), responsible for the DNA damage surveillance.

When DNA damage occurs, an organized and complex pathway of post-translational modifications (PTMs), prompted by several enzymes, regulates the fine orchestration of the repair machinery. PTMs are reversible modifications that are known to be essential to dynamically coordinate the signalling networks thanks to their ability to diversify the protein functions without the need of *de novo* protein synthesis. Moreover, PTMs generate docking sites on target proteins at the damaged site, contributing to the formation of supramolecular structures named DDR *foci*. Among the PTMs, ubiquitination stands up as a key modification that influences almost all aspects of DDR. The peculiarity of ubiquitination as a signalling device lies in the fact that ubiquitin (ub) can form different polymeric chains through the addition of ub molecules on any of the eight amine groups (the N-terminus (M1), K6, K11, K27, K29, K33, K48, and K63) present in the first monomer added to the target protein. The resulting chains will show a distinct topology that leads to different outcomes. Until now, the type of ub chain largely associated to genome maintenance has been the K63-linked that mediates protein-protein interaction in DDR.

Currently our knowledge about the mechanisms behind the maintenance of genome integrity is predominantly based on the action of the ubiquitin ligases. It is well known the fundamental role of the two E3s RNF8 and RNF168, which induces extensive chromatin ubiquitination by targeting histones H2A and H2A.X. Interestingly, we recently discovered that RNF168 prompts an unusual

ubiquitination signalling by targeting the bidentate site K13/15 at the N-terminal tail of histones belonging to H2A family. This important finding demonstrates that RNF168 marks chromatin around DSBs using a unique ubiquitin mark, different from that promoted by Ring1b/Bmi1 at the C-terminal of the same histones.

Starting from these unprecedented findings, which highlighted the peculiarity of the DSBs-induced RNF168-activity towards chromatin, our work focused on two main aspects. First: we aimed to better characterize the specific activity of RNF168 in the context of DDR. Indeed, we demonstrated that RNF168 induces the atypical K27-linked ubiquitination on chromatin and that this modification is the major chromatin-ubiquitin mark after DSBs. In addition, we showed that RNF168-dependent K27-linked ubiquitination targets the bidentate site of the histone H2A.X. Secondly, we strived to demonstrate that the RNF168-dependent K27-ubiquitination is fundamental for the signal spread on chromatin. We found that K27-linked ubiquitination is strictly required for the proper activation of the DNA repair: it recruits, together with the K63-ubiquitination, 53BP1 and BRCA1 to the DDR *foci*. Moreover, we found that the DDR proteins recruited in RNF168-dependent manner are able to recognize K27-linked ubiquitin chains, providing further evidence of the pivotal role of this linkage in the ub-based events that regulate DDR.

Afterwards, considering the emerging relevance of RNF168 in this process, we focused on the identification and characterization of its novel interacting partners and substrates. To this aim, we performed a quantitative proteomic screening based on the SILAC method, which identified several proteins involved in various biological processes. Interestingly, among them we found a poorly characterized deubiquitinating enzyme (DUB), involved in cancer biology, namely USP10. DUBs, which are enzymes able to reverse ubiquitination and process all types of ub-chains, have a crucial role in many cellular processes, including DDR. Thus, we

concentrated our research activity on the characterization of the role of USP10 in the regulation of the RNF168-induced signalling, thereby participating in the DNA damage response. Our findings demonstrate that USP10 constitutively interacts with RNF168 and, in particular, we discovered that it is able to recognize the region on RNF168 that encompasses its RING-finger domain, which is responsible for the ubiquitin ligase activity, suggesting a possible modulatory role of USP10 on the E3 ub ligase. Indeed, we found that the deubiquitinase affects the ubiquitination status of RNF168 but does not affect its stabilization. Although at the moment the mechanism of action is not fully understood, our results suggest a catalytically-independent regulation of the activity of RNF168 by USP10. At last, we functionally characterized USP10 as a necessary player for the correct activation of DDR and DNA repair.

Overall, our findings highlighted the intricate as well as fascinating DDR-signalling cascade, clearly indicating the importance of RNF168 as a key enzyme of the pathway. The characterization of the RNF168-dependent ubiquitination provides an additional signalling network able to reinforce the transmitted signal and give an idea on the potential of ub-crosstalk as a critical regulatory mechanism for genome maintenance. Moreover, our evidence on the action of USP10 on RNF168 opened a new scenario which promises to shed light on the mechanisms that regulate the activation of RNF168. Thus it is of fundamental importance to investigate the fine-tuning of the pathway of USP10 in order to unravel the molecular interplay that lies behind the ub cascade in DDR.

RNF168 PROMOTES NON-CANONICAL K27 UBIQUITINATION TO SIGNAL DNA DAMAGE

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Published in *Cell Reports*, 2015 Jan 13; Vol. 10, Issue 2, p226-238. Epub 2015 Jan 8.

SUMMARY

Ubiquitination regulates numerous cellular processes by generating a versatile communication system based on eight structurally and functionally different chains linked through distinct residues. Except for K48 and K63, the biological relevance of different linkages is largely unclear. Here, we show that RNF168 ubiquitin ligase promotes non-canonical K27-linked ubiquitination both *in vivo* and *in vitro*. We demonstrate that residue K27 of ubiquitin (ubK27) is required for RNF168-dependent chromatin ubiquitination, by targeting histones H2A/H2A.X, and that it is the major ubiquitin-based modification marking chromatin upon DNA damage. Indeed, ubK27 is strictly required for the proper activation of the DNA damage response (DDR) and is directly recognized by crucial DDR mediators, namely 53BP1, Rap80, RNF168, and RNF169. Mutation of ubK27 has dramatic consequences on DDR activation, preventing the recruitment of 53BP1 and BRCA1 to DDR *foci*. Similarly to the DDR, atypical ubiquitin chains could play unanticipated roles in other crucial ubiquitin-mediated biological processes.

INTRODUCTION

Ubiquitination is a post translational modification widely used to regulate protein function in a dynamic and reversible manner. It is a multistep process involving an ubiquitin activating enzyme (E1) that activates the C terminus of free ubiquitin,

which in turn is passed to an E2-conjugating enzyme and finally, with the help of an E3 ubiquitin ligase, targets a Lys residue of the substrate. After the first ubiquitin monomer, additional ubiquitin molecules can be attached to the target protein through any of the eight amine groups in the first molecule - the N terminus (M1), K6, K11, K27, K29, K33, K48, and K63 - to form poly-ubiquitin chains. These different linkages increase the complexity of the ubiquitin system, giving rise to ubiquitin chains with distinct topology, providing structural flexibility that result in a multitude of functional outcomes. The functional roles of K48- and K63-linked ubiquitin chains have been widely investigated; K48- poly-ubiquitination is extensively utilized to target proteins for 26S proteasomal degradation, whereas K63-linked ubiquitination mediates protein-protein interaction in different processes (Chen and Sun, 2009, Jackson and Durocher, 2013, Woelk et al., 2007). Besides the relevance of K11 and M1 linkages in cell cycle regulation and NF-κB activation (Iwai and Tokunaga, 2009, Wickliffe et al., 2009), respectively, little is known about the writers (ubiquitin ligases), the readers (ubiquitin receptors), and the functional consequences of the other non-canonical ubiquitin chains. Recently, different groups reported the essential role of ubiquitination in the DNA damage response (DDR) and in several DNA repair mechanisms. A paradigmatic example of DDR coordination via ubiquitination is represented by the signalling pathway triggered by DNA double-strand breaks (DSBs). Activation of ATM induced by DSBs elicits a cascade of phosphorylation and ubiquitination events that promote the formation of supramolecular complexes, namely the DDR *foci*, which function in integrating and amplifying the signal to downstream effectors. These ubiquitination events are initiated by two ubiquitin ligases, RNF8 and RNF168, which modify chromatin ubiquitinating histones H2A and H2A.X in the proximity of the damage. The K63-specific E2-conjugating enzyme Ubc13 has also been consistently implicated in these

processes (Doil et al., 2009, Huen et al., 2007, Kolas et al., 2007, Pinato et al., 2009, Stewart et al., 2009, Wang and Elledge, 2007). Interestingly, it has been recently reported that the ubiquitin tag generated on chromatin by RNF168 upon genotoxic stress targets histone H2As on the unprecedented bidentate K13/K15 site at its N-terminal tail (Gatti et al., 2012, Mattiroli et al., 2012). This intricate ubiquitin-based network drives the formation of signalling platforms to facilitate the recruitment to DDR *foci* of fundamental components of the pathway, such as 53BP1 and BRCA1, required for the activation of downstream effectors (Huen et al., 2007, Kolas et al., 2007, Mailand et al., 2007). To ensure the fine-tuning of this ubiquitin-based communication system, a number of different mechanisms have evolved to constrain the activity of RNF8 and RNF168. These include the inhibitory effect of different deubiquitinating enzymes. Specifically, USP3 and USP16 counteract H2A ubiquitination (Weake and Workman, 2008), BRCC36 displays selectivity for K6 and K63 modulating the signals generated by apical ubiquitin ligases (Sobhian et al., 2007), and OTUB1 suppresses RNF168-mediated ubiquitination independently of its catalytic activity, by inhibiting UBC13 (Nakada et al., 2010). An additional case of negative regulation of DDR signalling is provided by the two HECT-type ubiquitin ligases TRIP12 and UBR5, which control the accumulation of RNF168 to DDR *foci*, thereby preventing excessive histone ubiquitination (Gudjonsson et al., 2012). Finally, an alternative example of negative regulator of DDR is offered by RNF169, an ubiquitin ligase related to RNF168, which functions at the DDR *foci* by competing with 53BP1 and RAP80/BRCA1 for the binding to RNF168-modified chromatin, thus limiting their recruitment to DSBs (Chen et al., 2012, Poulsen et al., 2012). The involvement of the ubiquitination system in the DDR has been mainly linked to the role played by K63-ubiquitination. This is partly due to the pivotal role of Ubc13 in many aspects of the DDR and to the consequent development of specific investigation reagents

(K63-ubiquitin chains, linkage-specific antibodies, etc.). However, a detailed study of the role played by different ubiquitin chain linkages in the regulation of DDR and DNA repair is still missing. In this study, we adopted biochemical and mass spectrometry approaches, together with small interfering RNA (siRNA)-based ubiquitin knockdown, to survey the functional relevance of different types of ubiquitination in DDR. Our results reveal that the atypical K27-linked ubiquitination is surprisingly the major ubiquitin mark targeting chromatin after DSB induction. This modification is promoted by RNF168 and addresses histone H2A and H2A.X on chromatin. Importantly, in addition to K63, K27 ubiquitination is strictly required for the proper activation of the DDR. Ubiquitin mutants lacking this residue are largely defective in the recruitment of 53BP1 and BRCA1 to the DDR *foci*, whereas ubiquitin forms carrying only K27 and K63 residues are able to restore 53BP1 localization to DDR *foci*. Finally, we found that the DDR proteins accumulating on damaged chromatin in RNF168-dependent manner - i.e., Rap80, 53BP1, RNF169, and RNF168 itself - are able to physically interact with synthetic K27-linked ubiquitin dimers. Overall, our data identify a crucial function for non-canonical K27 ubiquitination in the DDR, determine the ubiquitin ligase that specifically builds K27 ubiquitin conjugates, and define key targets and readers of this modification.

MATERIALS AND METHODS

Cell culture and RNAi

All culture media were supplemented with 10% fetal bovine serum and 2 mM L-glutamine. U2OS cell line expressing the RNF168-targeting shRNA in a doxycycline-inducible manner, kindly provided by J. Lukas, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum tetracycline free (BioWest), 2 mM L-glutamine, 1 µg/ml puromycin (Sigma), and 5 µg/ml blasticidin

S (Sigma). Depletion of endogenous RNF168 was obtained by treating U2OS cells with 0.1 μg/ml doxycycline (Sigma) for 96 hr. RNAi is detailed in Supplemental Experimental Procedures.

Targeted mass spectrometric analysis by SRM

Sample preparation, development of SRM assay, and the detailed protocol are included in the Supplemental Experimental Procedures.

Shotgun mass spectrometric analysis

Detailed protocol for shotgun analysis is given in the Supplemental Experimental Procedures.

Pull-down assays with synthetic di-ub

Pull-downs were performed by incubating 1 μM of recombinant proteins with 0.25 μg of ubiquitin dimers for 1 hr in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, and 1 mM EGTA. Samples were washed four times using high-salt buffer (500 mM NaCl), equilibrated in 150 mM NaCl, and then analyzed by SDS-PAGE and immunoblotting. Ubiquitin immunoblotting was performed using polyvinylidene difluoride membranes (Sigma-Aldrich), denatured in guanidinium chloride and immunoblotted using anti-ubiquitin (P4D1; Santa Cruz Biotechnology).

In vitro *ubiquitination assay*

Recombinant proteins were expressed as described in the Supplemental Experimental Procedures. For the reaction, 5 μg of purified RNF168 construct was incubated with 0.1 μg human recombinant E1 ub-activating enzyme, 0.2 μg of purified His-Ubc5Hc (kindly provided by E. Maspero), and 1 μg of ubiquitin in 25 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 100 mM NaCl, 1 μM dithiothreitol, and 2 mM

ATP (Sigma-Aldrich) at 30°C for 1 hr. ATP regeneration system (Sigma-Aldrich) was used to recycle ATP.

In vivo *detection of ubiquitinated chromatin and histones*

HEK293T cells expressing different ubiquitin mutants 48 hr after transfection were collected in PBS, containing protease inhibitor cocktail (Sigma-A;drich), 1 mM phenylmethylsulfonyl fluoride, and 20 µM N-ethylmaleimide. One-tenth of the samples were separately processed for protein normalization, whereas the remaining was subjected to acidic extraction. Then, samples were either directly analyzed by SDS-PAGE and western blotting or subjected to a second step of purification, by immunoprecipitating using the FLAG-resin, in the presence of 0.1% SDS. Samples were then eluted by glycine and processed for mass spectrometry analysis or by SDS-PAGE and immunoblot as indicated.

Immunofluorescence analysis

U2OS and HEK293T cells subjected to different transfections (plasmids and siRNA oligonucleotides) and drug treatments (etoposide) were processed as previously described (Pinato et al., 2011). Images were acquired by confocal scanning laser microscopy (Leica TCS2; Leica Lasertechnik).

RESULTS

RNF168 remodels chromatin by promoting K27-dependent ubiquitination

It has been previously shown that expression of the histone ubiquitin ligase RNF168 induces extensive chromatin ubiquitination. However, the use of K63-specific antibodies revealed that these ubiquitin signals are only partially explained by the formation of K63 ubiquitin chains on histone H2As (Doil et al., 2009, Pinato et al., 2009, Stewart et al., 2009), suggesting that additional non-K63-linked ubiquitination events are induced by RNF168 to remodel chromatin

structure. To further characterize RNF168-dependent chromatin ubiquitination, we first adopted a biochemical approach by using a panel of ubiquitin mutants carrying a single K/R mutation on each of the seven lysines in the ubiquitin sequence, potentially involved in the formation of poly-ubiquitin chains. These FLAG-tagged constructs were expressed in human embryonic kidney 293T (HEK293T) cells together with RNF168, in order to determine the Lys residue(s) critical for RNF168-induced chromatin ubiquitination. Analysis of chromatin after acidic extraction revealed that the expression of K63R mutant marginally altered the amount of ubiquitinated proteins in the extracts (see ***Figure 1A***), suggesting that K63 is largely dispensable for RNF168-induced nucleosome ubiquitination. Although similar results were obtained with most other lysines, a significant reduction of chromatin ubiquitination is instead observed by using an ubiquitin mutant lacking K27 (K27R; ***Figure 1A***). To further investigate the possible role of K27 linkage, we then asked whether the K27 residue of ubiquitin is by itself sufficient to sustain chromatin ubiquitination. We used a reciprocal series of mutants, where all the seven lysines of ubiquitin were converted to arginine, except one (K-only mutants). By using this approach, we observed that the ubiquitin construct depleted of all the lysines (K0 mutant) strongly impaired the formation of ubiquitin signal on chromatin, although it partially retained the ability to generate multi-mono-ubiquitination, by conjugating to target proteins via the C-terminal Gly76 (ub1 and ub2; ***Figure 1B***). Remarkably, we found that the presence of the single K27 was sufficient to rescue the ubiquitination signal on chromatin at comparable levels to the wild-type protein, clearly indicating that RNF168 uses primarily this atypical ubiquitin linkage to target chromatin ***(Figure 1B)***. In these experiments, we observed variability in the incorporation of different ubiquitin mutants, which is intrinsic to the system because mutants affecting distinct Lys residues could be differently conjugated into proteins ***(Figures S1A***

and S1B). To exclude that the differences in ubiquitination signal were due to uneven expression/conjugation of the mutants, we normalized the samples in respect to the mono-ubiquitinated form of histones ***(Figures S1C and S1D)***. Also, in this case, the relevance of ubK27 is evident, both using the K/R and the K-only mutants of ubiquitin. To exclude that the K27R mutant might prevent per se ubiquitin conjugation, thereby resulting in an indirect reduction of the ubiquitination signal, we carried out two different experiments. First, we performed *in vitro* ubiquitination assays using different combinations of enzymes that specifically generate either K63- (Ubc13/Mms2 and UbcH5c/Nedd4; ***Figures S2A and S2B***) or K48-linked chains (Ube2R1/Nedd4; ***Figure S2C***; Maspero et al., 2011), in the presence of ubiquitin wild-type, K0, K27R, K63R, or K48R mutants. In all cases, K27R mutant was able to generate ubiquitin conjugates at level comparable to wild-type protein. Then, to further support the functionality of K27R, we expressed either FLAG-tagged K48-only or K63-only mutants, in the presence of hemagglutinin (HA)-tagged wild-type, K27R, K63R, or K48R mutants in the context of ubiquitin depletion ***(Figures S2D and S2E)***. We observed that both K48 and K63 conjugates are formed in the presence of the K27R mutant construct ***(Figure S2E)***. Overall, these results indicate that ubK27 is largely responsible for RNF168-mediated chromatin ubiquitination and that the low incorporation observed when expressing the K27R mutant is not due to its intrinsic deficiency in ubiquitin conjugation.

***RNF168/UbcH5c complex forms K27-linked ubiquitin chains* in vitro**

Although these results unambiguously support the relevance of the ubK27 for RNF168-dependent chromatin ubiquitination, they do not prove direct ability of RNF168 to promote K27-linked ubiquitination. To address this point, we performed *in vitro* ubiquitination assays with purified recombinant proteins, using

bacterially expressed RNF168 as E3 ubiquitin ligase, in the presence of wild-type ubiquitin or different mutants, i.e. K0, K63 only, and K27 only. As E2-conjugating enzyme, we opted for the use of UbcH5c, due to its low selectivity toward E3s and to its broad linkage specificity. Using this system, we ascertained that RNF168 is able to induce extensive auto-ubiquitination in the presence of wild-type ubiquitin ***(Figure 1C)***. In contrast, the K0 mutant highly impaired the formation of poly-ubiquitin chains, generating a modest signal likely due to mono-ubiquitination on different sites of RNF168 itself. Notably, when the sole K27 was present, RNF168 ubiquitination was completely recovered and comparable to wild-type levels ***(Figure 1C)***. The K63-only mutant retained the capacity to induce ubiquitination but at significantly lower levels than wild-type or K27-only ubiquitin. Overall, these results indicate that UbK27 is the favorite Lys residue utilized by RNF168 to induce non-canonical ubiquitination both *in vivo* and *in vitro*.

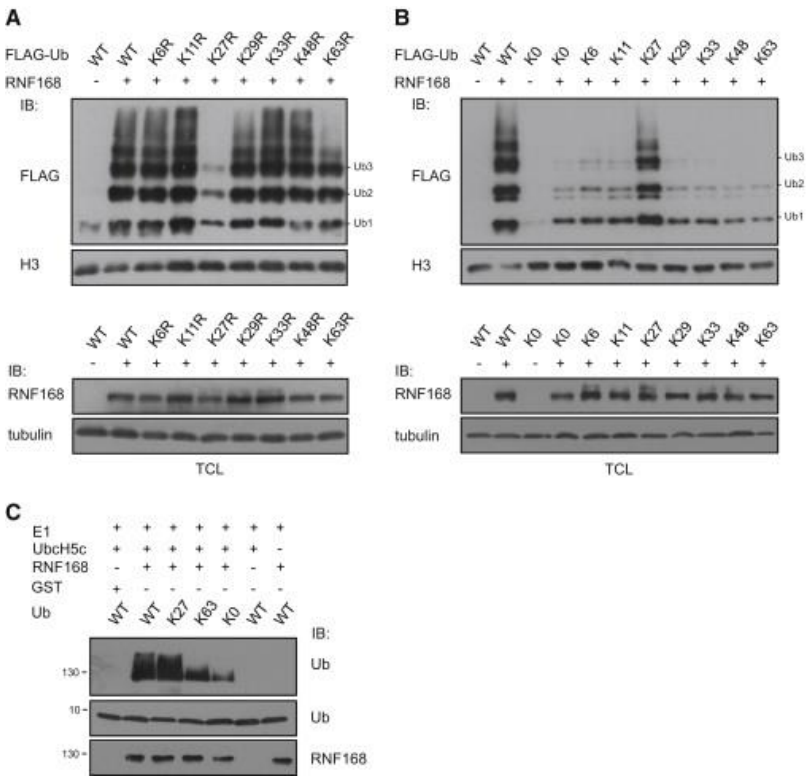


***FIGURE 1: RNF168 INDUCES NONCANONICAL K27-LINKED UBIQUITINATION BOTH* IN VIVO *AND* IN VITRO. (A)** *In vivo* ubiquitination of chromatin was evaluated in HEK293T cells expressing exogenous RNF168, together with FLAG-tagged ubiquitin mutants carrying a single K/R substitution, as indicated. After acid extraction of core nucleosomes, samples were analyzed by SDS-PAGE. Immunoblotting (IB) with anti-FLAG revealed the presence of higher-molecular-weight proteins compatible with mono-, di-, and tri-ubiquitinated forms of histones (ub1, ub2, and ub3, respectively). Protein loading was detected by anti-H3 (on chromatin extracts) and anti-tubulin (on total cell lysates; TCL). RNF168 expression was verified by anti-RNF168 IB. WT, wild-type. **(B)** *In vivo* ubiquitination of chromatin was evaluated as in (A) by using the K-only set of ubiquitin mutants; samples were analyzed as in (A). **(C)** *In vitro* auto-ubiquitination assay was performed using purified E1, E2 (UbcH5c), and E3 (glutathione S-transferase [GST]-RNF168), in combination with the indicated ubiquitin mutants. IB was performed as indicated. Signals observed with ubiquitin (ub) IB correspond to ubiquitinated forms of RNF168 (top) and to free ubiquitin (bottom). See also Figures S1 and S2.

Targeted mass spectrometric analysis reveals that RNF168 promotes the formation of K27-ubiquitinated conjugates into chromatin

We next took a mass-spectrometry-based approach to test whether K27 non-canonical ubiquitination normally occurs in RNF168-expressing cells. Therefore, we set up an analysis based on liquid chromatography coupled to selected reaction-monitoring mass spectrometry (LC-SRM; Maiolica et al., 2012) to unambiguously identify and quantify the tryptic di-Gly signature peptide indicative for K27-linked ubiquitination (TITLEVEPSDTIENVK[GlyGlyAK]; ***Figure 2A***). To build the required specific and sensitive SRM assay for the K27-linked ubiquitin peptide, we digested synthetic K27-ramified di-ubiquitin with trypsin and analysed the resulting peptides. SRM identifies the targeted molecular entity based on a peak group formed by multiple co-eluting signal traces that are generated from fragment ions derived from the target molecule (transitions), in this case, the ubK27 di-Gly peptide ***(Figure 2A)***. The integrated peak area of all transition signals determines the quantity of the targeted peptide. To determine whether RNF168 induces K27-linked ubiquitination, we expressed either wild-type FLAG-tagged

ubiquitin alone or together with RNF168 in HEK293T cells, extracted chromatin fraction, and enriched the FLAG-tagged ubiquitinated proteins ***(Figures 2B and 2C)***. The resulting protein samples were treated with trypsin and analysed by SRM as described above. Remarkably, we found that the ubK27 di-Gly peptide is highly represented in samples where RNF168 is expressed but barely detectable when only the wild-type FLAG-ubiquitin is present ***(Figures 2D and 2E)***. This result clearly indicates that K27 ubiquitination is induced in cells by RNF168.

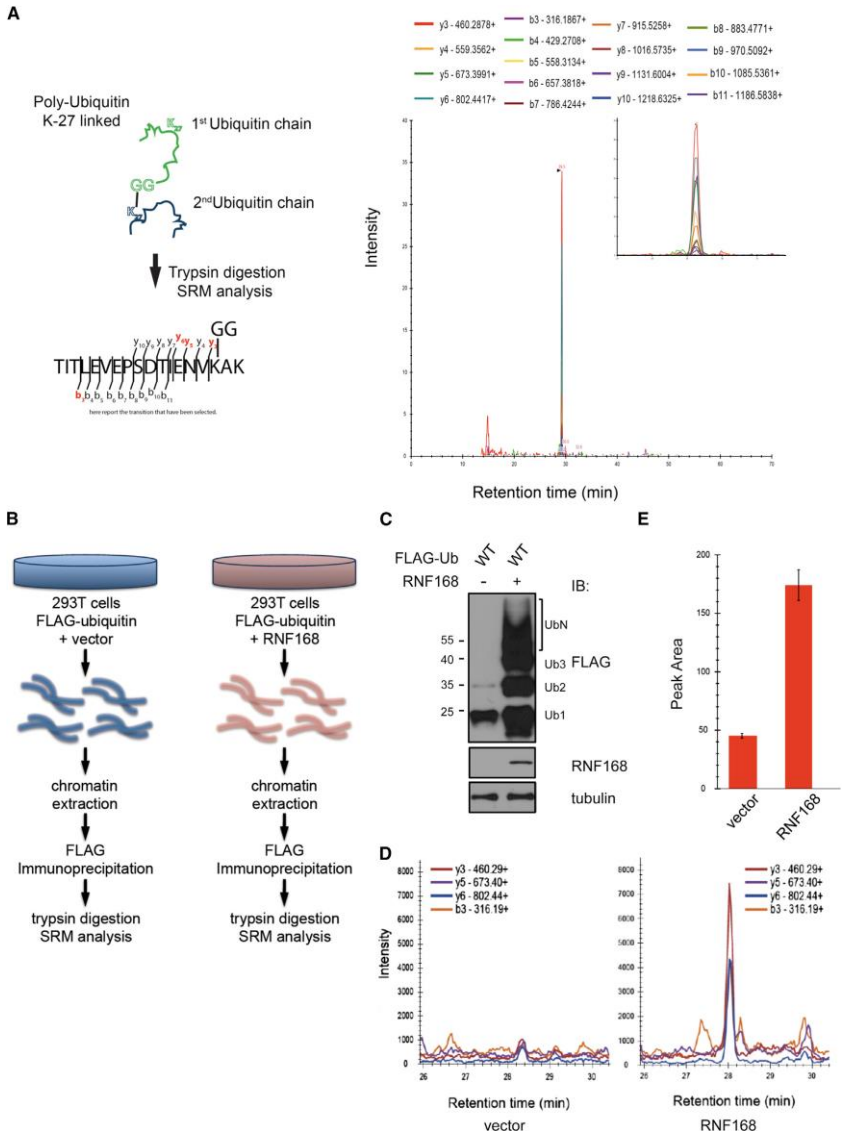


FIGURE 2: SRM ANALYSIS REVEALS THE FORMATION OF K27 CONJUGATES ON CHROMATIN IN CELLS EXPRESSING RNF168. **(A)** Development of the SRM assay for K27-ubiquitinated peptide detection. K27 di-ubiquitin was digested with trypsin and analysed by LC-SRM. Fifty-one transitions were monitored; the four most intense transitions corresponding to the ions y3, y5, y6, and b3 were selected for label-free quantification of the chromatin samples. **(B)** Schematic representation of the experimental procedure. HEK293T cells were co-transfected with RNF168 or empty vector, together with FLAG-tagged ubiquitin wild-type; 72 hr after transfection, cells were subjected to chromatin purification followed by FLAG immunoprecipitation (IP). **(C)** After glycine elution, samples were in part analysed by IB as indicated and in part subjected to trypsin digestion and processed for SRM analysis, as described in the Supplemental Experimental Procedures. **(D)** Representative chromatograms of ubiquitinated K27 peptide measured in digested chromatin samples. **(E)** SRM quantification of the chromatin samples. The bars in the graph indicate the sum of the areas of each of the four transitions measured to quantify the K27-ubiquitinated peptide in chromatin samples. Error bars represent SD. See also Figure S3.

Histones are substrates of RNF168-induced K27 ubiquitination

It has been shown that histones belonging to the H2A family are ubiquitinated by RNF168 (Doil et al., 2009, Gatti et al., 2012, Mattiroli et al., 2012, Pinato et al., 2009, Stewart et al., 2009). Given that we now observed that RNF168 remodels chromatin *via* ubK27, we asked whether histones are also targets of this modification. We transfected HEK293T cells with FLAG-tagged K27-only ubiquitin mutant, together with RNF168 or the vector alone ***(Figure S3A)***, and applied the experimental protocol used in Figure 2B. This allowed us to highly enrich the sample with K27-linked ubiquitin conjugates, as indicated by the amount of K27 di-Gly peptides detected ***(Figures S3B and S3C)***. Then we analysed these samples by shotgun mass spectrometry, in order to identify, among all the proteins pulled down with K27 conjugates, those that are subjected to ubiquitination, as indicated by the presence of the di-Gly signature within their sequence (see ***Table 1***). As expected, we obtained a significant number of peptides, conjugated to di-Gly mark and corresponding to different histones, including H2As, together with other

putative targets of K27 ubiquitination. Due to their fundamental role in the activation of DDR, we focused on histone H2A and H2A.X for further analysis.

Peptide Prophet Probability	Peptide	Protein
0.99	K.LLGGVTIAQGGVLPNIQAVLLPKK[GlyGly].T	H2A (Q8IUE6)
0.99	K.VTIAQGGVLPNIQAVLLPK[GlyGly]K.T	H2A (P0C0S8)
1	K.VTIAQGGVLPNIQAVLLPKKTESHHK[GlyGly].A	H2A
0.99	K.AVTK[GlyGly]YTSSK.-	H2B (P33778)
0.99	R.EIAQDFK[GlyGly]TDLR.F	H33 (P84243)
0.99	M.PEPAK[GlyGly]SAPAPK.K	H2B (P06899)
1	R.K[GlyGly]ASGPPVSELITK.A	H13 (P10412)
1	R.TLSDYNIQK[GlyGly]ESTLHLVLR.L	ubiquitin (P0CG47)
0.99	R.LIFAGK[GlyGly]QLEDGR.T	ubiquitin (P0CG47)
1	G.K[GlyGly]PEPPAMPQPVPTA.-	RS3 (P23396)
0.96	R.VQCCLYFIAPSGHGLK[GlyGly]PLDIEFMK[GlyGly]R.L	SEPT7 (Q16181)
0.91	R.VAGGPVTPRK[GlyGly]GPPK.F	CNRG (P18545)
0.91	K.SGK[GlyGly]YVLGYK.Q	RL30 (P62888)
0.89	N.K[GlyGly]PGPYSSVPPPSAPPPK.K	NOLC1 (Q14978)
0.86	K.VLK[GlyGly]YAGHPPFEHSPIR.F	PER3 (P56645)
1	A.DQLTEEQIAEFK[GlyGly]EAFSLFDKDGDGTITTK.E	CALM HUMAN

0.92	K.K[GlyGly]ISSSGALMALGV.-	MYLK2 (Q9H1R3)
0.95	K.AYIDK[GlyGly]EIEALQDK[GlyGly]IK.N	SULF1 (Q8IWU6)
0.94	K.EFVK[GlyGly]SSVACK.W	CD37L (Q7L3B6)
0.91	K.LVEALDLFERQMLK[GlyGly].E	PTCD1 (O75127)

TABLE 1: IDENTIFICATION OF UBIQUITINATED PROTEINS DERIVED FROM K27-UBIQUITIN-ENRICHED CHROMATIN EXTRACTS. A selected list of di-Gly-marked peptides, identified by mass spectrometry analysis (see Experimental Procedures for details), reveals the presence of a high number of peptides corresponding to histone proteins.

More specifically, we aimed to verify if H2As are substrates of K27 ubiquitination and to assess the specificity for ubK27 in respect to other chain linkages. We expressed a panel of K-only mutants of ubiquitin in HEK293T cells, together with RNF168, and performed biochemical analysis. Ubiquitinated proteins derived from chromatin extracts were immunopurified by using FLAG resin and subjected to immunoblotting using antibodies directed to histones H2A and H2A.X ***(Figure 3A)***. Notably, we found that, in the presence of wild-type and K27-only ubiquitin, both histones H2A and H2A.X are markedly ubiquitinated. Conversely, the expression of the mutants K0, K63-only, and K33-only did not assist RNF168-mediated ubiquitination of H2As. Overall, these results indicate that ubK27 is required to ubiquitinate histone H2As *in vivo*. We next asked whether inactivation of the K13/K15 site - specifically targeted by RNF168 - prevents ubK27-mediated histone ubiquitination. We tested different mutants of H2A.X targeting either the N-terminal (K13/15Q) or the C-terminal (K118/119Q) ubiquitination site ***(Figure 3B)***. Extending previous reports (Gatti et al., 2012, Mattiroli et al., 2012), we found that inactivation of K13/K15 site abolished K27-dependent ubiquitination induced by RNF168, which is instead recovered by the mutant affecting the C-terminal site.

This result indicates that RNF168-dependent K27 ubiquitination is required for histone ubiquitination at K13/K15 site. However, because histone H2As have at least four different Lys that can be theoretically simultaneously targeted by ubiquitin, the detected ubiquitin conjugates could result from multi-mono-ubiquitination on different sites, poly-ubiquitination, or a combination of the two. To elucidate this aspect, we generated mutants of H2A.X where a single Lys of the K13/K15 site is present (see ***Figure 3C***) and expressed them together with wild-type and the K27-only ubiquitin. Then, chromatin fractions were subjected to FLAG-immunoprecipitation in order to enrich the samples with ubiquitinated nucleosomes. The multi-mono-ubiquitination hypothesis would predict that these mutants (no. 2 and no. 3; ***Figure 3D***) undergo only mono-ubiquitination (ub1). Instead, we also clearly observed the di-ubiquitinated form (no. 3; ub2-H2A.X; ***Figure 3D***). Although in principle it is possible that alternative Lys residues are ubiquitinated by RNF168, in addition to K13/K15/K118/K119, this is improbable because the ubiquitination signal is almost abolished when all the sites are substituted (no. 4; ***Figure 3D***). Similarly, we can exclude that the ubiquitin signal observed derives from ubiquitination of different histones - i.e., H2B - or other tightly bound proteins, because we performed FLAG-ubiquitin immunoprecipitation followed by HA-H2A.X immunoblot. This result likely indicates that histone H2As are targets of K27 ubiquitination *in vivo*.

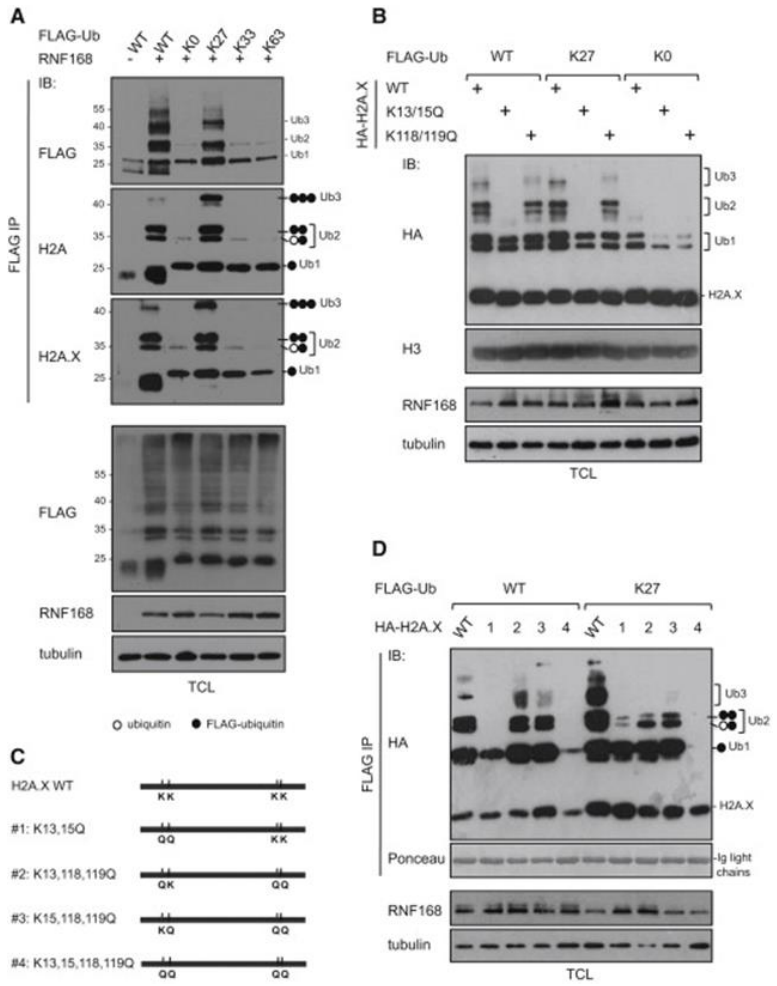


FIGURE 3: HISTONES H2A AND H2A.X ARE TARGETS OF RNF168-INDUCED UBK27. **(A)** Chromatin fractions derived from HEK293T cells expressing exogenous RNF168 together with the indicated K-only ubiquitin mutants were immunoprecipitated with the FLAG resin. FLAG, H2A, and H2A.X IB revealed the presence of higher-molecular-weight proteins compatible with mono-, di-, and tri-ubiquitinated forms (ub1, ub2, and ub3) of histones. Empty and full circles represent the incorporation of endogenous and exogenous (FLAG-tagged) ubiquitin, respectively. Protein loading was verified by FLAG, tubulin, and RNF168 IB on TCL. **(B)** *In vivo* ubiquitination assay of histone H2A.X was evaluated in HEK293T cells expressing exogenous RNF168 and indicated FLAG-tagged ubiquitin constructs, together with HA-H2A.X wild-type and its N- and C-terminal mutants (K13,15Q and K118,119Q, respectively). Forty-eight hours post-transfection, samples were subjected to acid extraction and analysed by SDS-PAGE. Anti-HA IB shows the ubiquitination status of the histone forms. Protein loading was normalized by anti-H3 IB on chromatin extracts (top) and by anti-tubulin on TCL. **(C)** Schematic representation of the H2A.X mutants used in (D). **(D)** HEK293T cells were co-transfected with either wild-type or K27-only mutant of

ubiquitin, together with the mutated forms of H2A.X, in the presence of RNF168. After 48 hr, cells were subjected to chromatin extraction followed by FLAG IP in the presence of SDS. Samples were subjected to IB as indicated.

Chromatin ubiquitination induced by DSBs is dependent on ubK27

As RNF168, together with RNF8, has been clearly implicated in DDR activation *via* chromatin ubiquitination, our findings suggested RNF168-dependent K27 ubiquitination of histones H2A and H2A.X as key molecular events in response to DNA damage. To investigate directly if K27 ubiquitination forms on chromatin upon genotoxic stress, we expressed the different ubiquitin mutants in HEK293T cells. We then induced the formation of DSBs and analysed the level of chromatin ubiquitination. As expected, cells exposed to etoposide (inhibitor of Topoisomerase II) markedly increased the ubiquitin signal on chromatin ***(Figure 4A)***. This effect is dependent on ubiquitin integrity, because the K0 mutant showed a very low level of ubiquitination, which is not altered by etoposide treatment. Although differences in ubiquitin incorporation are detected using different mutants, we observed a clear induction of chromatin ubiquitination with K27-only ubiquitin, slightly lower than with wild-type ubiquitin ***(Figure 4A)***. Conversely, the presence of the sole K63 failed to complement chromatin ubiquitination, suggesting that this type of modification by itself is not sufficient to induce nucleosome ubiquitination upon DNA damage. We further observed that the ubiquitin mutant carrying both K27 and K63 residues was able to fully recover chromatin ubiquitination ***(Figure 4A)***, thereby suggesting a synergistic effect between K27 and K63 in regulating ubiquitination events on chromatin upon DSBs. Similar conclusions can be drawn from the analysis of the K/R mutants ***(Figure 4B)***. Mutants lacking K27 (K27R and K27,63R) were unable to sustain DNA-damage-induced chromatin ubiquitination, whereas mutation of the other Lys residues (K63R and K33R) did not exert any significant effect. Overall, this set of

data clearly shows that chromatin is mainly remodeled upon DNA damage by K27-dependent ubiquitination. Next, to assess whether etoposide-induced K27 ubiquitination depends on the well-characterized ATM/RNF168-signalling pathway, we tested the ubiquitination status of damaged chromatin upon RNF168 depletion and ATM inhibition. Importantly, knockdown of RNF168 obtained by siRNA transfection ***(Figure 4D)*** remarkably impaired the ubK27 signal on chromatin, as shown in Figure 4C. Similar abrogation of ubiquitin signals was obtained in cells pretreated with the ATM inhibitor (KU55933; ***Figure 4E***), although the signal corresponding to phosphorylated form of H2A.X (γ-H2A.X) is still high because of the presence of other kinases of the family (DNA-PK and ATR) not inhibited by KU55933 and the high dose of etoposide we used (30 μM).

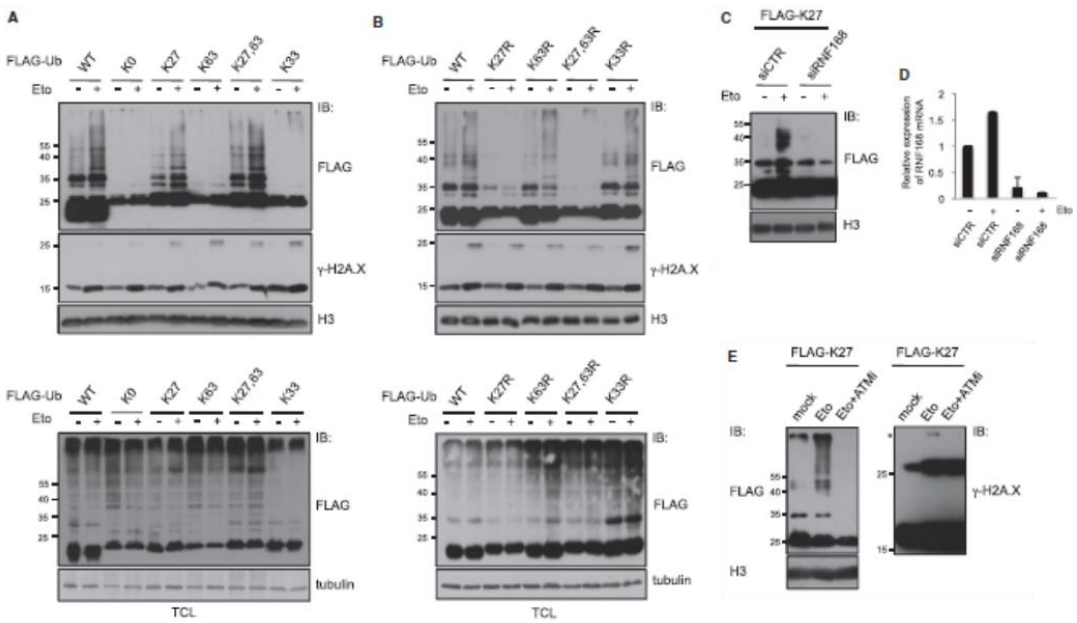


FIGURE 4: K27 UBIQUITINATION IS INDUCED ON CHROMATIN UPON FORMATION OF DSBS.

FIGURE 4. K27 UBIQUITINATION IS INDUCED ON CHROMATIN UPON FORMATION OF DSBS **(A and B)** HEK293T cells expressing FLAG-tagged ubiquitin K/R or K-only mutants were treated with etoposide (30 μM) for 1 hr. After 3 hr, chromatin was extracted from cells and analyzed by IB as indicated. Equal loading and H2A.X phosphorylation (γ-H2A.X) were verified by IB to H3 and to phospho-Ser139 of H2A.X, respectively (top). Expression of ubiquitin mutants was detected by FLAG IB on TCL (bottom). **(C)** HEK293T cells were transfected with siRNA-targeting RNF168 and control siRNA together with FLAG-tagged ubiquitin K27 only. Ninety-six hours after siRNA transfection, cells were treated with etoposide (30 μM) for 1 hr. After 3 hr, cells were subjected to chromatin extraction. IB was performed as indicated. **(D)** Expression of RNF168 mRNA detected by real-time PCR. Total RNA was extracted from HEK293T cells processed as in (C) and subjected to quantitative real-time PCR. The amount of RNF168 RNA relative to 18 s rRNA is shown. Results were expressed relative to the siRNA control in absence of etoposide (siCTR−) that were arbitrarily assigned a value of 1.0. Error bars represent SD. **(E)** HEK293T cells expressing FLAG-tagged K27 only were pretreated with ATMi (KU55933; 10 μM) for 30 min. Cells were then treated and processed as in (C). Asterisk (∗) indicates the di-ubiquitinated form of H2A.X, abrogated by ATMi.

Ubiquitin knockdown impairs the recruitment of 53BP1 and BRCA1 to DDR foci

Chromatin ubiquitination is required for proper formation of DDR *foci* and for activation of the signalling cascade. Because we found that ubK27 is essential for both RNF168- and DNA-damage-induced ubiquitination, we reckoned that it could be required to effectively trigger this process. To assess the role of the different Lys residues of ubiquitin in the formation of DDR *foci*, we set up experimental conditions to markedly reduce ubiquitin expression levels in U2OS cells while retaining cell viability. By using a combination of two different siRNAs, targeting the ubiquitin precursors UBA52 and RPS27A (Adam et al., 2013), we achieved a significant reduction in the formation of ubiquitin conjugates ***(Figures S4A and S4B)*** and tested how the ubiquitin knockdown impacts the formation of DDR *foci*. Upon etoposide treatment, we obtained comparable levels of H2A.X phosphorylation and MDC1 accumulation at DDR *foci* in cells transfected with the siRNAs targeting ubiquitin and with control siRNAs ***(Figures S4C, S4D, and S4G)***. Conversely, 53BP1 and BRCA1 recruitment to DDR *foci* - which are reportedly

dependent on chromatin ubiquitination - were dramatically reduced ***(Figures S4E - S4G)***. To corroborate these results, we reintroduced a siRNA-resistant form of ubiquitin (WTRes) and largely restored proper localization of 53BP1 and BRCA1 to DDR *foci* (Figures S4E–S4G). Albeit predictable from the literature, this represents a direct demonstration that siRNA-based ubiquitin depletion selectively impairs the recruitment of DDR factors downstream of the ubiquitin-dependent step, without affecting upstream, phosphorylation-dependent events.

***UbK27 is strictly required for the proper formation of DDR* foci**

Next, we employed the ubiquitin knockdown system to explore the functional relevance of the different Lys residues of ubiquitin in the activation of the DDR pathway, by using the siRNA-resistant form of the K/R ubiquitin mutants. In keeping with the reported involvement of the K63-specific ubiquitin-conjugating enzyme Ubc13 (Huen et al., 2007, Kolas et al., 2007, Stewart et al., 2009, Wang and Elledge, 2007), expression of the K63R mutant failed to restore 53BP1 and BRCA1 recruitment to the sites of lesions ***(Figures 5A and 5B)***. However, the single substitution K27R - which does not affect K63 ubiquitination - showed an equally marked defect in restoring these DDR events, and the double substitution displayed only marginal additional defects ***(Figures 5A and 5B)***. This effect is highly specific because the other mutants tested (K48R and K33R) performed as wild-type ubiquitin, indicating that they are not directly involved in the accumulation of DDR proteins at the sites of damage. Then we asked whether the use of the K27R and K63R ubiquitin mutants *per se* altered the ability of cells to respond to genotoxic agents, impacting also upstream events induced by DSBs. In our experimental conditions, we found that both MDC1 recruitment and H2A.X phosphorylation were similar in all K/R mutants and comparable to control cells, confirming that the phosphorylation-dependent events induced by DNA damage

upstream of RNF168-induced ubiquitination were not affected by the expression of mutant forms of ubiquitin ***(Figures S5A and S5B)***. Similar conclusions were reached by an independent set of results. We transfected the FLAG-tagged ubiquitin constructs in HEK293T cells in order to obtain an elevated expression of the proteins, aiming to compete with the endogenous form. Using this system, we confirmed that overexpression of the ubiquitin mutants carrying either K27R or K63R substitutions impaired the accumulation of 53BP1 and BRCA1 at DDR *foci* ***(Figures S5C - S5F)***.

***K27,63-only mutant partially rescues the localization of 53BP1 to DDR* foci**

The above results indicate that K27 and K63 linkages are required for the generation of ubiquitin-dependent signals upon DSB formation. Hence, we asked whether they are sufficient to support the formation of DDR *foci*. To this purpose, we expressed K27-only, K63-only, and K27,63-only mutants in the context of ubiquitin depletion, and we assessed the recruitment of 53BP1 and BRCA1 to the sites of damage. Remarkably, the simultaneous presence of K27 and K63 of ubiquitin was sufficient to significantly restore etoposide-induced 53BP1-positive *foci*, whereas the sole presence of K27 or K63 had incomplete effects ***(Figure 5C)***. However, none of these constructs was able to restore BRCA1-positive *foci* (data not shown), suggesting the contribution of additional ubiquitin linkages, besides K27 and K63, in its recruitment. Consistent with the data in Figure 4E, we found that the K27,63-dependent formation of 53BP1 *foci* upon etoposide treatment is prevented by ATM inhibition ***(Figure 5D)***. Similarly, by using U2OS cells conditionally expressing RNF168-targeting small hairpin RNA (shRNA) (Doil et al., 2009), we found that depletion of RNF168 completely abolished the accumulation of 53BP1 to DDR *foci*, even in the presence of K27,63-only mutant ***(Figure S6A)***, and reduced the formation of ubiquitin conjugates within the nucleus ***(Figure***

S6B). Collectively, our results indicate that K63 and K27 linkages are necessary, and sufficient in combination, to recruit key players of the DDR (i.e., 53BP1) and that these ubiquitin-mediated events are dependent on ATM and RNF168 activity.

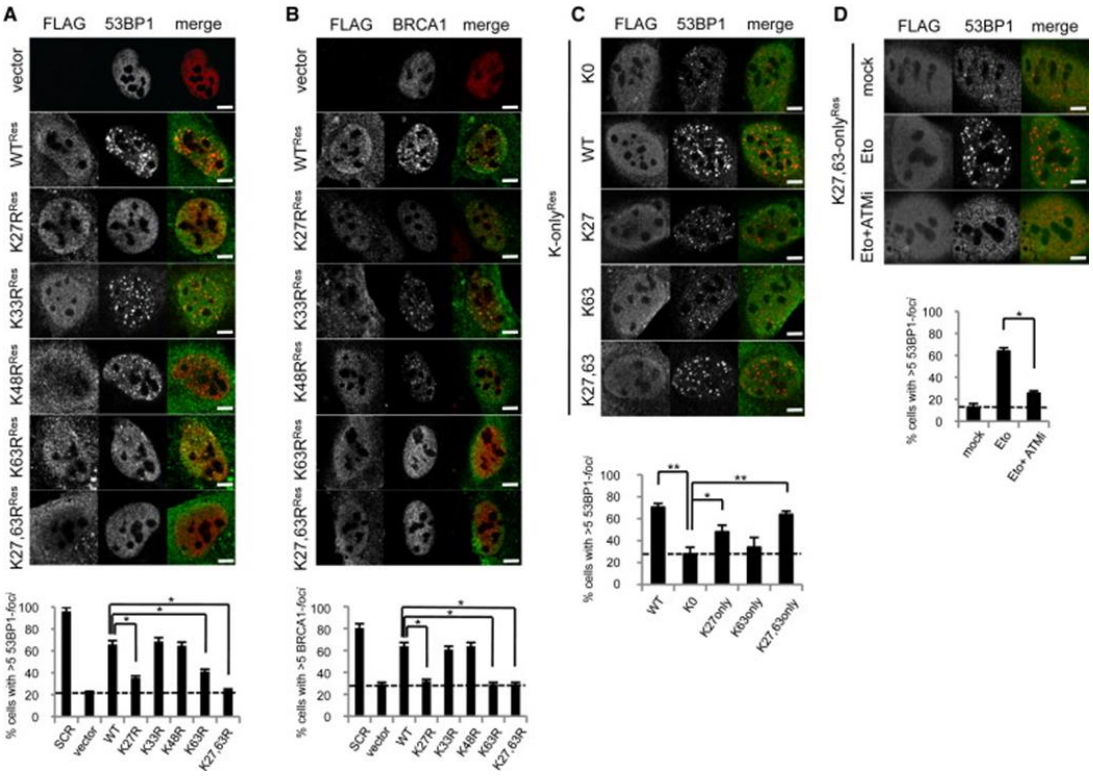


***FIGURE 5: UBK27 IS ESSENTIAL FOR THE ASSEMBLY OF DDR* FOCI. (A and B)** U2OS cells were transfected with siRNA-resistant forms of wild-type ubiquitin and a panel of K/R mutants, as indicated. Ubiquitin knockdown was obtained by transfecting two siRNAs targeting ubiquitin in a 1:1 combination (see the Supplemental Experimental Procedures). Eighteen hours later, cells were treated with etoposide (5 μM) before fixing. Immunostaining was performed with the indicated antibodies. **(C)** U2OS cells were transfected with siRNA-resistant FLAG-tagged ubiquitin WT and K-only mutants and then transfected with ubiquitin siRNAs, as in (A) and (B). Cells were treated with etoposide (5 μM) for 1 hr before fixing. Immunostaining was performed with FLAG and 53BP1 antibodies. **(D)** U2OS cells expressing siRNA-resistant FLAG-tagged K27,63-only ubiquitin were transfected with two siRNAs targeting ubiquitin for 18 hr and then treated or not with etoposide as in (A)–(C) (top and middle) and in the presence of ATM inhibitor (ATMi KU55933; 10 μM) before fixing. Immunostaining was performed as in (C). (A–D) Quantification of the FLAG-positive cells with more than five *foci* labeled with the indicated antibodies. At least 50 cells per

condition were counted. The graphs are a summary of three independent experiments; each value represents the mean ± SD of three separated experiments. ∗p < 0.05; ∗∗p < 0.01. The scale bars represent 1 μm. See also Figures S2 and S4–S6.

K27 ubiquitination is recognized by DDR proteins - Rap80, 53BP1, RNF168, and RNF169

Our experiments collectively indicate a pivotal role for K27 linkage in the ubiquitin-based events regulating the DDR, promoting the accumulation of 53BP1 and BRCA1 to the damage sites. We would thus expect that these proteins be recruited to DDR *foci via* ubK27 signal. It has been described that BRCA1 is relocated to DNA damage by means of Rap80, which contains two adjacent ubiquitin-binding domains (UBDs) named UIM1-UIM2 or tandem UIMs (Sato et al., 2009, Sobhian et al., 2007). We thought that, although Rap80-UIMs show specificity for K63 over K48 chains, they might be able to interact with additional types of chains, as previously shown (K6) (Sobhian et al., 2007). Thus, we tested the ability of the Rap80-UIMs to recognize K27-ubiquitinated proteins in a purified system. We performed a pull-down assay using the synthetic K27-linked ubiquitin dimers (diubK27), besides diubK63 and diubK48 as positive and negative reference, respectively. As control of specificity, we used the defective mutant of UIMs (Rap80-UIM∗∗) carrying two aminoacid substitutions (A88S and A113S). As shown in ***Figure 6A***, we found that the Rap80-UIMs interact with diubK27, whereas the mutant does not. Next, we focused on another crucial DDR protein, namely 53BP1, whose recruitment to DDR *foci* is dependent on two different histone modifications, methylation on K20 of histone H4 and ubiquitination on K15 of histone H2A (H2AK15ub). The binding to H2AK15ub is dependent on the ubiquitination-dependent recruitment (UDR) motif of 53BP1, which recognizes the ubiquitinated H2A on K15 but does not directly interact with ubiquitin monomer

(Fradet-Turcotte et al., 2013). By performing similar experiments as in Figure 6A, we tested the UDR motif of 53BP1 and a mutant form unable to localize to DDR *foci* (L1619A and R1627A; Fradet-Turcotte et al., 2013) in a pull-down assay with ubiquitin dimers ***(Figure 6B)***. Strikingly, we found that the sole UDR motif is able to bind diubK27 and diubK63, but not diubK48, whereas the mutant UDR** completely failed to bind diubK27 and reduced the interaction with diubK63. Finally, we tested two additional DDR proteins that are recruited/stabilized on chromatin upon DNA damage by interacting with RNF168-dependent ubiquitin conjugates; i.e., RNF168 itself and the closely related RING finger protein RNF169. As shown in ***Figures 6C*** and ***6D***, the two ubiquitin ligases can interact with the K27-linked ubiquitin dimeric peptide. Overall, these results show that K27 ubiquitination is widely recognized by crucial players of the DDR.

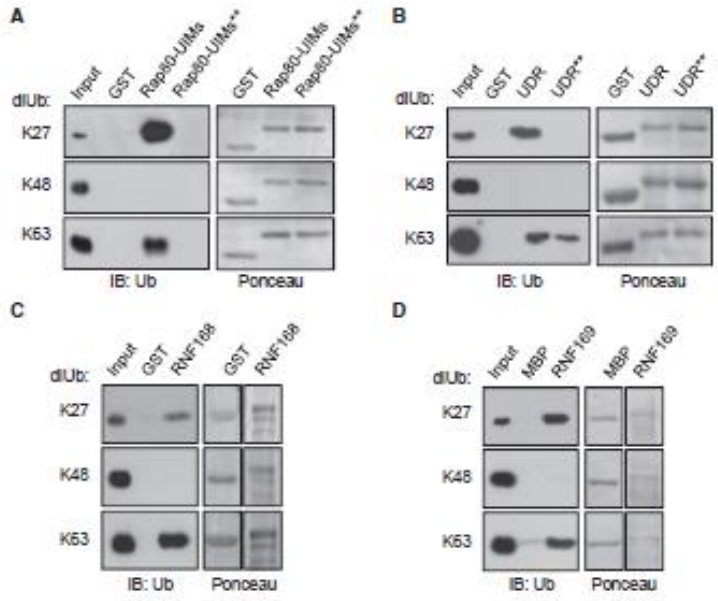


FIGURE 6: 53BP1, RAP80, RNF169, AND RNF168 DIRECTLY INTERACT WITH K27-LINKED UBIQUITIN DIMERS. **(A–D)** Pull-down assays performed using different recombinant proteins incubated with synthetic K27, K48, and K63-linked ubiquitin dimers (diub); in (A), GST-

tagged Rap80-UIMs and Rap80-UIMs** (A88S and A113S); in (B), GST-tagged 53BP1-UDR wild-type or and GST-53BP1-UDR** mutant (L1619A and R1627A); in (C) and (D), GST-tagged RNF168 and MBP-tagged RNF169 as full-length proteins. Proteins were solved by SDS-PAGE, and IB was performed with ubiquitin antibody (P4D1). Ponceau staining revealed equal loading.

DISCUSSION

In this study, we report a key biological function for the non-canonical K27 ubiquitin linkage. We show that ubK27 is required to promote chromatin ubiquitination following DNA damage, and this is strictly dependent on the activity of the ubiquitin ligase RNF168. Moreover, we find that histones belonging to the H2A family are targets of this modification on the K13/K15 site and that crucial players of DDR - including Rap80 and 53BP1 - directly interact with the K27-linked ubiquitin mark.

Role of K27 ubiquitination in the cellular response to DNA damage

Here, we disclose the biological relevance of K27 ubiquitination, being essential for the proper activation of the signalling cascade induced by genotoxic stress. We show that ubiquitin mutants lacking ubK27 exhibit dramatic defects in the recruitment of DDR proteins, which act downstream of the ubiquitination-dependent step (i.e., 53BP1 and BRCA1). In keeping with the literature, we confirmed an essential role for K63 linkage in the activation of the DDR pathway. However, our studies reveal that this modification is only moderately involved in RNF168- and DNA-damage-induced ubiquitination of core histones and thus presumably targets other important mediators of the DDR. The essential role of K27 and K63 linkage in DDR is further corroborated by the observation that ubK27 and UbK63 in combination are sufficient to rescue the formation of 53BP1-positive *foci*. Interestingly, we did not observe the same result with BRCA1, implying additional complexity in its mode of recruitment to chromatin, possibly

involving K6 linkage (Nishikawa et al., 2004, Wu-Baer et al., 2003). Overall, our results highlight the importance to generate specific and univocal signals on chromatin to alert cells to genotoxic stress. Others and we recently reported that, upon DSBs, RNF168 ubiquitinates a site on the N-terminal tail of histone H2As (K13/K15; Gatti et al., 2012, Mattiroli et al., 2012), which is distinct from the canonical K118/K119 site. We now provide additional mechanistic insight into the emerging theme of ubiquitin diversity, by demonstrating that RNF168 marks chromatin histones with an atypical ubiquitin linkage, which could act as a different language to reinforce the peculiarity of the transmitted signal. These findings redefine a crucial step of the cellular mechanisms maintaining genome stability and will likely foster further molecular investigations in this area.

Targets and readers of K27-linked ubiquitin mark on chromatin

Even though it has been reported that K27 ubiquitination can be assembled in different cellular contexts (Ben-Saadon et al., 2006, Peng et al., 2011, Zucchelli et al., 2010), its functional relevance in specific processes has been elusive. Similarly, a direct connection between enzymes promoting this modification and their putative targets could not be established to date. In this respect, our finding that the DDR ubiquitin ligase RNF168 specifically induces K27 ubiquitination on chromatin histones is highly relevant, encourages new studies in this direction, and opens a number of crucial questions. First, it is predictable that, aside from H2A and H2A.X, other factors are targets of RNF168-mediated K27 ubiquitination. One candidate to be investigated is 53BP1, because it has been recently demonstrated to be targeted by RNF168's activity (Bohgaki et al., 2013). Another interesting candidate is the polycomb protein Ring1b. Ciechanover and colleagues demonstrated that Ring1b promotes auto-ubiquitination through mixed poly-ubiquitin chains (K6, K27, and K48) and this is a prerequisite for Ring1B to mono-

ubiquitinate *in vitro* the histone H2A (Ben-Saadon et al., 2006). In our experiments, we observed that the substitution of K27 of ubiquitin markedly affects ubiquitin chains but also the formation of mono-ubiquitinated histone H2As. A tantalizing hypothesis is that RNF168 could modulate the activity of Ring1b by promoting its K27-linked ubiquitination, drawing a direct connection between RNF168 activity and the regulation of gene silencing. A multitude of ubiquitin-interacting motifs play an essential role in this ubiquitin-based communication system, allowing the recruitment of DDR proteins to damaged chromatin by binding to the products of RNF168 activity (Doil et al., 2009, Fradet-Turcotte et al., 2013, Panier et al., 2012, Penengo et al., 2006, Pinato et al., 2009, Pinato et al., 2011, Sato et al., 2009, Stewart et al., 2009). In this study, we provide clear evidence that different DDR proteins - Rap80, RNF169, and RNF168 itself - directly interact with K27-linked ubiquitin dimers. These data indicate that the histone ubiquitin code is far more complex than previously predicted and that a number of ubiquitin-dependent events in the DDR can be driven directly by K27 ubiquitination. Another relevant observation relates to 53BP1. It has been recently demonstrated that the UDR domain of 53BP1 (Fradet-Turcotte et al., 2013) binds to ubiquitinated histone H2A on K15. Now, we show that the UDR is sufficient to directly interact with K27 and K63 ubiquitin dimers. This is a remarkable finding because it reveals that 53BP1 UDR is *bona fide* a UBD, further supporting its crucial role as reader of chromatin ubiquitination induced by DNA damage.

RNF168 as a writer of K27 ubiquitination

K27 linkage is used by RNF168 to induce extensive chromatin ubiquitination. Nevertheless, this K27 ubiquitin signal does not target proteins for proteasomal degradation (Figure S7) but rather generates the docking sites for different

downstream signalling effectors. Remarkably, our data show that RNF168 significantly increases the level of mono-ubiquitinated and multi-mono-ubiquitinated histones, indicating that ubK27 affects the general ubiquitination status of chromatin. In line with this, it has been suggested that the stable accumulation of RNF168 to DDR *foci* depends on the binding of its UBDs to ubiquitin conjugates promoted by RNF168 itself (Panier et al., 2012). Thus, RNF168, by promoting chromatin ubiquitination, generates the docking sites for itself and stabilizes its accumulation at DDR *foci* in a positive feedback loop. Our identification of atypical K27 ubiquitination as key mediators of a central biological process like the DDR will likely encourage a thorough, unbiased analysis of different ubiquitin linkages also in other crucial cellular pathways regulated by ubiquitin, where alternative modifications may have as yet escaped systematic *in vitro* and *in vivo* analysis.

AUTHOR CONTRIBUTIONS

M.G., S.P., F.R., and M.G.P. performed biochemical studies using the ubiquitin mutants, F.R. analyzed histone ubiquitination, S.P. generated constructs and performed binding assay with K27-linked conjugates and DDR proteins, M.G. performed ubiquitin and RNF168 knockdown and prepared samples for MS analysis, A.M. and R.A. designed and performed the LC-SRM analysis and provided intellectual support, M.G. and L.P. performed immunofluorescence analysis, and L.P. planned the project, designed the experiments, and wrote the manuscript. All the authors discussed the data and commented on the manuscript.

ACKNOWLEDGMENTS

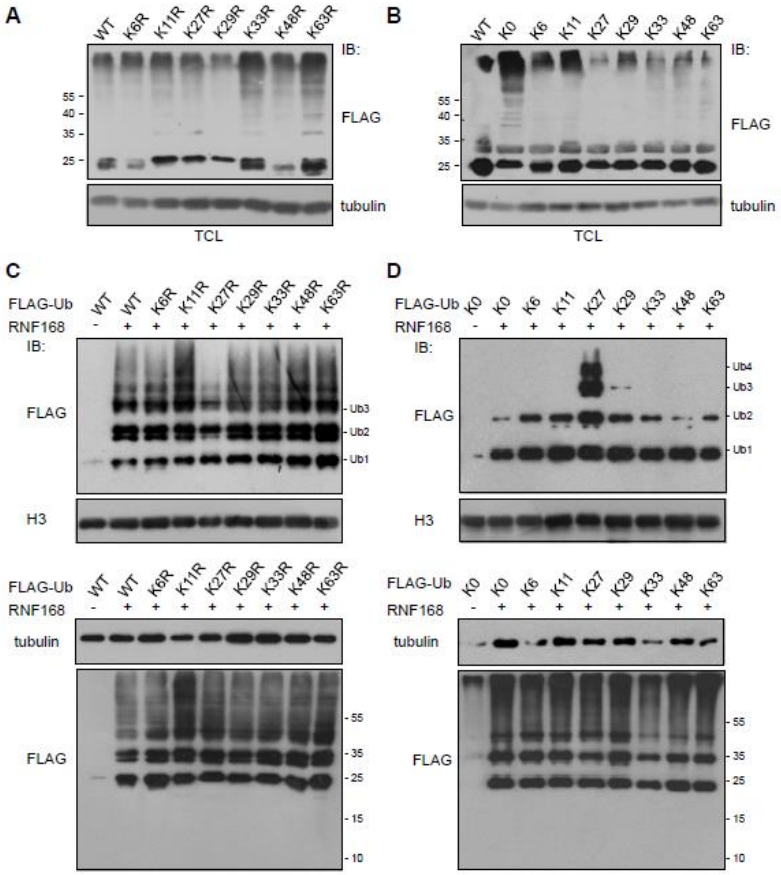
We are grateful to Massimo Lopes and Simona Polo for critical reading of the manuscript. This work was supported by Associazione Italiana per la Ricerca sul Cancro (IG: 11979) and Fondazione Cariplo (grant 2013-0594) to L.P., by

Fondazione Italiana per la Ricerca sul Cancro to S.P. and M.G. (Rif. 14885), and by an EMBO long-term fellowship to A.M.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

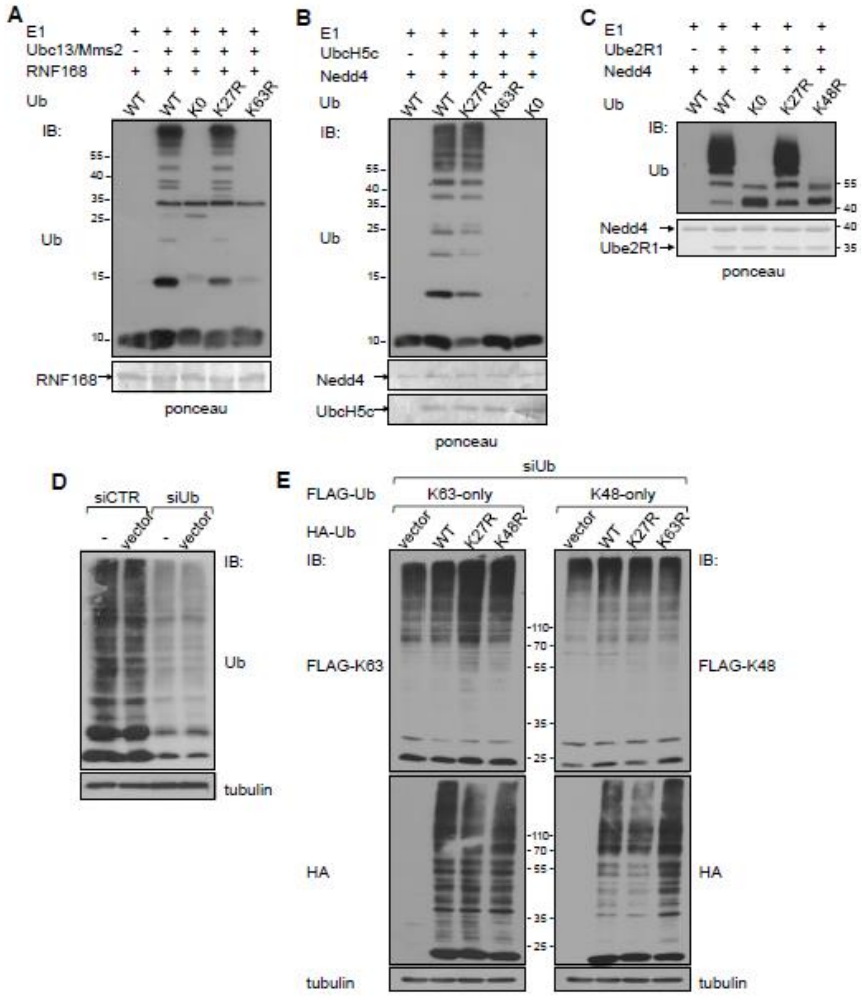
FIGURE S1: RELATED TO FIGURE 1. EXPRESSION LEVELS OF THE DIFFERENT UBIQUITIN MUTANTS.



(A and B) One tenth of HEK293T cells expressing exogenous RNF168, together with FLAG-tagged K/R (A) and K-only (B) mutants of ubiquitin, were lysed directly with Laemmli buffer and analyzed by SDS-PAGE. IB with anti-FLAG antibody revealed the level of expression of different ubiquitin mutants (upper panels). Protein loading was visualized by anti-tubulin IB. **(C and D)** Chromatin extracts from HEK293T cells expressing exogenous

RNF168, together with FLAG-tagged K/R (C) and K-only (D) mutants of ubiquitin, were normalized based on the signal corresponding to the mono-ubiquitinated forms of histones, instead of on the quantity of chromatin extracts loaded on the gel. IB with anti-FLAG antibody revealed the level of expression (TCL, lower panels) and incorporation into chromatin (chromatin extracts, upper panels) of different ubiquitin mutants. Protein loading was visualized by anti-H3 and anti-tubulin IB.

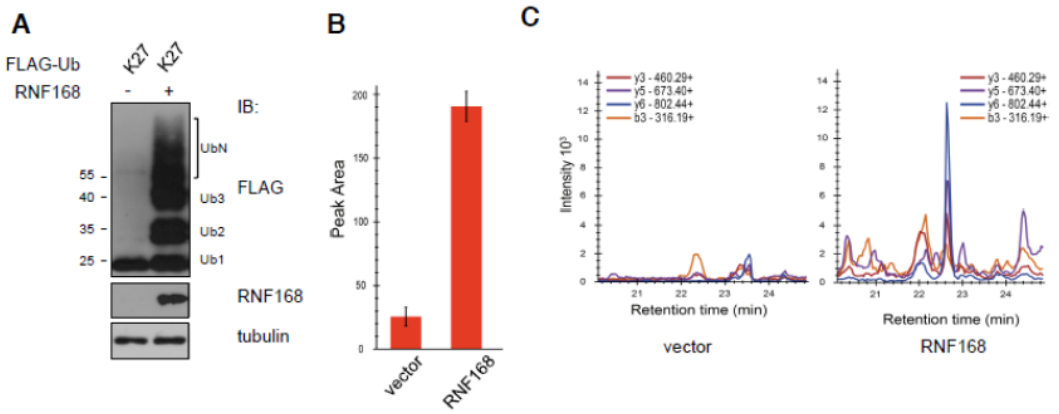
***FIGURE S2: RELATED TO FIGURE 1 AND 5. K27R DOES NOT HAMPER THE FORMATION OF K63 AND K48 POLY-UBIQUITIN CHAINS* IN VITRO *AND* IN VIVO.**



(A-C) *In vitro* ubiquitination assay performed using different pairings of E2/E3 in combination with the indicated ubiquitin constructs. (A) Ubc13/Mms2 and GST-RNF168; (B) UbcH5c and HECT domain of Nedd4; (C) Ube2R1 and HECT domain of Nedd4. In all cases ubiquitin conjugates were revealed by ubiquitin IB. **(D)** HEK293T cells were

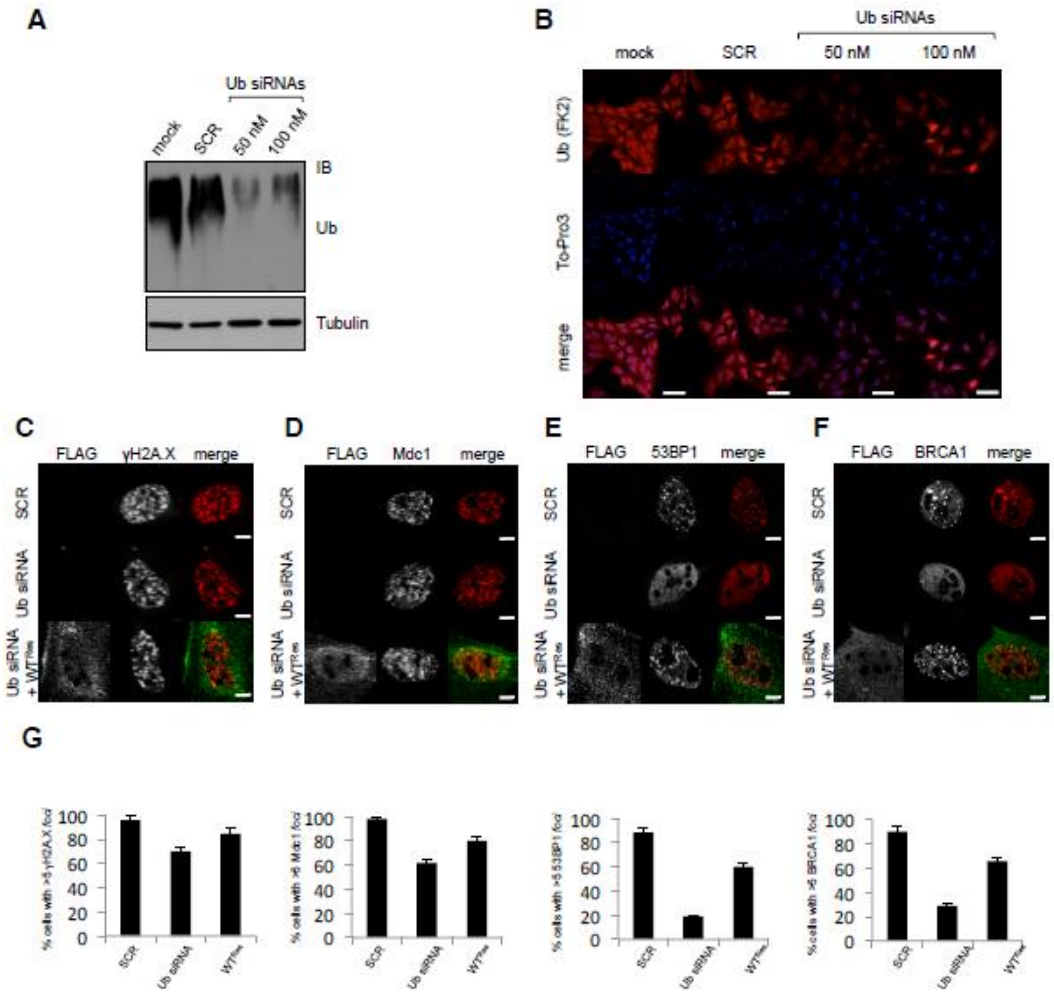
transfected with siRNAs targeting ubiquitin (siUb) or with control siRNA (siCTR), and lysed after 20 hours in RIPA buffer. **(E)** HEK293T cells treated with ubiquitin siRNAs were transfected with the indicated constructs and processed as in (D). IB was performed using FLAG IB to reveal the formation of K63 and K48 conjugates. Expression/conjugation of K/R mutants are revealed by HA IB (lower panels). Tubulin was used as loading control.

FIGURE S3: RELATED TO FIGURE 2. SRM ANALYSIS ON CELLS EXPRESSING K27-ONLY MUTANT OF UBIQUITIN.



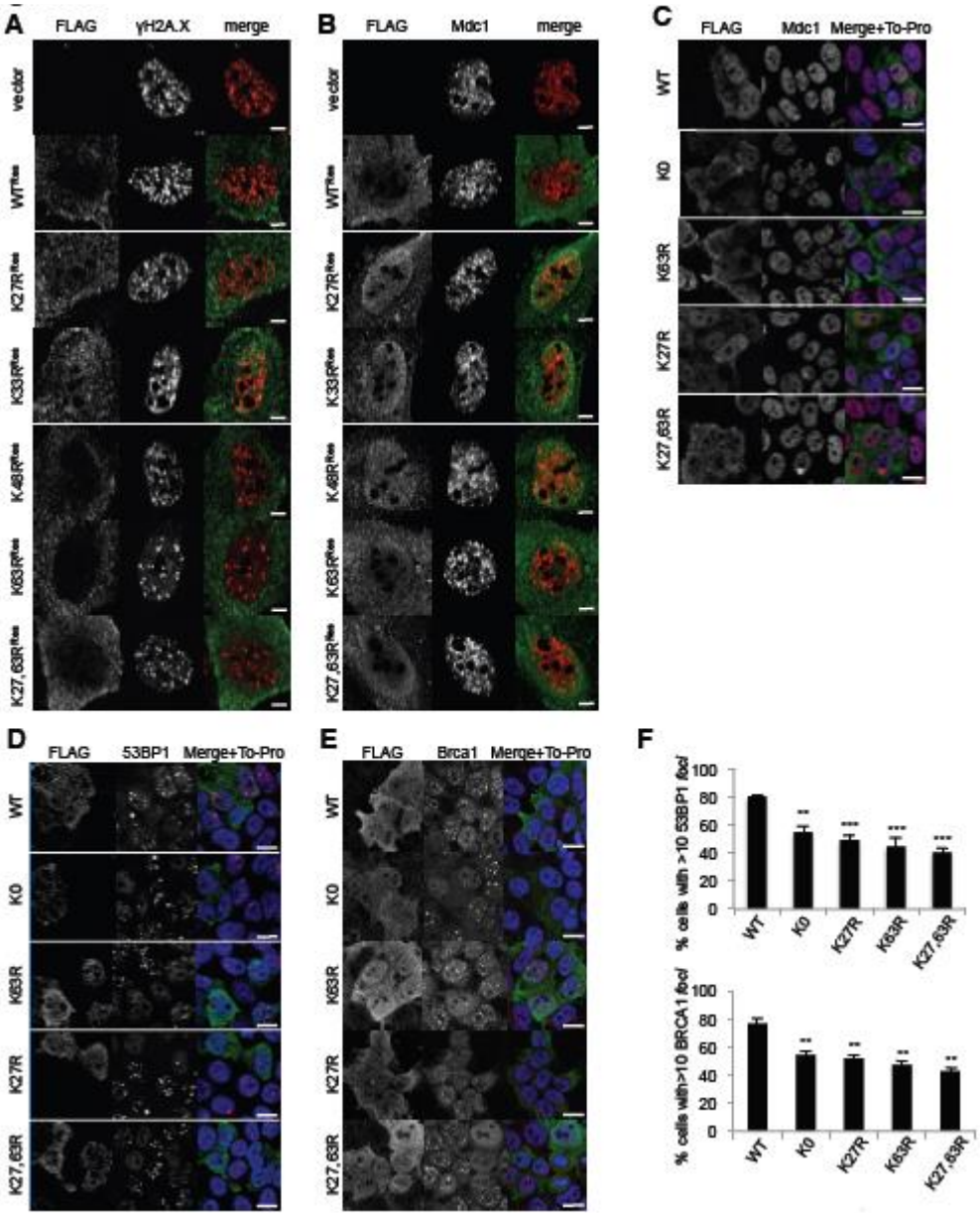
(A) HEK293T cells expressing exogenous RNF168 together with FLAG-Ubiquitin K27-only were subjected to chromatin extraction followed by FLAG IP. Samples were then eluted with glycine and subjected either to SDS-PAGE and IB as indicated, or to trypsin digestion and processed for SRM analysis. See Extended Experimental Procedures for details. **(B)** SRM quantification of the chromatin samples. The bars in the graph indicate the sum of the areas of each of the four transitions measured to quantify the K27 ubiquitinated peptide in chromatin samples. Error bars represent SD. **(C)** Representative chromatograms of ubiquitinated K27 peptide measured in digested chromatin samples.

FIGURE S4: RELATED TO FIGURE 5. UBIQUITIN KNOCKDOWN, OBTAINED BY USING SIRNA OLIGONUCLEOTIDES, HIGHLY AFFECTS THE DDR SIGNALLING CASCADE.



(A) U2OS cells were transiently transfected with two different siRNAs targeting ubiquitin at two different concentrations (50 nM and 100 nM). After 18 hours, cells were lysed in RIPA buffer and protein extracts were subjected to SDS-PAGE and IB using anti-ubiquitin antibody (P4D1). **(B)** U2OS cells transfected as in (A) were treated with etoposide (5 μM) for 1 hour before fixing and immunostained using anti-ubiquitin antibody (FK2); nucleus were stained with To-Pro3. Scale bars, 10 μm. **(C-F)** siRNA-resistant FLAG-tagged ubiquitin (WTRes) was expressed in U2OS cells. Cells were then transfected with siRNAs to induce ubiquitin knockdown. After 18 hours cells were treated with etoposide (5 μM) before fixing. Immunostaining was performed as indicated. Scale bars, 1 μm. (G) Quantitation of cells expressing FLAG-ubiquitin with more than 5 foci positive for the indicated DDR proteins. The graphs are a summary from three independent experiments; error bars represent SD.

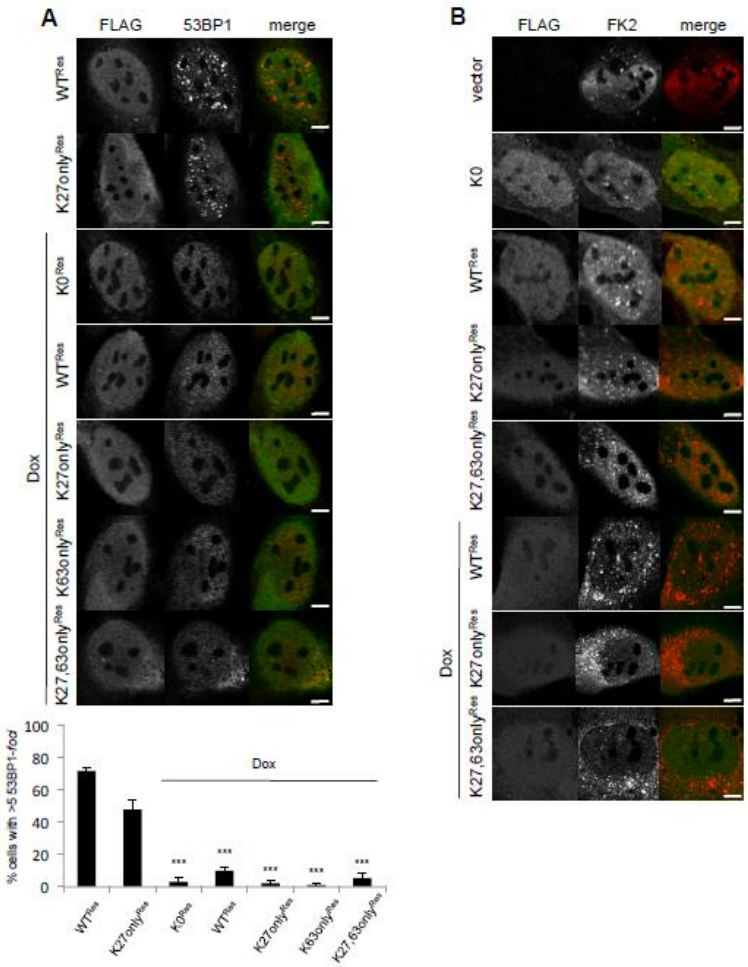
FIGURE S5: RELATED TO FIGURE 5. K27R MUTANT DOES NOT AFFECT THE PHOSPHO-DEPENDENT EVENTS UPSTREAM OF RNF168 LIGASE, BUT IMPAIRS THE RECRUITMENT OF 53BP1 AND BRCA1 AT DDR FOCI IN HEK293T CELLS.



(A and B) U2OS cells were transfected with siRNA-resistant forms of wild-type ubiquitin and K/R mutants, as indicated. Ubiquitin knockdown was obtained by transfection of siRNAs targeting ubiquitin. After 18 hours, cells were treated with etoposide (5 μM) before fixing. Immunostaining was performed with the indicated antibodies. Scale bars, 1 μm. **(C-F)** HEK293T cells were transfected with the indicated FLAG-tagged ubiquitin K/R

mutants. 48 hours after transfection cells were treated for 1 hour with etoposide (5μM) before fixing. Immunostaining was performed as indicated. Scale bars, 5 μm. (F) At least 150 cells per condition were counted. The graphs are a summary of three independent experiments; each value represents the mean ± SD of three separated experiments. **P<0.01, ***P<0.001.

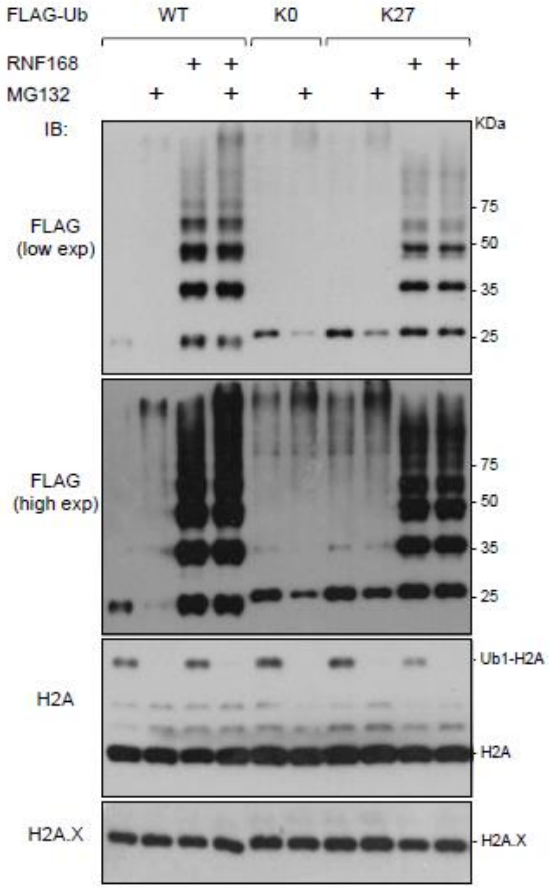
FIGURE S6: RELATED TO FIGURE 5. RNF168 DEPLETION AFFECTS UBK27- AND UBK63-DEPENDENT RECRUITMENT OF 53BP1 TO DDR FOCI AND REDUCES THE SIGNAL OF UBK27 CONJUGATES WITHIN THE NUCLEUS.



(A and B) U2OS cells conditionally expressing RNF168 targeting shRNA, treated or not with doxycycline (Dox) to induce RNF168 depletion, were transfected with the indicated siRNAresistant forms of FLAG-tagged wild-type ubiquitin (WTRes) and K-only mutants, and then subjected to ubiquitin knockdown. After 18 hours, cells were treated with

etoposide (5 µM, 1 hour) and fixed. Immunostaining was performed using FLAG, 53BP1 and ubiquitin (FK2) antibodies. At least 50 cells per condition were counted. The graph is a summary of three independent experiments; each value represents the mean ± SD. ***P<0.001. Scale bars, 1 µm.

FIGURE S7: RELATED TO FIGURE 5. K27 UBIQUITINATION DOES NOT TARGET HISTONES FOR PROTEASOMAL DEGRADATION.



HEK293T cells expressing exogenous RNF168, together with the indicated FLAG-tagged ubiquitins, were treated or not with MG132 (10 µM) for 4 hours, and lysed. FLAG IB revealed the formation of ubiquitin conjugates in cells upon transfection of the ubiquitin constructs. Anti-H2A and anti-H2A.X IB revealed the expression level of histones in cells in different experimental conditions. The mono-ubiquitinated form of H2A (Ub1-H2A) is reduced upon proteasome inhibition.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids and constructs

The full-length cDNA of RNF168 (clone IRATp970F1053D) was cloned into pcDNA3.1 (Invitrogen) and pGEX6P2. Ubiquitin cDNA derived from UBC gene (ID: NM_021009.5). K/R mutants of ubiquitin were generated by site-directed mutagenesis. K-only and K0 were kindly provided by E. Maspero and S. Polo. Plasmids encoding HA-H2A.X were already described (Pinato et al., 2009). Point mutations were introduced by site-specific mutagenesis. The RAP80-UIMs were amplified by PCR from the full-length protein (a gift of S. Elledge) and cloned into pGEX6p2 vector. The aminoacid substitutions A88SA113S were introduced by site directed mutagenesis. The cDNA of 53BP1-UDR was synthesized by Eurofins and cloned into pGEX6P2. The L1619A and R1627A mutations were introduced by site-specific mutagenesis. MBP-RNF169 full-length was kindly provided by D. Durocher. Oligonucleotides sequences are given below. All constructs were sequence verified.

In vitro *ubiquitination assay*

Each reaction was performed in a buffer containing 25 mM Tris-HCl pH 7.4, 5 mM MgCl2, 100 mM NaCl, 1 µM dithiothreitol (DTT), and 2 mM ATP (Sigma). ATP regeneration system (Sigma) was used to recycle ATP. Purified ubiquitin wild-type and mutants (1 µg) were added to the reaction mixture. For each specific reactions, we incubated 5 µg of purified RNF168 with 0.1 µg human recombinant E1 Ub-activating enzyme, 0.25 µg each of purified Ubc13/Mms2, at 30°C for one hour. Purified HECT domain of Nedd4 (1 µg) was incubated with E1 (0.1 µg), UbcH5c (0.25 µg) at 37°C for 30 minutes. Alternatively, purified HECT domain of Nedd4 (1 µg) was incubated with E1 (0.1 µg) and with purified Ube2R1 (0.8 µg) for one hour at 37°C.

Sample preparation for mass spectrometric analyses

Protein Disulfide bonds were reduced with TCEP (Thermo) at a final concentration of 10 mM at room temperature for 1 hour. Free thiols were alkylated with 10 mM iodoacetamide at room temperature for 30 min in the dark. Protein samples were digested with trypsin (1:50 w/w) for 10 hours at 35°C (Promega) in a solution containing 50mM ammonium bicarbonate (Sigma), 1 M UREA (Sigma) at pH 8. Peptides were desalted on a C18 Sep-Pak cartridge (Waters), dried under vacuum and reconstituted with a solution containing 2% acetonitrile (AcN) and 0.1% formic acid (FA) and analyzed by LC-SRM. Chromatographic separation of peptides was carried out with an Eksigent (Eksigent Technologies) and NanoLC system connected to a 15-cm fused-silica emitter with 75-µm inner diameter (BGB

Analytik) packed in-house with a Magic C18 AQ 3-µm resin (Michrom BioResources).

Targeted mass spectrometric analysis by selected reaction monitoring (SRM)

For assay development, the optimal transitions for the detection of the K27 ubiquinated peptide (TITLEVEPSDTIENVK[GlyGly]AK) were determined by SRM analysis of the tryptic digested K27 poly-ubiquitin chain. The peptide samples were analyzed by LCSRM analysis using a linear gradient ranging from 95% solvent A (98% H20,2% acetonitrile, 0.1% formic acid) to 35% solvent B (98% acetonitrile, 2% H2O, 0.1% formic acid) over 40 min at a flow rate of 300 nl/min. Mass spectra were acquired in SRM mode on triple quadrupole/ion trap mass spectrometer (5500QTrap, ABSciex, Concord, Canada) where Q1 and Q3 operated at unit resolution and with a dwell time of 50 and 250 ms for assay development and chromatin sample measurements, respectively. We experimentally tested in SRM mode 51 transitions, calculated using the tool Skyline (MacLean et al., 2010) and corresponding to singly charged y- b-ions from doubly or triply charged precursors. The most four intense transitions were selected for the analysis of the chromatin samples (y3+, y5+ y6+, b3+). Collision energies were calculated as suggested by the vendor. Accurate retention time of the ubiquitinated K27 peptide was determined using the iRT concept as described in (Escher et al., 2012). Duplicate injections were performed for each chromatin sample and the sum of the areas of each transition were used for peptide quantification.

Shotgun mass spectrometric analysis

Shotgun analysis of the chromatin samples was carried out on LTQ-Orbitrap XL mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source (Thermo Scientific). Chromatographyx separation of the peptides was achieved with a Proxeon (Proxeon Biosystems) NanoLC system connected to a 15-cm fused-silica emitter with 75-µm inner diameter (BGB Analytik) packed in-house with a Magic C18 AQ 3-µm resin (Michrom BioResources). The digested chromatin samples were analyzed by LC–tandem MS (LC-MS/MS) with a linear gradient ranging from 95% solvent A (98% H20,2% acetonitrile, 0.1% formic acid) to 35% solvent B (98% acetonitrile, 2% H2O, 0.1% formic acid) over 90 min at a flow rate of 300 nl/min. Mass spectra were acquired in a data-dependent manner, with an automatic switch between MS and MS/MS scans. Highresolution MS scans were acquired in the Orbitrap (60,000 FWHM, target value 106) to monitor peptide ions in the mass range of 350–1,650 m/z, followed by collision-induced dissociation MS/MS scans in the ion trap (minimum signal threshold 150, target value 104, isolation width 2 m/z) of the five most intense precursor ions. The precursor ion masses of scanned ions were dynamically excluded from MS/MS analysis for 10 s.

Singly charged ions and ions with unassigned charge states were excluded from triggering MS2 events.

Database searching of shotgun mass spectrometric data

Raw data were converted to the open mzXML format with ReAdW (version 4.3.1). mzXML files were searched by the SEQUEST via Sorcerer Software 4.2.0 against UniProtKB/Swiss-Prot protein databases (release 2012_11, containing 20,243 proteins) concatenated with reverse sequences. For in silico digestion, trypsin was used as the protease and was assumed to cleave after lysine (K) and arginine (R) unless followed by proline (P). Two missed cleavage sites and one non- tryptic terminus were allowed per peptide. The precursor ion tolerance was set to 50 parts per million (ppm), and fragment ion tolerance was set to 0.5 dalton. The data were searched allowing ubiquitination of lysine, as a variable modification and carboxy-amidomethylation of cysteine residues as a fixed modification. Finally, The identification results were statistically analyzed with the PeptideProphet algorithm (v 4.6). For peptide identification the false discovery rate (FDR) was maintained below 1%.

Antibodies

Antibodies used in this study included mouse monoclonal anti-ubiquitin (P4D1, Santa Cruz Biotechnology) and FK2 (Stressgen Bioreagents), mouse monoclonal anti-FLAG (M2, Sigma), rabbit polyclonal anti-FLAG (Sigma), mouse monoclonal anti-HA.11 (16B12, Covance), rabbit polyclonal anti-HA (Abcam), anti-phospho-histone H2AX (Ser139, γ-H2A.X, Upstate), rabbit polyclonal anti-histone H2A (Abcam), rabbit polyclonal anti-histone H3 (Abcam), rabbit polyclonal anti-histone H2A.X (Abcam), rabbit polyclonal anti-53BP1 (Abcam), mouse monoclonal anti-BRCA1 (Santa Cruz Biotechnology), mouse monoclonal anti-MDC1 (Abcam), mouse monoclonal anti-α-Tubulin (Sigma). The linkage-specific antibodies directed to K48 (Apu2.07) and K63 (Apu3.A8) were from Genentech. Mouse anti-53BP1 was a gift from T. Halazonetis and anti-RNF168 polyclonal antibody was made in house.

Expression of recombinant proteins

Recombinant GST-fusion proteins (RNF168, UDR and RAP80 UIM) were expressed in E. coli strain BL21 pLys by a 16 hours induction with 1 mM IPTG at 18°C and produced

as previously described (Pinato et al., 2011). The GST-UDR were extracted by using 8M Urea and re-folded by four dialysis steps. Recombinant MBP fusion protein was expressed in E. coli strain BL21 pLys by a three hours induction with 1 mM IPTG at 37°C in Luria Bertani broth enriched with 0,2% glucose and purified using

amylose resin (New England Biolabs). Ube2R1, Ubc13, Mms2 and the HECT domain of Nedd4 were kindly provided by E. Maspero and S. Polo.

RNA interference

The siRNAs (Invitrogen) targeting UBA52 and RPS27A precursors of ubiquitin were used for ubiquitin knockdown. siRNA-resistant form of the different ubiquitin mutants were obtained by site-directed mutagenesis and sequence verified. Transfections of siRNAs (targeting ubiquitin or RNF168) were performed using oligofectamine reagent (Invitrogen) in U2OS cells and jetPRIME® Transfection Reagent (Polyplus) in HEK293T cells. All the siRNAs and oligonucleotide sequences are included below.

siRNA sequences

Ubiquitin UBA52: ACACCATTGAGAATGTCAA
Ubiquitin RPS27A: AGGCCAAGATCCAGGATAA
RNF168: CGTGGAACTGTGGACGATAATTCAA

Quantitative real-time PCR

Total RNA was extracted from HEK293T cells using TRIzol® (Invitrogen). To eliminate residual genomic DNA, the samples were treated with DNase I (Promega). The cDNA was synthesized from 1 µg of total RNA using ImProm-IITM Reverse Transcription System (Promega) according to the manufacturer's instructions in an amplification cycler. Sixteen nanograms of each cDNA sample were used as template for the amplification reaction that was performed using SYBR® Green Mastermix (BIO-RAD). PCR amplifications were performed in triplicates and the ΔΔCT method was used to calculate the relative quantity; 18S rRNA was used for normalization.

RT-PCR primers

RNF168 for-TCAGCCAGTTCGTCTGCTCAGT
RNF168 rev-TCTTCTTCCTCGCTGGCCCGT
18S for-TGCGAGTACTCAACACCAACA
18S rev-CTGCTTTCCTCAACACCACA

Oligonucleotides for mutagenesis

H2A.X: K13Q for-GCAAGGCCCGCGCCCAGGCCAAGTCGCGCTCG
H2A.X: K15Q for-CCGCGCCAAGGCCCAGTCGCGCTCGTCGCGC
H2A.X: K13,15Q for -CGGCAAGGCCCGCGCCCAGGCCCAGTCGCGCTCGTCGCGC
H2A.X: K118,119Q for-GCCGTGCTGCTGCCCCAGCAGACCAGCGCCACCGTG
RAP80-UIMs: A88S for-GAACAGTTTGCTCTGTCTCTCAAAATGAGTGAG

RAP80-UIMs: A113S for-GAGCTCTTGAGGAAATCCATTGCTGAAAGCCTG
53BP1-UDR: L1619A for-GCAGCGGACATTAGCGCGGATAACCTGGTGGAA
53BP1-UDR: R1627A for-CTGGTGGAAGGTAAGGCCAAACGTCGCTCGTAA

Oligonucleotides for the generation of siRNA-resistant forms of ubiquitin
Ub siRNA-resistant (UbRes) on K6R, K11R, K33R, K48R and K63R:
for-GAGCCGAGTGATACGATCGAAAACGTCAAGGCAAAATCCA
Ub siRNA-resistant (UbRes) on K27R:
for-GAGCCGAGTGATACGATCGAAAACGTCCGCGCAAAGATCCA
Ub siRNA-resistant (UbRes) on K29R:
for- GAGCCGAGTGATACGATCGAAAACGTCAAGGCACGCATCCA

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USP10 REGULATES THE RNF168-DEPENDENT SIGNALLING BY AFFECTING ITS UBIQUITINATION STATUS

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SUMMARY

RNF168 is an E3 ubiquitin ligase that induces extensive chromatin ubiquitination after double strand breaks (DSBs) induction. The RNF168-dependent ubiquitination is a critical event in DNA damage response (DDR) by promoting the focal accumulation of the downstream DDR proteins to DSBs-flanking chromatin. Our current knowledge is predominantly based on the RNF168-mediated signalling, but how this E3 is fine-tuned is not yet clear and under intense investigation. Here we report that a poorly characterized deubiquitinating enzyme (DUB), named USP10, is a new interactor of RNF168. We show that USP10 constitutively interacts with RNF168 and recognizes the RING-finger domain of the ubiquitin ligase. We demonstrate that USP10 regulates the ubiquitination status of RNF168, without affecting its stability. Interestingly, chromatin ubiquitination is also highly regulated by USP10. Indeed, USP10 depletion markedly reduces RNF168-mediated chromatin ubiquitination, while its ectopic expression promotes it. In line with these results, we observe that in cells depleted of USP10, the recruitment of 53BP1 to DDR *foci* is significantly impaired. These findings revealed a novel role of USP10 in the fine-tuning of chromatin ubiquitination, through the regulation of RNF168 ubiquitination status.

INTRODUCTION

Genome integrity is essential to prevent genetic alterations that can lead to carcinogenesis[1, 2, 3]. DNA is continuously subjected to random changes but only

few of them accumulate as mutations in the DNA sequence, thanks to the preserving action of a complex network called DNA damage response (DDR). DDR is responsible for the DNA damage surveillance that includes a set of processes such as DNA repair, chromatin remodelling, cell-cycle checkpoints and apoptosis[4]. When a damage occurs on DNA, the initial trigger of DDR is the phosphorylation of the histone H2A.X (that in this form is known as γ-H2A.X)[5] by the action of a family of kinases named PIKKs, which includes ATM (for Double Strand Breaks), ATR (for Single Strand Breaks)[3, 6, 7, 8]. Phosphorylation is one of the reversible modifications known as post-translational modifications (PTMs)[9, 10]. PTMs have a crucial role in DDR in virtue of their ability to both alter the activity of an existing protein and to generate docking sites on target proteins at the damaged sites, contributing to the formation of supramolecular structures named DDR *foci*[9, 10]. While phosphorylation initiates the pathway, ubiquitination, another PTM, is fundamental to sustain the signalling[11, 12, 13]. A major breakthrough in recent years has been that ubiquitination gives rise to diverse polymeric chains with distinct topology leading to an intricate code[14, 15]. Ubiquitination is a three steps reaction that involves the concerted action of ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). In DDR RNF8 and RNF168, two E3 ubiquitin ligases, target histone H2A and H2A.X allowing the efficient local assembly of DNA damage-repair factors[16, 17, 18, 19]. RNF8 is quickly recruited to the damaged chromatin and its recruitment is in turn fundamental for the focal accumulation of RNF168. As we recently reported, RNF168 promotes the non-canonical K27-ubiquitination on histones H2A and H2A.X at the bidentate K13/15 site at their N-terminal[18, 19] by providing to uniquely mark the substrates. The RNF168-mediated ubiquitination is necessary for the recruitment of the effectors of the pathway, such as BRCA1 and 53BP1, which contain ubiquitin binding domains able to recognize and use ubiquitin as a docking site to stay

anchored to chromatin[16, 20, 21]. Although a lot is known about the effects of RNF168-mediated ubiquitination, less is known about its activation after DSBs induction. Recently, different groups reported the importance of the deubiquitinating enzymes in the modulation of DDR. These DUBs were found actively involved in the regulation of the recruitment of the RNF168-responsive factors, in checkpoint recovery and DSB-induced transcriptional silencing[22, 23, 24, 25, 26, 27]. Moreover, it was discovered their involvement as regulatory components in the RNF8/RNF168-mediated ubiquitination[28, 29, 30].

In this study we describe the identification of USP10, a deubiquitinating enzyme known to regulate p53 activation[31], as a new interactor of RNF168. We show that USP10 forms a complex with RNF168 and modulates its ubiquitination status. We provide data suggesting that USP10 is recruited onto chromatin after DNA damage, where it is able to regulate RNF168-dependent ubiquitination. Consistent with these data, we demonstrate that USP10 positively regulates the recruitment of 53BP1 to DDR *foci*.

MATERIALS AND METHODS

SILAC combined MS

HeLa cells were grown in MEM containing $^{13}C_6{}^{15}N_4$ L-Arginine (H-heavy isotope), $^{13}C_6{}^{14}N_4$ L-Arginine (M-medium isotope) or non-labeled $^{12}C_6{}^{14}N_4$ L-Arginine (L-light isotope). Then cells maintained in M and H medium were transiently transfected with FLAG- tagged RNF168 while cells maintained in L medium were transfected with the empty vector, both using Lipofectamine 2000 reagent (ThermoFisher). 24 hours after transfection cells were treated with 30μM etoposide (Sigma) for 1 hour or left untreated. 3 hours after treatment cells were subjected to cellular fractionation in order to separate the nuclear from the cytosolic fraction. Then the two fractions from the diverse arginine labelling cell cultures were mixed in a

1:1:1 ratio. The FLAG immunocomplexes were isolated using the anti-FLAG affinity gel (M2, Sigma) for 3 hours at 4°C and washed one time with NETN buffer and 3 times with HNTG buffer (20mM Hepes pH7.5, 150mM NaCl, 0.1% Triton X-100, 10% Glycerol 10%) and then eluted by glycine. Afterwards, samples were digested with trypsin and processed to LC-MS analysis.

Cell culture and transfection

HeLa cells were grown in MEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2mM L-Glutamine (Sigma), 1mM Sodyum Pyruvate (Sigma) and 1% non-essential amino acid solution (Sigma). HEK293T cells were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco), 2mM L-Glutamine (Sigma). U2OS cell line was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma). U2OS cell line stably transfected with pAc-GFP-RNF168 was cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum tetracycline free (BioWest), 2 mM L-glutamine (Sigma), 300µg/ml G418 disulfate salt solution (Sigma) and 1% PEN-STREP (BioWhittaker). Plasmid transfections were performed using Lipofectamine 2000 reagent (ThermoFisher) in HeLa cells, Calcium-Phosphate in HE293T cells and FuGENE reagent (Roche) in U2OS cell lines. siRNAs-targeting USP10 transfections were performed using Oligofectamine reagent (Life Technologies) in U2Os cell lines and JetPRIME Transfection Reagent (Polyplus).

DNA constructs and antibodies

The full-length human RNF168 cDNA was purchased from RZPD (clone IRATp970F1053D) and cloned into pGEX6P2 (GE Helthcare) and FLAG-pcDNA3.1 (Invitrogen). The truncated forms of RNF168 constructs were generated by PCR

amplification followed by cloning into pGEX6P2 vector. The oligonucleotide sequences are available upon request. The full-length human USP10 cDNA was kindly provided by Zhenkun Lou and cloned into pcDNA3.1 (Invitrogen). Its catalytic mutant was obtained by site-direct point-mutation using the following oligonucleotides: forward: AAT AAA GGG AAC TGG GCC TAC ATT AAT GCT ACA; reverse: TGT AGC ATT AAT GTA GGC CCA GTT CCC TTT ATT. HA-tagged ubiquitin cDNA derived from UBC gene (ID: NM_021009.5) and kindly provided by Josef Yarden was cloned into pcDNA3-TOPO (invitrogen). All constructs were sequence verified. Antibodies used: rabbit polyclonal anti-USP10, anti-53BP1 and anti-HA-tag (Abcam); rabbit-polyclonal anti-FLAG (TM) and anti-FLAG affinity gel (M2) (Sigma). Monoclonal-mouse anti-γ-H2A.X (Ser139, Millipore), mouse-monoclonal anti-GFP and anti-p53 (DO-1; sc-126) (SantaCruz).

RNA interference

The siRNAs-targeting USP10 (Invitrogen) were used for USP10 knockdown. Transfections of siRNAs were performed using oligofectamine reagent (Invitrogen) in U2OS cells and jetPRIME® Transfection Reagent (Polyplus) in HEK293T cells. All the siRNAs and oligonucleotide sequences are included below.

siRNA sequences

siRNA SCR: UAACGACGAGUCACGAAAGAGAGGG

siRNA #1: UAAAGAGCCACUAAAGAGAGGCGGG

siRNA #2: UGCAAAGGGAGAAAGAGUUUCUCUC

siRNA #3: GAGAAACUCUUUCUCCCUUUGCAAA

GST pull-down assays

Recombinant GST fusion proteins were expressed in *E*. coli strain BL21 pLys by a 3 hours induction with 1mM IPTG at 37°C. Bacterial cells were harvested,

resuspended in PBS supplemented with Protease Inhibitor Cocktail (Sigma) and 1mM PMSF and sonicated. Lysates were incubated with 1% Triton X-100 for 30 min at room temperature and then centrifuged (14000rpm for 30 min a 4°C). GST-tagged proteins were purified with Glutathione-Sepharose resin (GE healthcare) as by manufacturer's instructions. For the pull-down experiments with cellular lysates, HeLa cells were lysed in NETN buffer (50mM TrisHCl pH 7.5, 500mM NaCl, 1mMSodium Pyruvate, 50mM NaF, 1mM Na_3PO_4, 20µM NEM, protease inhibitor cocktail (Sigma), 80 U/ml benzonase), and clarified by centrifugation at 13000rpm for 30 min at 4°C. 1µM of GST fusion proteins, immobilized onto GSH beads, were incubated with 500µg of lysates for 2 hours at 4°C. Specifically bound proteins were resolved on SDS-PAGE (10%) and transferred onto nitrocellulose membranes and analysed by immunoblotting as indicated.

Co-immunoprecipitation and immunoprecipitation

For the co-immunoprecipitation experiments FLAG-tagged RNF168 was transiently expressed in HeLa cells using Lipofectamine 2000 reagent (ThermoFisher). 24 hours after transfection cells were treated with 30µM etoposide (Sigma) for 1 hour or left untreated. Then, cells were lysed in NETN buffer (50mM TrisHCl pH 7.5, 500mM NaCl, 1mMSodium Pyruvate, 50mM NaF, 1mM Na_3PO_4, 20µM NEM, protease inhibitor cocktail (Sigma), 80 U/ml benzonase), and clarified by centrifugation at 13000rpm for 30 min at 4°C. Lysates were incubated with the anti-FLAG affinity gel (M2, Sigma) for 3 hours at 4°C and washed one time with NETN buffer and 3 times with HNTG buffer (20mM Hepes pH7.5, 150mM NaCl, 0.1% Triton X-100, 10% Glycerol 10%). The bound proteins were released by boiling in Laemmli sample buffer and examined by Western blotting. For the immunoprecipitation experiments FLAG-tagged RNF168 was transiently expressed in HEK293T cells together with HA-tagged ubiquitin and USP10, or its catalytic

mutant using Calcium-Phosphate; or USP10 was depleted from cells using jetPRIME® Reagent (Polyplus) transfection of siRNAs-targeting USP10. 48 hours after, FLAG-tagged RNF168 was transiently expressed in cells together with HA-tagged ubiquitin by 48-hours transfection using Calcium-Phosphate. Then, cells were lysed in RIPA buffer (50mM TrisHCl pH 7.5, 500mM NaCl, 1% 1mM Sodium Pyruvate, 50mM NaF, 1mM Na_3PO_4, 20µM NEM, protease inhibitor cocktail (Sigma), 80 U/ml benzonase), and clarified by centrifugation at 13000rpm for 30 min at 4°C. Lysates were incubated with the anti-FLAG affinity gel (M2, Sigma) for 3 hours at 4°C and washed one time with NETN buffer and 3 times with HNTG buffer (20mM Hepes pH7.5, 150mM NaCl, 0.1% Triton X-100, 10% Glycerol 10%). The bound proteins were released by boiling in Laemmli sample buffer and examined by Western blotting.

Immunofluorescence

U2OS cell line transfected with siRNAs-targeting USP10 or Scramble siRNA (Invitrogen) were grown on glass coverslips. 72 hours after transfection, cells were treated with etoposide (5µM, 1 hour) and left for another 24 hours before fixing and staining them; or 96 hours after transfection, cells were treated with etoposide (5µM, 1 hour) and then fixed and stained. Cells were fixed in 4% paraformaldehyde, permeabilized by a 10-minutes treatment with 0.5% Triton X-100 in BSA, blocked with PBG (PBS, BSA, gelatin) for 1 hour and immunoprobed with the appropriate antibody for 1 hour at RT. Incubation with secondary antibodies (Alexa fluor 488 anti-mouse IgG, Alexa Fluor 555 anti-rabbit IgG (Invitrogen)), was performed for 30 min at RT. Nuclei were stained with 0.2µM To-PRO3 for 10 minutes. Images were acquired by confocal scanning laser microscope (Leica TCS2; Leica Lasertechnik, Heidelberg, Germany).

In vivo *detection of ubiquitinated chromatin*

HEK293T cells treated at different time points with 30μM Etoposide for 1 hour, or expressing different constructs were collected in PBS, containing protease inhibitor cocktail (Sigma-A;drich), 1 mM phenylmethylsulfonyl fluoride, and 20 mM N-ethylmaleimide. One-tenth of the samples were separately processed for protein normalization, whereas the remaining were subjected to acidic extraction as previously described (Citterio, E. et al.; Mol Cell Biol, 2004). Then, samples were either directly analysed by SDS-PAGE and western blotting.

RESULTS

USP10 directly interacts with RNF168

As extensively described, RNF168 is a key enzyme in DDR, which induces chromatin ubiquitination at DSBs[18, 19, 25]. Considering its importance in genome integrity, we decided to gain more insights into the comprehension of this process by searching for new RNF168-interacting proteins. Thus, we performed a quantitative proteomic analysis based on the SILAC method. As shown in ***figure 1A***, we cultured HeLa cells in media supplemented with Arginine containing different isotopes of carbon and nitrogen, $^{13}C_6{}^{15}N_4$ (H-heavy isotope), $^{13}C_6{}^{14}N_4$ (M-medium isotope) or non-labeled $^{12}C_6{}^{14}N_4$ (L-light isotope). Then, cells maintained both in M and H medium were transfected with FLAG-tagged RNF168, while cells grown in L medium were transfected with the empty vector. Afterwards we treated cells with etoposide (inhibitor of Topoisomerase II) to induce DSBs or left them untreated as indicated in ***figure 1A***. Consequently, the cells from the diverse arginine labelled cultures were subjected to fractionation, in order to separate the nuclear from the cytosolic fraction, and therefore mixed in a 1:1:1 ratio. FLAG immunocomplexes were purified from the nuclear fractions and, after glycine elution ***(Fig. 1B)***, were analysed by mass spectrometry. The screening identified

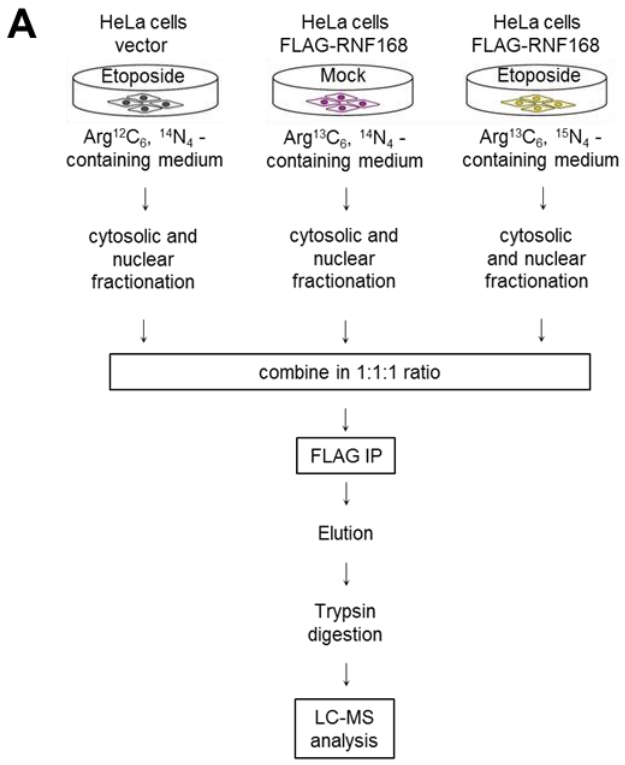
proteins involved in various biological processes, including several already known to be associated to RNF168 ***(Tab.1)*** thereby proving the sensitivity and reliability of the screen. Interestingly, among the identified interactors we found a poorly characterized deubiquitinating enzyme (DUB), namely USP10, a 798 amino acids long protein that is localized in the cytoplasm where it regulates p53 homeostasis; after genotoxic stress a fraction of USP10 translocates to the nucleus to stabilize and activate p53[31]. The tumour suppressor p53 is involved in a variety of cellular functions, including DNA repair. Due to its deubiquitinating activity and its involvement in cancer biology as p53 regulator, we considered USP10 a good candidate to regulate the RNF168-based signalling, thereby participating in the DNA damage response.

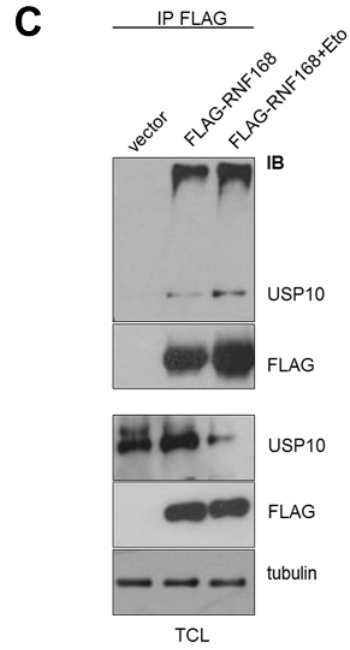
id	Protein Name	Gene Name	Peptides (seq)	Sequence Coverage [%]	Mol. Weight [kDa]	Ratio M/L	Ratio H/L	Ratio H/M
56	Histone H2B	HIST2H2BA	2	14,5	18,804	1,0656	1,364	1,2179
188	E3 ubiquitin-protein ligase RNF8	RNF8	5	11,5	55,462	3,7266	4,3078	0,9255
191	Valosin-containing protein p97	VCP	11	14,8	89,321	1,0547	1,5207	1,248
322	Histone H1.5	HIST1H1B	3	17,3	22,58	1,3109	1,4512	1,1736
368	**Ubiquitin carboxyl-terminal hydrolase 10**	**USP10**	**2**	**2,8**	**87,133**	**1,3508**	**1,9082**	**1,1384**
380	DNA-dependent protein kinase catalytic subunit	PRKDC	4	1	469,08	1,2611	1,4762	1,1257
462	Tripartite motif-containing protein 28;Nuclear corepressor KAP-1	TRIM28;KAP1	3	4,3	88,549	1,7511	1,5983	0,87361
481	Nucleolin	NCL	26	29,2	76,613	1,8734	2,2822	1,1182
486	DNA repair protein XRCC6	XRCC6; KU70	3	5,7	69,842	1,0663	1,2608	0,95379

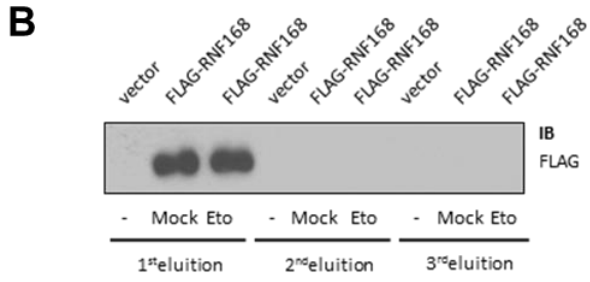
TABLE 1: IDENTIFICATION OF RNF168 INTERACTORS FROM RNF168-ENRICHED NUCLEAR AND CYTOSOLIC FRACTIONS. A selected list of interactors of RNF168, identified by SILAC-combined mass spectrometry analysis; $^{13}C_6{}^{15}N_4$ L-Arginine (H-heavy isotope), $^{13}C_6{}^{14}N_4$ L-Arginine (M-medium isotope) or non-labeled $^{12}C_6{}^{14}N_4$ L-Arginine (L-light isotope).

In order to confirm that USP10 is an interactor of RNF168, we adopted a biochemical approach by expressing FLAG-tagged RNF168 in HeLa cells and treated or not with etoposide. Then cells were lysed in mild conditions and protein extracts were subjected to FLAG immunoprecipitation. USP10 immunoblotting (IB) clearly reveals that endogenous USP10 is able to interact with

RNF168 *in vivo* ***(Fig.1C)***. Remarkably, as displayed in ***figure 1C***, USP10 is able to interact with FLAG-RNF168 under both genotoxic stress and physiological conditions. To further demonstrate that USP10 directly interacts with RNF168, we performed an *in vitro* pull-down assay using recombinant GST-tagged RNF168 expressed in bacteria. As shown in ***figure 1D***, GST-RNF168 was able to pull-down USP10 derived from HeLa cell extracts.







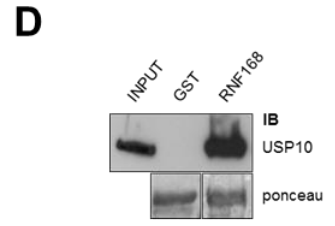


***FIGURE 1: USP10 INTERACTS WITH RNF168 BOTH* IN VIVO *AND* IN VITRO. (A)** Schematic representation of the experimental procedure. HeLa cells were grown in medium supplemented with Arginine containing the indicated isotopes of carbon and nitrogen. Afterwards, cells were transfected with FLAG-RNF168 or the empty vector, treated or not with etoposide for 1 hr and subjected to fractioning in order to separate the cytosolic fraction from the nuclear components. Then, samples were mixed in a 1:1:1 ratio followed by FLAG immunoprecipitation (IP). **(B)** After glycine elution, samples were in part analyzed by IB as indicated and in part subjected to trypsin digestion and processed for LC-MS analysis. **(C)** *In vivo* validation of the USP10/RNF168 interaction. HeLa cells expressing the indicated constructs were treated or not with etoposide (30 μM) for 1 h. After 3 hrs, cells were lysated and subjected to co-immunoprecipitation (Co-IP) with FLAG resin; IB was performed using the indicated antibodies. **(D)** Demonstrating the direct interaction between USP10 and RNF168. *In vitro* pull-down assay was perfomerd using GST-tagged RNF168 and HeLa cells lysates. IB was performed with anti-USP10.

USP10 interacts with multiple regions within RNF168 in vitro

To further characterize the physical association between USP10 and RNF168, we mapped the region(s) within RNF168 that interact with USP10. To this aim, we produced a panel of different GST-RNF168 deletion mutants encompassing the whole protein ***(Fig. 2A)*** that were incubated with HeLa cells extracts in an *in vitro* pull down-assay. The results obtained revealed that USP10 is able to interact with full-length GST-tagged RNF168-construct, while the GST-tagged RNF168-deleted construct lacking the RING-finger domain (57-571$^{(\Delta 57)}$) weakly interacts with USP10 ***(Fig. 2B)***. Interestingly, also the C-terminus of the RNF168 (439-571$^{(MIU2\text{-}Cterm)}$) seems to retain the ability to associate with USP10. In addition, as shown in ***figure 2B***, the GST-tagged RNF168 sequence between aminoacids 168 and 462 (168-462$^{(MIU1\text{-}MIU2)}$), which contains the two ubiquitin binding domains (UBDs), MIU1 and the MIU2, also interacts with USP10, although more weakly than the other constructs, suggesting that the two UBDs might be involved. Unexpectedly, the GST-tagged mutant encompassing the RING-finger domain and the MIU1 (1-180$^{(RF\text{-}MIU1)}$) does not associate with USP10 ***(Fig. 2B)***. Trying to further characterize the interaction, we performed the pull-down using the GST-tagged deletion

constructs in the RING-finger domain of RNF168. Notably, as shown in ***figure 2C*** we found that USP10 binds the isolated RING finger domain (1-57) but not its fragments (1-15 and 15-57). These results show that both regions are strictly required for the recognition by USP10, suggesting a specific mechanism where the short loop (1-15) and the RING finger form a functional domain able to drive the interaction between these two proteins.

Overall these results indicate that USP10 can interact with multiple regions within RNF168, including the RING-finger domain and the C-terminal suggesting a possible dual mode of interaction between the two proteins.

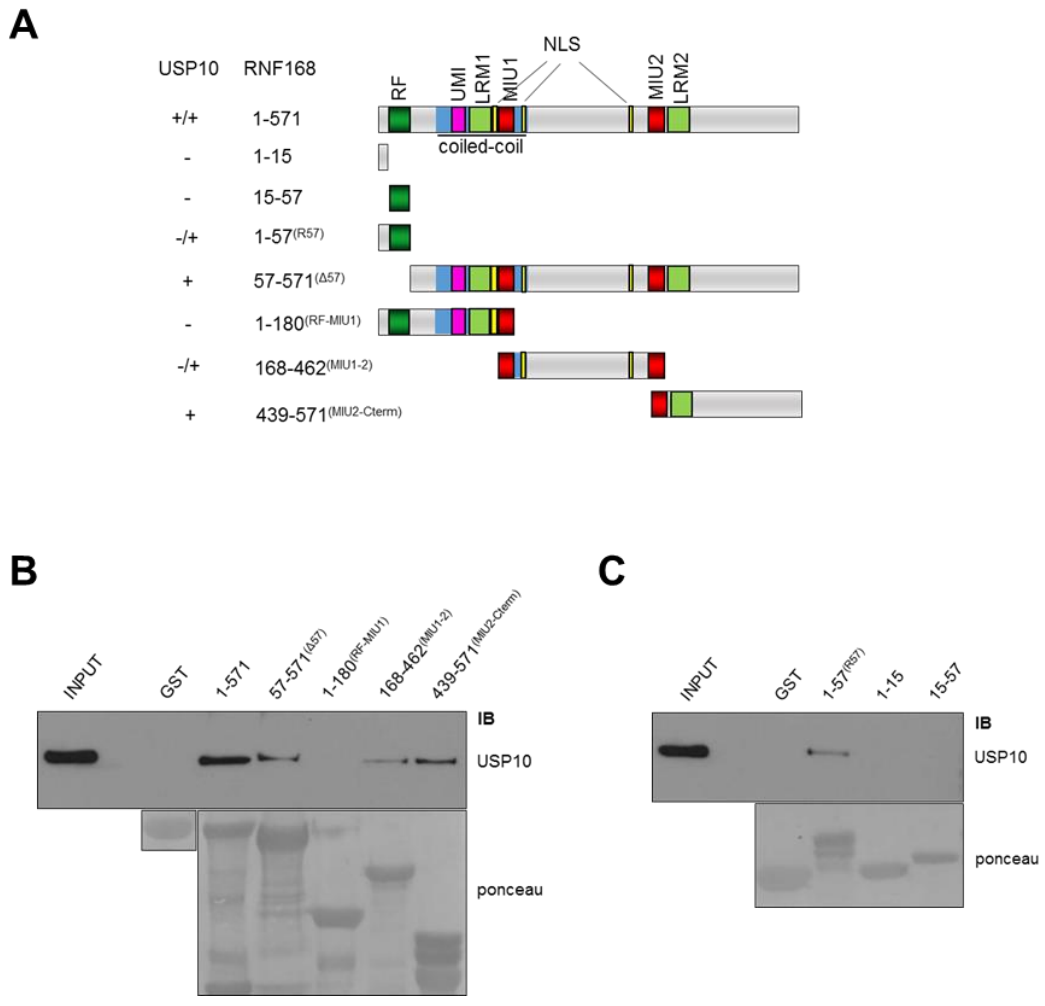


***FIGURE 2:* IN VITRO *USP10 INTERACTS WITH MULTIPLE REGIONS ON RNF168, INCLUDING ITS RING-FINGER DOMAIN.* (A)** Schematic representation of RNF168 and its deleted constructs used in pull-down experiments (numbers refer to the aminoacid position within the sequence; RF, RING-finger domain; NLS, nuclear localization sequences; UMI, UIM and MIU-related ub binding domain; MIU1 and MIU2, motifs interacting with ub 1 and 2; LRM1 and LRM2, LR-motifs 1 and 2). **(B-C)** Mapping the region(s) of RNF168 recognized by USP10. *In vitro* pull-down assay was performed using the indicated GST-tagged deleted constructs of RNF168 and HeLa cells lysates. The GST alone was used as control. IB was performed with anti-USP10.

After DNA damage, USP10 is recruited on chromatin in a time-dependent manner

Yuan and colleagues demonstrate that USP10 is a cytoplasmic protein that, following DNA damage, translocates into the nucleus in an ATM-dependent manner[31]. Since we found that RNF168 and USP10 form a complex, we asked ourselves whether USP10 is loaded onto damaged chromatin following genotoxic stress. To address this point, HEK293T cells were treated or not at different time points with etoposide as indicated in ***figure 3***, and then subjected to chromatin extraction. Immunoblotting revealed that, after DNA damage, USP10 is recruited to chromatin in a time-dependent manner, similarly to 53BP1 ***(Fig. 3 upper panels)***. Interestingly, both proteins display a unexpected biphasic recruitment, suggesting an additional mechanism able to regulate the access of the DDR protein to chromatin. Moreover, we found that a little portion of USP10 is also localised into chromatin in untreated conditions.

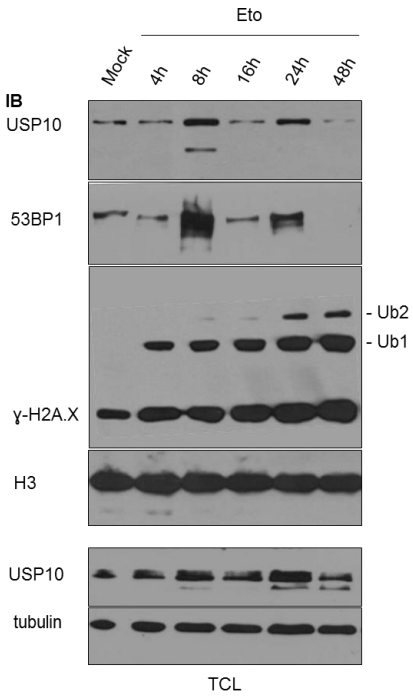


FIGURE 3: AFTER DSBS-INDUCTION USP10 IS LOADED ONTO CHROMATIN IN A BIPHASIC-FASHION. Evaluation of USP10 chromatin loading after genotoxic stress. HEK293T cells were treated with etoposide (30μM) for 1h and then harvested at the indicated time points. Samples were subjected to chromatin extraction and separated by SDS-PAGE. IB was performed as indicated.

USP10 depletion induces an aberrant recruitment of 53BP1 to DDR foci

Upon genotoxic stress, the phosphorylation of histone H2A.X and the accumulation of 53BP1 form characteristic DDR *foci* at the site of DNA damage[16, 20, 32, 33, 34], which are easily detectable by immunofluorescence techniques. We took advantage of this in order to explore the functional relevance of USP10 in both DDR *foci* formation and DNA repair. First, we set up USP10 knockdown in U2OS cells by using different siRNA oligonucleotides ***(Fig. 4A)***. Afterwards, we evaluated the impact of USP10 depletion in the activation of DDR. Cells were treated with etoposide at two different time points to measure both the early activation of the DDR (1 hr) and the DNA repair (24 hrs), and then fixed and

subjected to immunofluorescence ***(Fig. 4B)***. Notably, after 1 hour of treatment we found that the USP10-defective cells show a significantly impaired focal recruitment of 53BP1 to the damaged chromatin ***(Fig. 4B; middle panel and Fig. 4C)***. Moreover, the same cells displayed a totally delocalized ɣ-H2A.X inside the nucleus and the shape of its DDR *foci* appears smaller compared to the *foci* in control cells. In line with this result, 24 hours after treatment USP10-depleted cells still display ɣ-H2A.X *foci* compared to the control cells, suggesting a mild delay in DNA repair ***(Fig. 4B, lower panel)***.

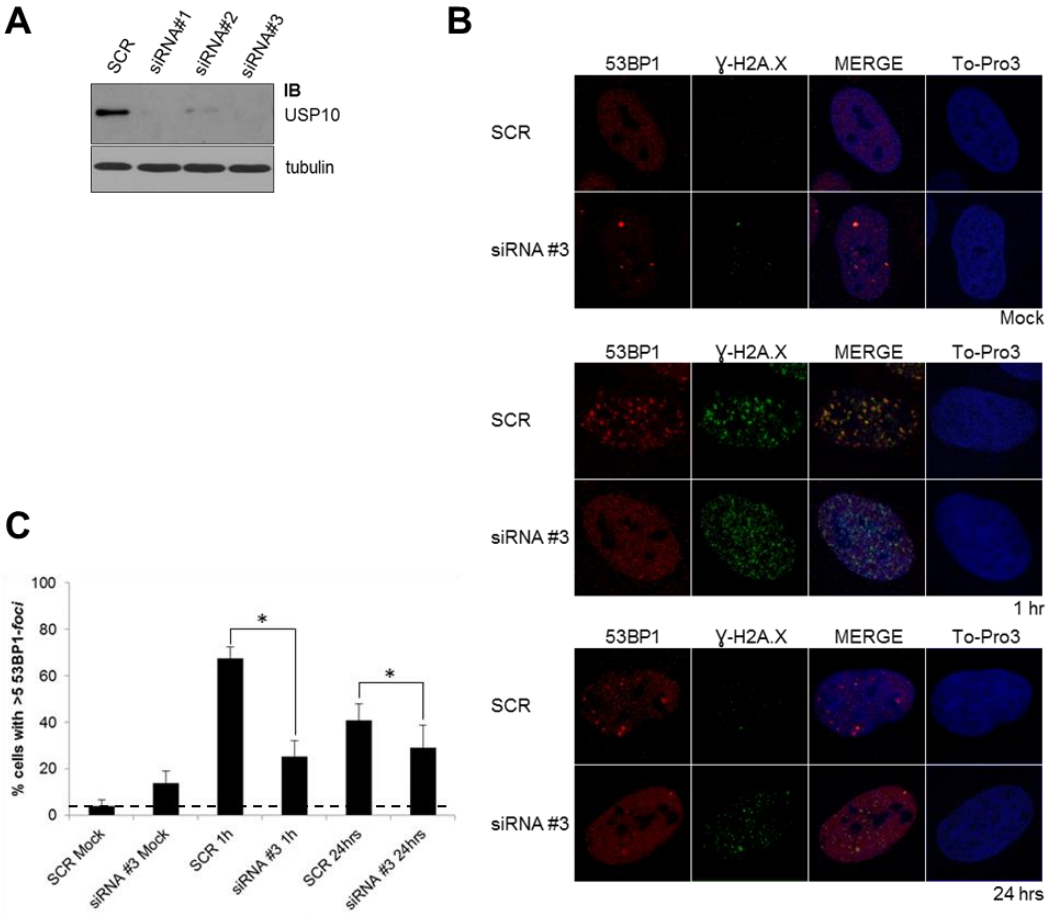


***FIGURE 4: USP10 IS ESSENTIAL FOR THE RECRUITMENT OF 53BP1 TO THE DDR* FOCI. (A-C)** Functional characterization of the role of USP10 in DDR. **(A)** Evaluation of USP10 expression in U2OS-depleted cells **(B)** U2OS cells were transfected with the indicated USP10-targeting siRNA. 72hrs after transfection, cells were treated with etoposide (5 μM) for 1 hr and fixed and stained or left for 24 hrs before fixing and staining them. Immunostaining was performed with the indicated antibodies. **(C)** Quantification of 53BP1 *foci*-positive cells (more than five *foci*). At least 100 cells per condition were counted. Each value represents the mean ± SD. *$p < 0.05$;

USP10 modulates the activity of RNF168

RNF168-dependent chromatin ubiquitination is required for the proper DDR-signalling[16, 20, 21]. Since we found that USP10 depletion in U2OS markedly impairs the recruitment of 53BP1 to the DDR *foci*, we hypothesized that USP10 might regulate RNF168-dependent chromatin-ubiquitination. Therefore we expressed USP10, or its catalytically-inactive mutant C42A, together with HA-tagged ubiquitin and FLAG-tagged RNF168 in HEK293T cells. Cells were then lysed and either total cell extracts ***(Fig. 5A)*** or chromatin fractions ***(Fig. 5C)*** were analysed by IB. Surprisingly, we found that the ectopic expression of USP10 induces an increase in ubiquitination, which seems to not be dependent upon its catalytic activity, as highlighted by the effect prompted by the catalytically-inactive mutant ***(Fig.5A and 5C)***. Conversely, we observed that depletion of USP10 significantly reduces the ubiquitination status of cells ***(Fig. 5B)***. The analysis of chromatin fractions reported in ***figure 5C*** confirmed that the expression of USP10 markedly enhanced chromatin ubiquitination induced by RNF168.

Overall, these results indicate that USP10 regulates RNF168-activity, likely in a non-catalytic manner.

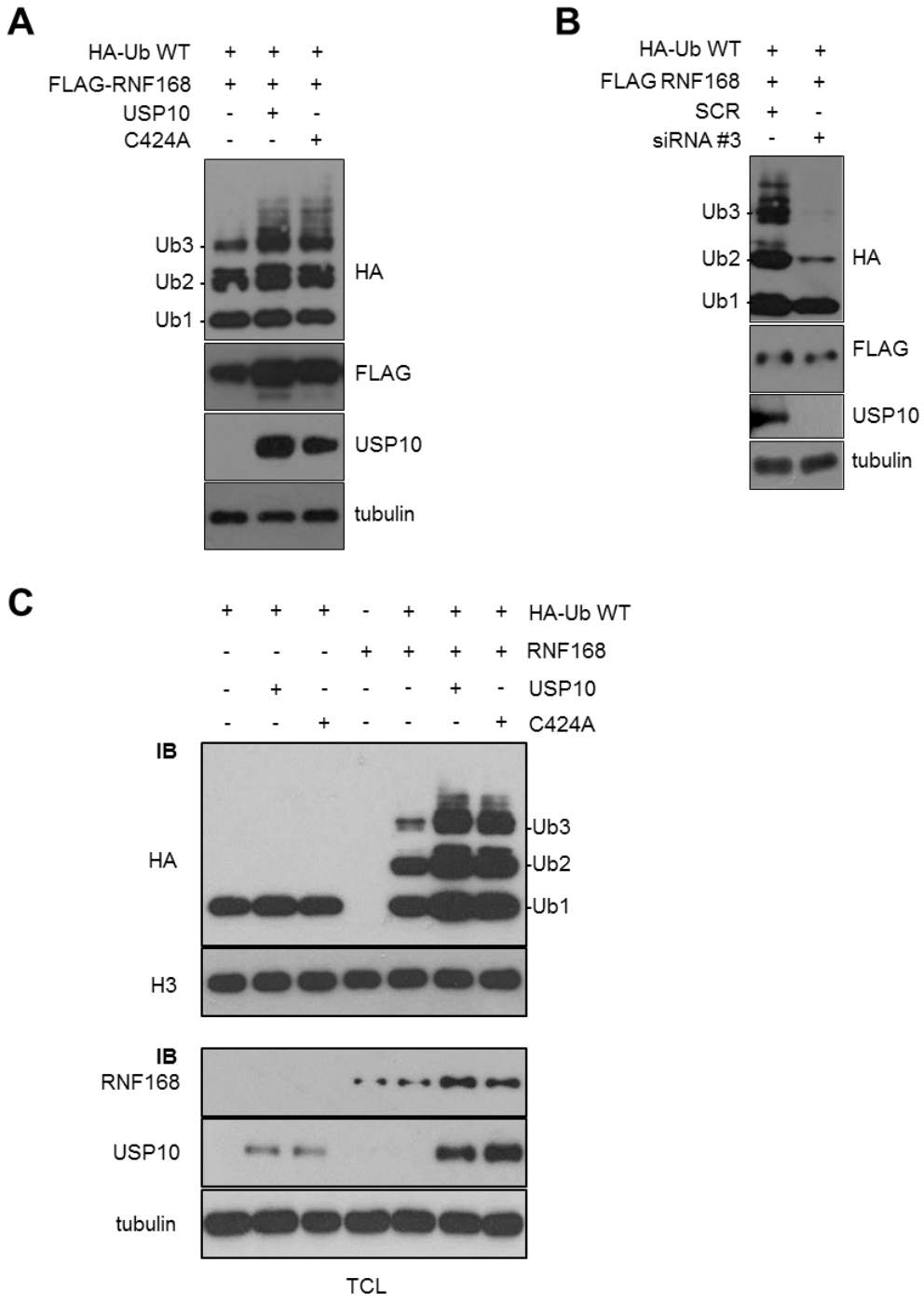


FIGURE 5: USP10 REMODELS CHROMATIN BY INDUCING RNF168-DEPENDENT UBIQUITINATION. **(A-B)** Assessment of the USP10 effects on RNF168-dependent ubiquitination status of cells. **(A)** HEK293T cells were transfected with the indicated constructs. After 48 hrs cells were

lysed and analysed by SDS-PAGE. IB was performed as indicated. **(B)** USP10 was depleted from HEK293T cells and then transfected the indicated constructs. 96 hrs after the siRNA-targeting USP10 transfection cells were lysed and analysed by IB as indicated. **(C)** *In vivo* evaluation of RNF168-mediated chromatin ubiquitination after USP10 expression. HEK293T cells were transfected with the indicated constructs. 48 hrs after transfection, chromatin was purified and analysed by SDS-PAGE. IB was performed as indicated.

USP10 induces an increase of the RNF168-ubiquitination status

Since USP10 is important for the correct DDR signal and affects RNF168-mediated chromatin ubiquitination, we next investigated if those effects are consequent to an USP10-dependent RNF168-stabilization. Thus, U2OS cells stably expressing GFP-tagged RNF168 were transfected with an increasing amount of USP10, lysed and subjected to biochemical analysis. Results demonstrate that overexpression of USP10 does not affect the expression levels of RNF168 ***(Fig. S1A)***. The same result was obtained by depleting USP10 from cells as well ***(Fig.S1B)***, demonstrating that USP10 is not involved in the protection of RNF168 from proteasome degradation. In virtue of the deubiquitinating activity of USP10 and considering its direct interaction with RNF168, we investigated if USP10 was able to affect the ubiquitination status of RNF168. To corroborate this hypothesis we either transfected HEK293T cells with HA-tagged ubiquitin and RNF168 together with USP10 ***(Fig.6A)***, or depleted it by using siRNAs ***(Fig.6B)***. Then the enriched FLAG-tagged RNF168 samples were subjected to IB to evaluate the ubiquitination status of RNF168 ***(Fig. 6A and 6B)***. Unexpectedly, we found that the expression of USP10 induces a marked increase of the poly-ubiquitinated forms of RNF168, while its depletion highly reduces them. Remarkably, as shows in ***figure 6A***, the catalytic mutant of USP10 also displays the ability to induce ubiquitination of RNF168, which further supports the non-catalytic regulation of USP10 on RNF168. To confirm these results in a more physiological system, we depleted USP10 from U2OS cells stably expressing GFP-tagged RNF168. Then lysates were subjected to

GFP-immunoprecipitation, in order to enrich samples with RNF168, and immunoblotted with anti-ubiquitin. Once again we observed a significant reduction of the RNF168-ubiquitination after USP10 depletion, clearly demonstrating that USP10 regulates the formation of the ubiquitin-conjugates on RNF168 ***(Fig.6C)***.

Taken together, these results demonstrate that USP10 is a new player in DDR, being recruited onto chromatin after DNA damage and involved in DDR *foci* formation. Moreover, we found that USP10 is an interactor of RNF168 able to regulate both its ubiquitination status and its chromatin-dependent ubiquitination.

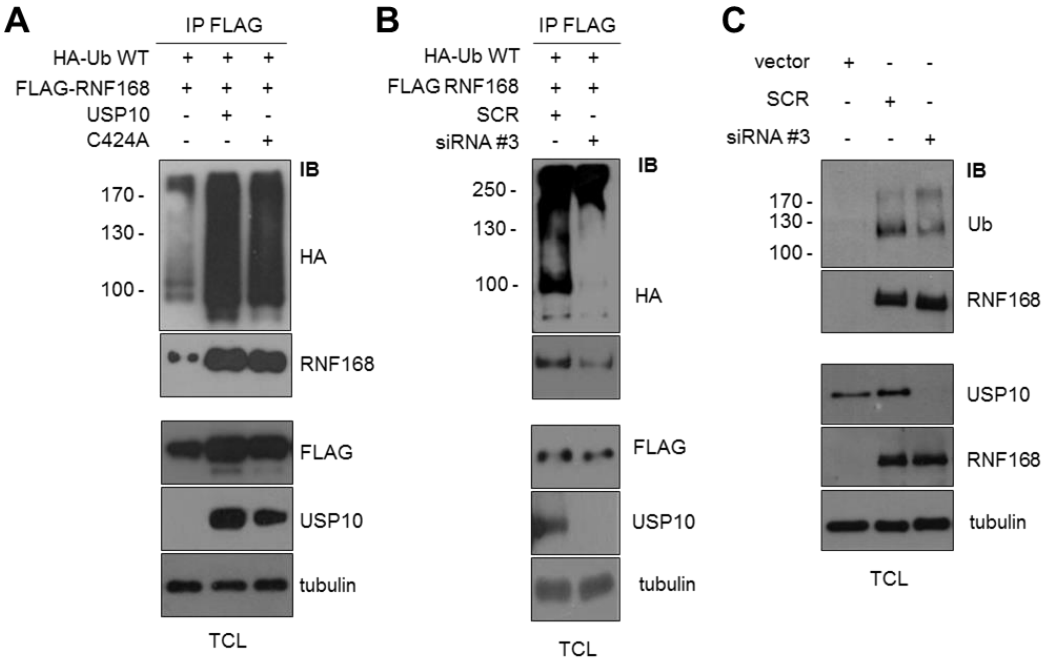


FIGURE 6: USP10 AFFECTS THE UBIQUITINATION STATUS OF RNF168. **(A-C**) Evaluation of RNF168 ubiquitination status. **(A-B)** HEK293T cells, transfected with the indicating constructs, were lysed and subjected to FLAG immunoprecipitation (IP). IB was performed using the indicated antibodies (C424A, USP10 catalytically-inactive mutant; the number is referred

to the catalytic Cys converted to Ala). **(C)** U2OS cells or U2OS cells stably expressing GFP-tagged RNF168 were transfected with the indicated constructs. Then cells were lysed and subjected to GFP-IP and analysed by SDS-PAGE. IB was performed as indicated.

DISCUSSION

Here we uncovered an USP10-dependent mechanism that provides a new level of regulation of RNF168-induced DNA damage signalling. USP10 was previously characterized as a cytoplasmic ubiquitin-specific protease that deubiquitinates p53, reversing Mdm2-induced p53 nuclear export and degradation[31]. Yuan and colleagues also discovered that USP10 translocates into the nucleus after ATM-dependent phosphorylation. Our report revealed a new intriguing role for USP10 in cellular homeostasis by integrating the previous model. Indeed we discovered that USP10 is loaded onto chromatin in untreated cells and its chromatin accumulation is enhanced upon induction of DSBs. We showed that its retention on chromatin follows a biphasic-fashion, as well as 53BP1. A similar mechanism was reported for both BRCA1[35, 36] and Bmi1[37], where their recruitment to DSBs is a kinetically biphasic process comprised of an early γ-H2A.X-independent stage and a late γ-H2A.X-dependent stage. Moreover, it was described by Miller and colleagues that histone acetylation changes also occur in a similar fashion following DSBs-induction, in order to regulate DNA repair[38]. It will be enthralling to investigate the relations between the chromatin states and the regulation of the protein assembling at the DSBs. In addition, we found that a small portion of USP10 lies on chromatin even in the untreated cells, which could be a *reservoir* ready to go into action following DNA damage-induction. Mechanistically we demonstrate that USP10 constitutively interacts with RNF168; this might indicate that the chromatin loaded USP10 is in complex with RNF168. The effect of this interaction is quite unexpected: we observed that USP10 overexpression induces extensive RNF168-dependent chromatin ubiquitination. *Au contraire*, USP10-

depleted cells show a marked reduction of their general ubiquitination status. RNF168 is fundamental for the focal accumulation of 53BP1 at the DDR *foci*, which in turn means that RNF168-disruption induces 53BP1 nuclear *focus* formation impairment[16, 20, 21, 39]. As revealed by the immunofluorescence analysis, we found that USP10-depleted cells show an aberrant focal recruitment of 53BP1 at the chromatin-flanking DSBs in the early steps of the pathway, suggesting an impairment in RNF168 function. These results point towards two possible hypotheses: the first is that USP10 induces stabilization of RNF168, the second entails a role for USP10 in RNF168 activation. As we demonstrated that USP10 does not induce the stabilization of RNF168, we can exclude the first hypothesis. Instead, our evidences suggest a USP10-mediated regulation on RNF168, which is independent on its proteolytic activity. In fact, the ability of the catalytically-inactive mutant of USP10 to induce RNF168-dependent ubiquitination similarly to the wild type clearly supports an alternative RNF168 regulation. Interestingly, this catalytically independent mechanism of action is also shared with another DUB acting in the DDR pathway. Indeed, Durocher and colleagues demonstrated that the deubiquitinating enzyme OTUB1 inhibits RNF168-dependent chromatin ubiquitination upon DSBs independently of its catalytic activity[30]. They showed that OTUB1 binds to and inhibits UBC13, an E2 conjugating enzyme crucial for the activation of DDR[30]. Conversely, here we propose that USP10 is directly involved in the activation of the ubiquitin ligase, in a catalytically inactive manner. In support of this, we found that the ubiquitin-conjugates on RNF168 are significantly increased in presence of ectopic USP10. Considering that once coupled with an E2 conjugating enzyme RNF168 is able to induce extensive auto-ubiquitination, we think that USP10 could regulate this phenomenon by stabilizing an E2, although we can not exclude an USP10-mediated modulation towards the RING-finger domain of RNF168. To our knowledge, no proves have been published to

corroborate both hypotheses while our mapping results only suggest an interaction model. Further investigations are required to unravel that mechanism.
In addition, USP10 possesses two domains: an ubiquitin carboxyl-terminal hydrolase (UCH; from the aminoacid 412 to the aminoacid 792[40]) and an ataxin 2-like carboxyl-terminal domain (Ataxin 2C; from the aminoacid 78 to the aminoacid 95[41]). While the UCH domain identifies thiol proteases that recognise and hydrolyse the peptide bond at the C-terminal glycine of ubiquitin, the Ataxin 2C domain, found in various eukaryotic proteins[41], interacts with poly(A)-binding proteins (PABPs), which are involved in gene expression[42]. It will be interesting to further examine the dynamics behind the function of both domains in order to understand whether USP10 may serve as a bridging molecule that functionally links the regulation of gene expression with DNA damage response.
In summary, our results identify USP10 as a new player in DDR by modulating RNF168-dependent ubiquitination at DSBs. These results reveal the existence of an additional layer of regulation in the ubiquitin-dependent signalling and highlight the complex dynamics behind the proteins involved in DDR in order to guarantee the maintenance of genome integrity.

AUTHOR CONTRIBUTIONS

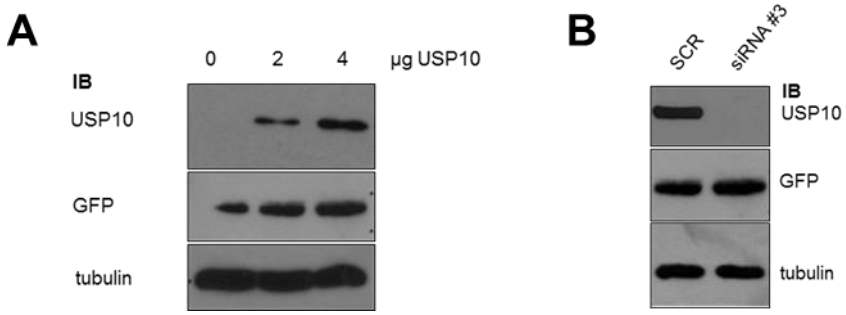
FR performed the biochemical and the immunofluorescence analysis. SP generated the GFP-tagged RNF168 depleted constructs. LP performed the SILAC analysis. LP and MG prepared samples for MS analysis. LP supervised the experiments. FR and LP designed the experiments and wrote the manuscript.

ACKNOWLEDGMENTS

Authors thank Prof Jiri Lukas for kindly providing U2OS stably expressing GFP-tagged RNF168 cell lines.

SUPPLEMENTAL INFORMATION

FIGURE S1. USP10 DOES NOT AFFECT RNF168 STABILIZATION



(A-B) Evaluation of RNF168 stabilization by USP10. **(A)** U2OS cells stably expressing GFP-RNF168 were transfected with the indicating µg of USP10. 48 hrs after transfection, cells were lysed and the GFP-RNF168 expression levels were analysed by SDS-PAGE. IB was performed as indicated. **(B)** USP10-targeting siRNA was transfected in U2OS stably expressing GFP-RNF168. After 96 hrs cells were lysed and analysed by immunoblotting as indicated.

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DISCUSSION

Nothing is more fundamental than the maintenance of genome integrity for the existence of all living beings. Recent discoveries revealed that changes in the human genome, regardless of being inherited or induced, can result in diseases that either significantly shorten lives (as seen in cancer) or dramatically affect the quality of lives (often seen in neurodegenerative diseases)[1, 2].

To maintain the integrity of their own genome, cells developed a complex network of surveillance processes, giving rise to the genome integrity network[3, 4, 5]. Among these processes, the one responsible for the proper processing of the different DNA damage, caused by various endogenous or exogenous agents, is the DNA damage response (DDR).

The function of the DDR network is to drive cells towards a decision: repair the damage or commit suicide, in order to counteract possible threats to the survival of the organism[6, 7, 8]. The damages that could occur on DNA are diverse but the most detrimental are the double strand breaks (DSBs) since they do not leave an intact complementary strand to be used as a template for DNA repair and can lead to chromosome breaks and translocations[1]. When a damage occurs on DNA, an organized and complex pathway of post-translational modifications (PTMs) regulates the fine-orchestration of the repair machinery allowing a rapid and dynamic recruitment of the DDR proteins to the damaged sites and therefore contributing to the formation of the DDR *foci*[9, 10].

Among the various types of PTMs, ubiquitination stands up as an important regulator of DDR; indeed it is able to both target key DDR proteins for proteasomal degradation and to function as a signalling device. Due to its eight amine groups - the N-terminus (M1), K6, K11, K27, K29, K33, K48, and K63 - present on its peptidic sequence, which are available for the addition of other

ubiquitin moieties to the first added to the substrate, ubiquitin (ub) has a powerful signalling potential.

Until now, the K48- and the K63-linked ub chains have been widely investigated. Indeed, it is well known in the "state of the art" that the K48- polyubiquitination is utilised to target proteins for 26S proteasomal degradation, while the K63-ubiquitination mediates protein-protein interaction in a plethora of processes, including DDR[11, 12, 13]. Conversely, little is known about the functional roles of other ub linkages, also referred as "non-canonical".

With the aim to explore the biological relevance of the different types of ub-linkages in DDR, we centred our research activity on the characterization of the RNF168-dependent K-linkage ubiquitination on chromatin. RNF168 is an E3 ubiquitin ligase, which is fundamental for the proper DNA repair after DSBs. Using biochemical and mass spectrometry approaches, together with small interfering RNA (siRNA)-based ubiquitin knockdown, we revealed that, following genotoxic stress, the major ub mark on chromatin is the non-canonical K27-linked ubiquitination and that this ubiquitination is prompted by RNF168. This result opened a novel scenario in the field, since it was assumed that RNF8/RNF168 cascade was based on the sole K63-linked ubiquitination[14, 15, 16, 17, 18]. We also added new crucial insights to the literature records by finding that this atypical ubiquitination functions in concert with the K63-linkage to sustain the DDR-signalling and to allow recruitment of 53BP1 to the DDR *foci*. These findings highlight the complexity of the system and the relevance of generating specific and univocal signals on chromatin to allow the correct DNA repair. This requirement of diversification is also emphasized, as we and others demonstrated, by the ability of RNF168 to ubiquitinate the bidentate K13/15 site at the N-terminal tail of histone H2A[19, 20], which is distinct from the Ring1b/Bmi1-dependent mono-ubiquitination on K118/119 site. Our latest findings revealed

that the bidentate K13/15 is targeted by the RNF168-dependent K27-ubiquitination, providing additional evidence in support of the highly specific regulation of chromatin ubiquitination. Moreover, considering the complexity of the system, which merges both the ub and the histone codes, it will be interesting to clarify the molecular events that define the DNA damage-dependent H2A ubiquitination orchestrated by RNF168 and Ring1b/Bmi1. The dimer Ring1b/Bmi1 is a portion of the polycomb group protein complex that acts as a transcriptional repressor and recently was found involved in DSBs repair[21, 22, 23, 24, 25]. Since we observed in our experiment a marked reduction of the histone H2A mono-ubiquitination when the K27 of ub was mutated, we suppose a possible modulatory role of RNF168 on Ring1b/Bmi1. This hypothesis could be an important step forward in the DDR riddle, connecting transcriptional regulation with RNF168-mediated remodelling activity. At this point it will be interesting to identify other possible targets of the RNF168-mediated K27-ubiquitination in order to better characterize the functional relevance of K27-linked ubiquitination in DDR. Until now there are indications of additional candidate targets; for instance Bohgaki and colleagues recently demonstrate that 53BP1 is targeted by RNF168[26]. 53BP1 is known to be a reader of the RNF168-dependent ubiquitination on K15 of histone H2A[27]. Indeed it was demonstrated that the UDR domain of 53BP1 recognizes the peculiar ub mark at the N-terminal tail of the histone H2A permitting its pull-down with the nucleosomes. In line with this, we showed that the UDR of 53BP1 is able to directly interact with both K27- and K63-ub dimers revealing that it is a *bona fide* UBD. Moreover we clearly demonstrated that the diubK27 is directly recognized by Rap80, RNF169 and RNF168 itself. Thus RNF168 accumulates to DDR *foci* not only by binding, through its UBDs, the RNF8-mediated ubiquitination, but also by recognizing ub conjugates that RNF168 generates on chromatin.

Taken together these results represent an unprecedented discovery that emphasizes the complexity of the "code" behind the DDR network, and places RNF168 as an important regulator of genome integrity.

Thus, to further investigate the DSBs-induced RNF168-signalling cascade and searching for RNF168 new interactors, we performed a quantitative proteomic screening, based on the SILAC method, which identified many proteins involved in various biological processes, such as transcriptional regulation, interferon signalling and RNA/DNA editing, suggesting the cross role of RNF168 into the cell. Among these interactors we found USP10, which is a poorly characterized deubiquitinase (DUB). In 2010, was demonstrated that USP10 is able to stabilize p53 by deubiquitinating it[28]. Since then USP10 was put in the middle of the discussion related to cancer biology. Using biochemical and immunofluorescence approaches, we found that USP10 is involved in the mechanism that regulates DNA repair. Indeed it is loaded onto chromatin after genotoxic stress and its depletion impairs the recruitment of 53BP1 to DDR *foci*. These findings integrate the model outlined by Yuan and colleagues, giving to USP10 an important role in the regulation of the cellular homeostasis. In addition we discovered that USP10 remodels the ub-conjugates on RNF168 and the ubiquitination status of chromatin as well. So far the USP10-mediated mechanism of action able to induce the phenotype is unclear and further investigations will be needed to clarify this point. Although we can not exclude a modulation of the activity of RNF168 through the recognition of its RING-finger by USP10, our evidences suggest an USP10-mediated non-catalytic regulation of RNF168, which through tethering could induce a conformational modification able to activate the E3 ubiquitin ligase[29, 30].

In last instance, it behoves us to mention that USP10, in addition to the UCH domain (ubiquitin carboxyl-terminal hydrolase), possesses an Ataxin 2C (ataxin 2-like carboxyl-terminal domain), which is identified in proteins involved in gene

expression[31, 32]. Given that, it will be interesting to investigate the possible involvement of USP10 in the regulation of gene expression and its possible role as a connector between this pathway and DNA repair.

Overall these results revealed that USP10 is a new player in DDR and uncovered a USP10-dependent mechanism that provides a novel regulatory level of the RNF168-induced signalling.

Our findings started to shed light on the complexity of the DDR network and provide some evidences that underlining proteins, such as USP10, could be used as translational pharmaceutical targets for better therapeutic interventions.

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LIST OF PUBLICATIONS

Gatti M, Pinato S, Maiolica A, Rocchio F, Prato MG, Aebersold R, Penengo L. (2015) *RNF168 Promotes Noncanonical K27 Ubiquitination to Signal DNA Damage*. Cell Reports. 2015 Jan; 10(13):1-13. doi: 10.1016/j.celrep.2014.12.021.