RNF168 promotes noncanonical K27 ubiquitination to signal DNA damage

Gatti, Marco ; Pinato, Sabrina ; Maiolica, Alessio ; Rocchio, Francesca ; Prato, Maria Giulia ; Aebersold, Ruedi ; Penengo, Lorenza

Abstract: 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 noncanonical 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.

DOI: https://doi.org/10.1016/j.celrep.2014.12.021

Originally published at:
Gatti, Marco; Pinato, Sabrina; Maiolica, Alessio; Rocchio, Francesca; Prato, Maria Giulia; Aebersold, Ruedi; Penengo, Lorenza (2015). RNF168 promotes noncanonical K27 ubiquitination to signal DNA damage. Cell Reports, 10(2):226-38.
DOI: https://doi.org/10.1016/j.celrep.2014.12.021

RNF168 promotes non-canonical K27 ubiquitination to signal DNA damage

Marco Gatti[1], Sabrina Pinato[1,5], Alessio Maiolica[2,5], Francesca Rocchio[1,5], Maria Giulia Prato[1], Ruedi Aebersold[2,3] and Lorenza Penengo[1,4*]

[1]Dept. of Pharmacological Sciences, University of Piemonte Orientale ''A. Avogadro'', 28100 Novara, Italy

[2]Department of Biology, Institute of Molecular Systems Biology, Eidgenössische Technische Hochschule (ETH) Zurich, Zurich, Switzerland

[3]Faculty of Science, University of Zurich, Zurich, Switzerland

[4]Institute of Molecular Cancer Research, University of Zürich, 8057 Zürich, Switzerland

[5]These authors contributed equally to this work

[*]Corresponding author. Mailing Address: Institute of Molecular Cancer Research, University of Zürich, 8057 Zürich, Switzerland.

Phone: +41 44 6353488, Fax: +41 44 6353484

E-mail: lorenza.penengo@uzh.ch

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 multi-step process involving a 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 polyubiquitin chains. These different linkages increase the complexity of the ubiquitin system, giving rise to ubiquitin chains with distinct topology, providing structural flexibility that results in a multitude of functional outcomes.

The functional roles of K48- and K63-linked ubiquitin chains have been widely investigated; K48 polyubiquitination is extensively utilized to target proteins for 26S proteasomal degradation, while K63-linked ubiquitination mediates protein-protein interaction in different processes (Chen and Sun, 2009; Jackson and Durocher, 2013; Woelk et al., 2007). Although the relevance of K11- and M1-linkages has been described in cell cycle regulation and NF-**κ**B activation, respectively (Iwai and Tokunaga, 2009; Wickliffe et al., 2009), 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 DDR and in several DNA repair mechanisms. A paradigmatic example of DDR coordination *via* ubiquitination is represented by the signaling 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 signaling 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 de-ubiquitinating 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 signaling 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, a 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 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*, while 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 interact both with synthetic K27-diUb and with K27-ubiquitinated nucleosomes purified from cells. Overall, our data identify a crucial function for non-canonical K27 ubiquitination in the DDR, determine the ubiquitin ligase that specifically builds K27 ubiquitin chains and define key targets and readers of this modification.

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 polyubiquitin chains. These FLAG-tagged constructs were expressed in 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. While 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-

monoubiquitination, by conjugating to target proteins via the C-terminal Gly76 (Ub1, 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 since mutants affecting distinct Lys residues could be differently conjugated into proteins (Figure S1A, 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 (Figure S2A, S2B). 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, UbcH5c/Nedd4, Figure S3A, S3B) or K48-linked chains (Ube2R1/Nedd4, Figure S3C)(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 HA-tagged wild-type, K27R, K63R or K48R mutants in the context of ubiquitin depletion (Figure S4A, S6A). We observed that both K48 and K63 conjugates are formed in the presence of the K27R mutant construct (Figure S4B). 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 specifically 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 towards 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 polyubiquitin chains, generating a modest signal likely due to monoubiquitination 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*.

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 analyzed 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 if RNF168 induces K27-linked ubiquitination, we expressed either wild-type FLAG-tagged ubiquitin alone or together with RNF168 in HEK293T cells, we extracted chromatin fraction and we enriched the FLAG-tagged ubiquitinated proteins (Figure 2B, 2C). The resulting protein samples were treated with trypsin and analyzed 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 (Figure 2D, 2E).

This result clearly indicates that K27 ubiquitination is induced in cells by RNF168.

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). Since 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 S5A), and we 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 (Figure S5B, S5C). Then, we analyzed 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.

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, since histone H2As has at least four different Lys that can be theoretically simultaneously targeted by ubiquitin, the detected ubiquitin conjugates could result from multi-monoubiquitination on different sites, polyubiquitination, 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 we 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-monoubiquitination hypothesis would predict that these mutants (#2, #3, Figure 3D) undergo only mono-ubiquitination (Ub1). Instead, we also clearly observed the di-ubiquitinated form (#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 since the ubiquitination signal is almost abolished when the all sites are substituted (#4, Figure 3D). Similarly, we can exclude that the signal observed derives from ubiquitination of another histone - i.e. H2B - or other tightly bound proteins, since 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*.

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 analyzed the level of chromatin ubiquitination. As expected, cells exposed to etoposide (an inhibitor of Topoisomerase II) markedly increased the ubiquitin signal on chromatin (Figure 4A). This effect is dependent on ubiquitin integrity, since 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, 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 signaling pathway, we tested the ubiquitination status of damaged chromatin upon RNF168 depletion and ATM inhibition. Importantly, depletion 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 pre-treated with the ATMi inhibitor (KU55933; Figure 4E), although the signal corresponding to phosphorylated form of H2A.X (γ-H2A.X) is still high, due to the presence of other kinases of the family (DNA-PK and ATR), not inhibited by KU55933, and to the high dose of etoposide we used (30 μM).

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

Chromatin ubiquitination is required for proper formation of DDR *foci* and the activation of the signaling cascade. Since 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 (Figure S6A, S6B) 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 (Figure S6C, S6D, S6G). Conversely, 53BP1 and

BRCA1 recruitment to DDR *foci* - which are reportedly dependent on chromatin ubiquitination - were dramatically reduced (Figure S6E, S6F, S6G). To corroborate these results, we reintroduced a siRNA-resistant form of ubiquitin (WT^{Res}) and largely restored proper localization of 53BP1 and BRCA1 to DDR *foci* (Figure S5E-G). Albeit predictable from the literature, this represents the first 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 (Figure 5A, 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 further defects (Figure 5A, 5B). This effect is highly specific since 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 (Figure S7A, S7B).

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* (Figure S8A-C).

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* induced, while 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.

In keeping 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 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 S9) and reduced the formation of ubiquitin-conjugates within the nucleus (Figure S10) .

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.

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 amino acid substitutions (A88S and A113S). As shown in Figure 6A, we found that the Rap80-UIMs interact with diUbK27, while 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 (H4K20me2) and ubiquitination on K15 of histone H2A (H2AK15ub). The binding to H2AK15ub is dependent on the UDR (Ubiquitination-Dependent Recruitment) 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, 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, while 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 Figure 6C, 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.

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 signaling 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 novel 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 K118/119 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, beside H2A and H2A.X, other factors are target of RNF168-

mediated K27 ubiquitination. One candidate to be investigated is 53BP1, since 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 polyubiquitin chains (K6, K27, and K48) and this is a prerequisite for Ring1B to monoubiquitinate *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 monoubiquitinated histone H2As. A tantalizing hypothesis is that RNF168 could modulate the activity of Ring1b by promoting its K27-linked ubiquitination, drawing an unprecedented 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., 2011; Pinato et al., 2009; 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 (K15ub-H2A). Now we show that the UDR is sufficient to directly interact with K27 and K63 ubiquitin dimers. This is a remarkable finding since 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 S11), but rather generates the docking sites for different downstream signaling effectors. Remarkably, our data show that RNF168 significantly increases the level of monoubiquitinated and multi-monoubiquitinated 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, Mol Cell 2012). Thus RNF168, by promoting chromatin ubiquitination, generates the docking sites for itself and stabilize 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.

Experimental Procedures

Cell culture and RNA interference

All culture media were supplemented with 10% fetal bovine serum and 2 mM L-glutamine. U2OS cell line expressing the RNF168-targeting short hairpin RNA (shRNA) in a doxycycline-inducible manner, kindly provided by J. Lukas, were cultured in DMEM supplemented with 10% fetal bovine serum tetracycline free (BioWest), 2 mM L-glutamine, 1 µg/ml puromycin (Sigma), 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 hours. RNA interference is detailed in Extended Experimental Procedures.

Targeted mass spectrometric analysis by selected reaction monitoring (SRM)

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

Shotgun mass spectrometric analysis

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

Pull-down assays with synthetic diUb

Pull-downs were performed by incubating 1 µM of recombinant proteins with 0.25 µg of ubiquitin dimers for 1 hour, in a buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 % Triton X-100, 10% Glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, protease

inhibitor cocktail (SIGMA), 20 μM NEM. 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 (PVDF) membranes (Sigma), denatured in guanidinium chloride and immunoblotted using anti-ubiquitin (P4D1, Santa Cruz).

In vitro *ubiquitination assay*

Recombinant proteins were expressed as described in Extended Experimental Procedures. GST-tag was removed from ubiquitin using PreScission protease (GE Healthcare). 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 $MgCl_2$, 100 mM NaCl, 1 μM dithiothreitol (DTT), and 2 mM ATP (Sigma) at 30°C for 1 hour. ATP regeneration system (Sigma) was used to recycle ATP.

In vivo *detection of ubiquitinated chromatin and histones*

HEK293T cells expressing different ubiquitin mutants 48 hours after transfection were collected in PBS, containing protease inhibitor cocktail (Sigma), 1 mM PMSF and 20 μM NEM. 1/10 of the samples were separately processed for protein normalization, while 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, Heidelberg, Germany).

Acknowledgments

We are grateful to Massimo Lopes and Simona Polo for critical reading the manuscript. This work was supported by Associazione Italiana per la Ricerca sul Cancro (IG: 11979) and by Fondazione Cariplo (grant n. 2013-0594) to LP; by Fondazione Italiana per la Ricerca sul Cancro to SP and MG (Rif. 14885); by EMBO long-term fellowship to AM.

Author contributions

MG, SP, FR and MGP performed biochemical studies using the ubiquitin mutants; FR analyzed histone ubiquitination; SP generated constructs and performed binding assay with K27-linked conjugates and DDR proteins; MG performed ubiquitin and RNF168 knockdown and prepared samples for MS analysis; AM and RA designed and performed the LC-SRM analysis and provided intellectual support; MG and LP performed immunofluorescence analysis; LP planned the project, designed the experiments and wrote the manuscript. All the authors discussed the data and commented on the manuscript.

Figure Legends

Figure 1. RNF168 induces non-canonical K27-linked ubiquitin chains 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 antibody revealed the presence of higher molecular weight proteins compatible with mono-, di- and tri-ubiquitinated forms of histones (Ub1, Ub2, 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.

(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 (GST-RNF168), in combination with the indicated ubiquitin mutants. IB was performed with the indicated antibodies. Ubiquitin conjugates observed with ubiquitin (Ub) IB correspond to ubiquitinated forms of 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 analyzed by LC-SRM. 51 transitions were monitored; the four most intense transitions corresponding to the ions y_3, y_5, y_6 and b_3 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 hours after transfection, cells were subjected to chromatin purification followed by FLAG immunoprecipitation (IP). After glycine elution, samples were in part analyzed by IB as indicated in panel (C), and in part subjected to trypsin digestion and processed for SRM analysis, as described in Extended 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.

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 immunoblot revealed the presence of higher molecular weight proteins compatible with mono-, di- and tri-ubiquitinated forms (Ub1, Ub2, 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 immunoblot 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). 48 hours post-transfection samples were subjected to acid extraction and analyzed 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 (upper panels) and by anti-tubulin on TCL.

(C) Schematic representation of the H2A.X mutants used in (D) and their possible ubiquitination pattern.
(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 hours cells were subjected to chromatin extraction followed by FLAG IP in the presence of SDS. Samples were subjected to IB with the indicated antibodies.

Figure 4. K27 ubiquitination is induced on chromatin upon formation of DSBs
(A) (B) HEK293T cells expressing FLAG-tagged ubiquitin K/R or K-only mutants were treated with etoposide (30 μM) for 1 hour. After 3 hours, chromatin was extracted from cells and analyzed by IB as indicated. Equal loading and H2A.X phosphorylation (g-H2A.X) were verified on chromatin extracts using antibodies directed to histone H3 and to the phosphorylated form of H2A.X (phospho-Ser139), respectively (upper panels). Expression of ubiquitin mutants was detected by FLAG IB on TCL (lower panels).
(C) HEK293T cells were transfected with siRNA targeting RNF168 and control siRNA together with FLAG-tagged ubiquitin K27-only. 96 hours after siRNA transfection cells were treated with etoposide (30 μM) for 1 hour. After 3 hours 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 18s 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 pre-treated with ATMi (KU55933, 10 μM) for 30 minutes. Cells were then treated and processed as in (C). Asterisk (*) indicates the di-ubiquitinated form of H2A.X, abrogated by ATMi.

Figure 5. UbK27 is essential for the assembly of DDR *foci*
(A) (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 cells with two siRNAs targeting ubiquitin in a 1:1 combination (see Extended Experimental Procedure). 18 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 hour before fixing. Immunostaining was performed with FLAG and 53BP1 antibody.

(D) U2OS cells expressing siRNA-resistant FLAG-tagged K27,63-only ubiquitin were transfected with two siRNAs targeting ubiquitin for 18 hours and then treated or not with etoposide as in (A-C) (upper and middle panels) and in the presence of ATM inhibitor (KU55933, 10 μM, lower panels) before fixing. Immunostaining was performed as in (C).

(A-D) Quantification of the FLAG-positive cells with more than 5 *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.

Figure 6. 53BP1, Rap80, RNF169 and RNF168 directly interact with K27-linked ubiquitin dimers

(A-D) Pull-down assays performed using different recombinant proteins; in A, GST-tagged Rap80-UIMs and Rap80-UIMs** (A88S and A113S); in B, GST-tagged UDR wild-type or GST-UDR** mutant (L1619A, R1627A); in C and D, GST-tagged RNF168 and MBP-tagged RNF169 as full-length proteins. Pull-down assays were performed using the indicated recombinant proteins incubated with synthetic K27, K48, K63-linked ubiquitin dimers (diUb). Proteins were solved by SDS-PAGE and IB was performed with anti-ubiquitin antibody (P4D1).

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.

References

Adam, S., Polo, S.E., and Almouzni, G. (2013). Transcription recovery after DNA damage requires chromatin priming by the H3.3 histone chaperone HIRA. Cell *155*, 94-106.

Ben-Saadon, R., Zaaroor, D., Ziv, T., and Ciechanover, A. (2006). The polycomb protein Ring1B generates self atypical mixed ubiquitin chains required for its in vitro histone H2A ligase activity. Mol Cell *24*, 701-711.

Bohgaki, M., Bohgaki, T., El Ghamrasni, S., Srikumar, T., Maire, G., Panier, S., Fradet-Turcotte, A., Stewart, G.S., Raught, B., Hakem, A.*, et al.* (2013). RNF168 ubiquitylates 53BP1 and controls its response to DNA double-strand breaks. Proc Natl Acad Sci U S A *110*, 20982-20987.

Chen, J., Feng, W., Jiang, J., Deng, Y., and Huen, M.S. (2012). Ring finger protein RNF169 antagonizes the ubiquitin-dependent signaling cascade at sites of DNA damage. J Biol Chem *287*, 27715-27722.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. Mol Cell *33*, 275-286.

Doil, C., Mailand, N., Bekker-Jensen, S., Menard, P., Larsen, D.H., Pepperkok, R., Ellenberg, J., Panier, S., Durocher, D., Bartek, J.*, et al.* (2009). RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. Cell *136*, 435-446.

Fradet-Turcotte, A., Canny, M.D., Escribano-Diaz, C., Orthwein, A., Leung, C.C., Huang, H., Landry, M.C., Kitevski-LeBlanc, J., Noordermeer, S.M., Sicheri, F.*, et al.* (2013). 53BP1 is a reader of the DNA-damage-induced H2A Lys 15 ubiquitin mark. Nature *499*, 50-54.

Gatti, M., Pinato, S., Maspero, E., Soffientini, P., Polo, S., and Penengo, L. (2012). A novel ubiquitin mark at the N-terminal tail of histone H2As targeted by RNF168 ubiquitin ligase. Cell Cycle *11*, 2538-2544.

Gudjonsson, T., Altmeyer, M., Savic, V., Toledo, L., Dinant, C., Grofte, M., Bartkova, J., Poulsen, M., Oka, Y., Bekker-Jensen, S.*, et al.* (2012). TRIP12 and UBR5 suppress spreading of chromatin ubiquitylation at damaged chromosomes. Cell *150*, 697-709.

Huen, M.S., Grant, R., Manke, I., Minn, K., Yu, X., Yaffe, M.B., and Chen, J. (2007). RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. Cell *131*, 901-914.

Iwai, K., and Tokunaga, F. (2009). Linear polyubiquitination: a new regulator of NF-kappaB activation. EMBO Rep *10*, 706-713.

Jackson, S.P., and Durocher, D. (2013). Regulation of DNA damage responses by ubiquitin and SUMO. Mol Cell *49*, 795-807.

Kolas, N.K., Chapman, J.R., Nakada, S., Ylanko, J., Chahwan, R., Sweeney, F.D., Panier, S., Mendez, M., Wildenhain, J., Thomson, T.M.*, et al.* (2007). Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. Science *318*, 1637-1640.

Maiolica, A., Junger, M.A., Ezkurdia, I., and Aebersold, R. (2012). Targeted proteome investigation via selected reaction monitoring mass spectrometry. J Proteomics *75*, 3495-3513.

Maspero, E., Mari, S., Valentini, E., Musacchio, A., Fish, A., Pasqualato, S., and Polo, S. (2011). Structure of the HECT:ubiquitin complex and its role in ubiquitin chain elongation. EMBO Rep *12*, 342-349.

Mattiroli, F., Vissers, J.H., van Dijk, W.J., Ikpa, P., Citterio, E., Vermeulen, W., Marteijn, J.A., and Sixma, T.K. (2012). RNF168 ubiquitinates K13-15 on H2A/H2AX to drive DNA damage signaling. Cell *150*, 1182-1195.

Nakada, S., Tai, I., Panier, S., Al-Hakim, A., Iemura, S., Juang, Y.C., O'Donnell, L., Kumakubo, A., Munro, M., Sicheri, F.*, et al.* (2010). Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. Nature *466*, 941-946.

Nishikawa, H., Ooka, S., Sato, K., Arima, K., Okamoto, J., Klevit, R.E., Fukuda, M., and Ohta, T. (2004). Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. J Biol Chem *279*, 3916-3924.

Panier, S., Ichijima, Y., Fradet-Turcotte, A., Leung, C.C., Kaustov, L., Arrowsmith, C.H., and Durocher, D. (2012). Tandem protein interaction modules organize the ubiquitin-dependent response to DNA double-strand breaks. Mol Cell *47*, 383-395.

Penengo, L., Mapelli, M., Murachelli, A.G., Confalonieri, S., Magri, L., Musacchio, A., Di Fiore, P.P., Polo, S., and Schneider, T.R. (2006). Crystal structure of the ubiquitin binding domains of rabex-5 reveals two modes of interaction with ubiquitin. Cell *124*, 1183-1195.

Peng, D.J., Zeng, M., Muromoto, R., Matsuda, T., Shimoda, K., Subramaniam, M., Spelsberg, T.C., Wei, W.Z., and Venuprasad, K. (2011). Noncanonical K27-linked polyubiquitination of TIEG1 regulates Foxp3 expression and tumor growth. J Immunol *186*, 5638-5647.

Pinato, S., Gatti, M., Scandiuzzi, C., Confalonieri, S., and Penengo, L. (2011). UMI, a novel RNF168 ubiquitin binding domain involved in the DNA damage signaling pathway. Mol Cell Biol *31*, 118-126.

Pinato, S., Scandiuzzi, C., Arnaudo, N., Citterio, E., Gaudino, G., and Penengo, L. (2009). RNF168, a new RING finger, MIU-containing protein that modifies chromatin by ubiquitination of histones H2A and H2AX. BMC Mol Biol *10*, 55.

Poulsen, M., Lukas, C., Lukas, J., Bekker-Jensen, S., and Mailand, N. (2012). Human RNF169 is a negative regulator of the ubiquitin-dependent response to DNA double-strand breaks. J Cell Biol *197*, 189-199.

Sato, Y., Yoshikawa, A., Mimura, H., Yamashita, M., Yamagata, A., and Fukai, S. (2009). Structural basis for specific recognition of Lys 63-linked polyubiquitin chains by tandem UIMs of RAP80. EMBO J *28*, 2461-2468.

Sobhian, B., Shao, G., Lilli, D.R., Culhane, A.C., Moreau, L.A., Xia, B., Livingston, D.M., and Greenberg, R.A. (2007). RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. Science *316*, 1198-1202.

Stewart, G.S., Panier, S., Townsend, K., Al-Hakim, A.K., Kolas, N.K., Miller, E.S., Nakada, S., Ylanko, J., Olivarius, S., Mendez, M.*, et al.* (2009). The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. Cell *136*, 420-434.

Wang, B., and Elledge, S.J. (2007). Ubc13/Rnf8 ubiquitin ligases control foci formation of the Rap80/Abraxas/Brca1/Brcc36 complex in response to DNA damage. Proc Natl Acad Sci U S A *104*, 20759-20763.

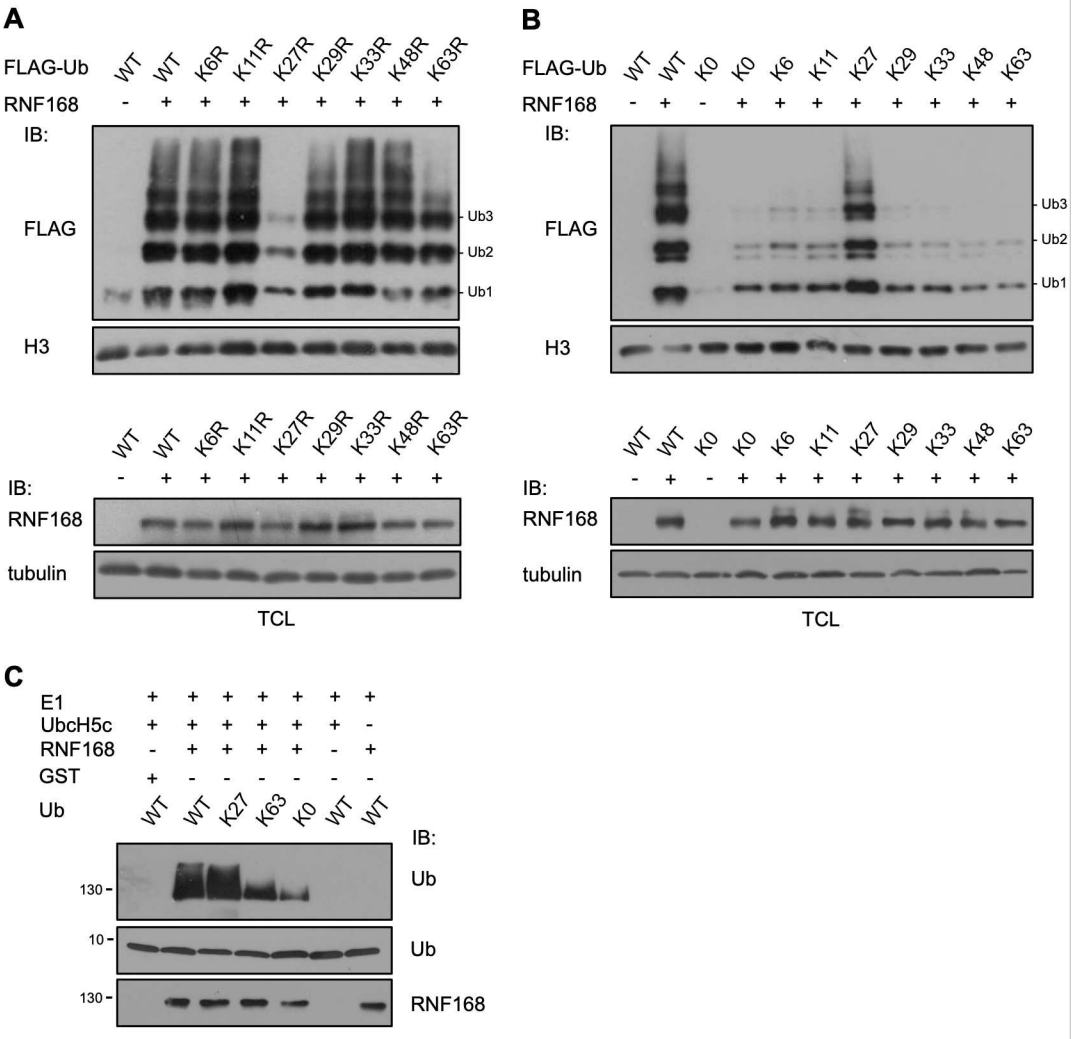
Wickliffe, K., Williamson, A., Jin, L., and Rape, M. (2009). The multiple layers of ubiquitin-dependent cell cycle control. Chem Rev *109*, 1537-1548.

Woelk, T., Sigismund, S., Penengo, L., and Polo, S. (2007). The ubiquitination code: a signalling problem. Cell Div *2*, 11.

Wu-Baer, F., Lagrazon, K., Yuan, W., and Baer, R. (2003). The BRCA1/BARD1 heterodimer assembles polyubiquitin chains through an unconventional linkage involving lysine residue K6 of ubiquitin. J Biol Chem *278*, 34743-34746.

Zucchelli, S., Codrich, M., Marcuzzi, F., Pinto, M., Vilotti, S., Biagioli, M., Ferrer, I., and Gustincich, S. (2010). TRAF6 promotes atypical ubiquitination of mutant DJ-1 and alpha-synuclein and is localized to Lewy bodies in sporadic Parkinson's disease brains. Hum Mol Genet *19*, 3759-3770.

Figure 1


A
FLAG-Ub
WT WT K6R K11R K27R K29R K33R K48R K63R
RNF168
- + + + + + + + +
IB:
FLAG
Ub3
Ub2
Ub1
H3
WT WT K6R K11R K27R K29R K33R K48R K63R
IB:
RNF168
tubulin
TCL
B
FLAG-Ub
WT WT K0 K0 K6 K11 K27 K29 K33 K48 K63
RNF168
- + - + + + + + + + +
IB:
FLAG
Ub3
Ub2
Ub1
H3
WT WT K0 K0 K6 K11 K27 K29 K33 K48 K63
IB:
RNF168
tubulin
TCL

C
E1
UbcH5c
RNF168
GST
Ub
WT WT K27 K63 K0 WT WT
IB:
130
Ub
10
Ub
130
RNF168

Figure 2

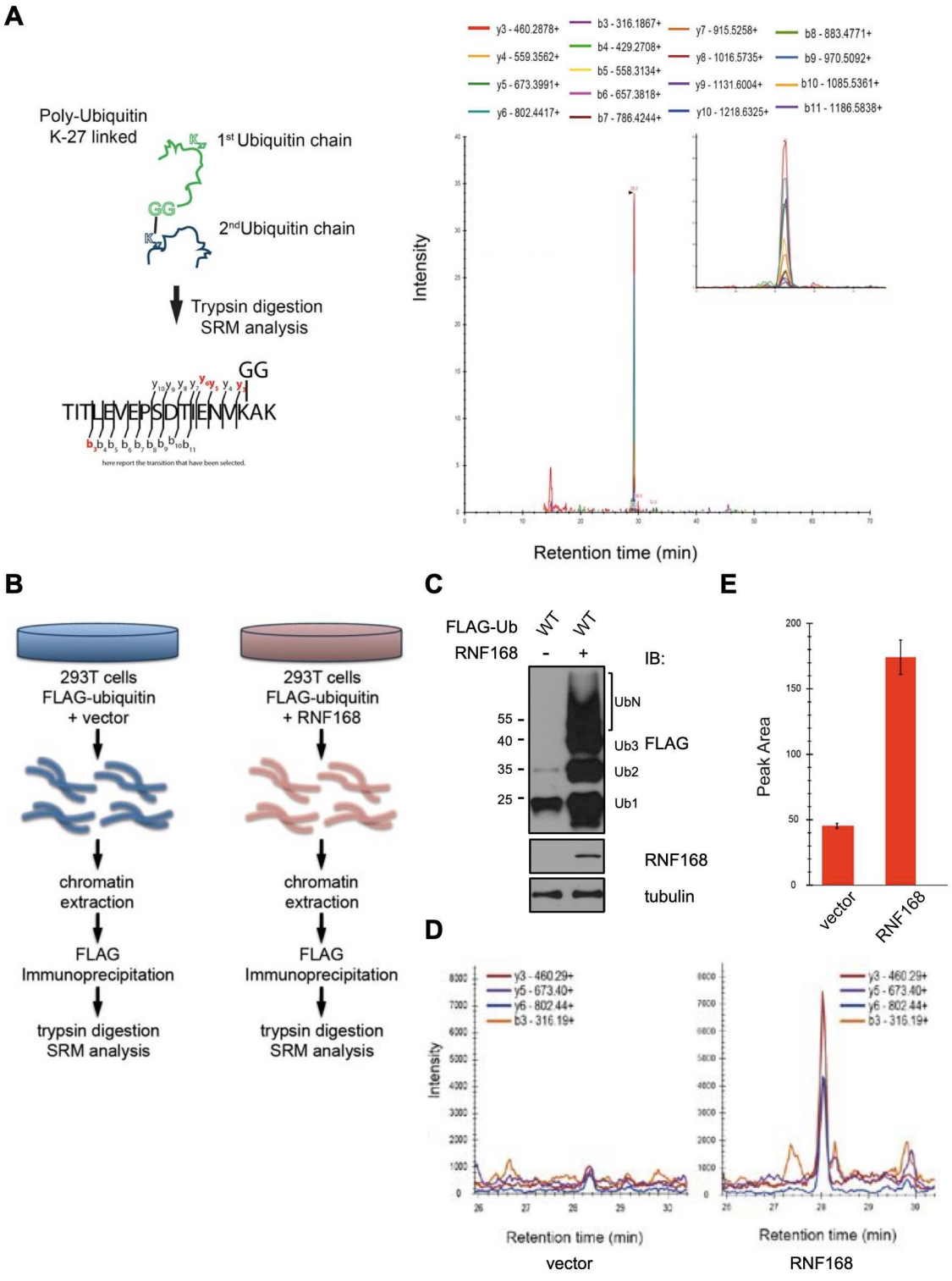

A
Poly-Ubiquitin
K-27 linked
1st Ubiquitin chain
2nd Ubiquitin chain
GG
Trypsin digestion
SRM analysis
TITLEVEPSDTIENVKAK
here report the transition that have been selected.
y3 - 460.2878+
y4 - 559.3562+
y5 - 673.3991+
y6 - 802.4417+
b3 - 316.1867+
b4 - 429.2708+
b5 - 558.3134+
b6 - 657.3818+
b7 - 786.4244+
y7 - 915.5258+
y8 - 1016.5735+
y9 - 1131.6004+
y10 - 1218.6325+
b8 - 883.4771+
b9 - 970.5092+
b10 - 1085.5361+
b11 - 1186.5838+
Intensity
Retention time (min)
B
293T cells
FLAG-ubiquitin
+ vector
293T cells
FLAG-ubiquitin
+ RNF168
chromatin
extraction
FLAG
Immunoprecipitation
trypsin digestion
SRM analysis
C
FLAG-Ub
RNF168
WT
WT
-
+
IB:
UbN
Ub3
Ub2
Ub1
FLAG
55
40
35
25
RNF168
tubulin
D
y3 - 460.29+
y5 - 673.40+
y6 - 802.44+
b3 - 316.19+
Intensity
Retention time (min)
vector
RNF168
E
Peak Area
vector
RNF168

Figure 3

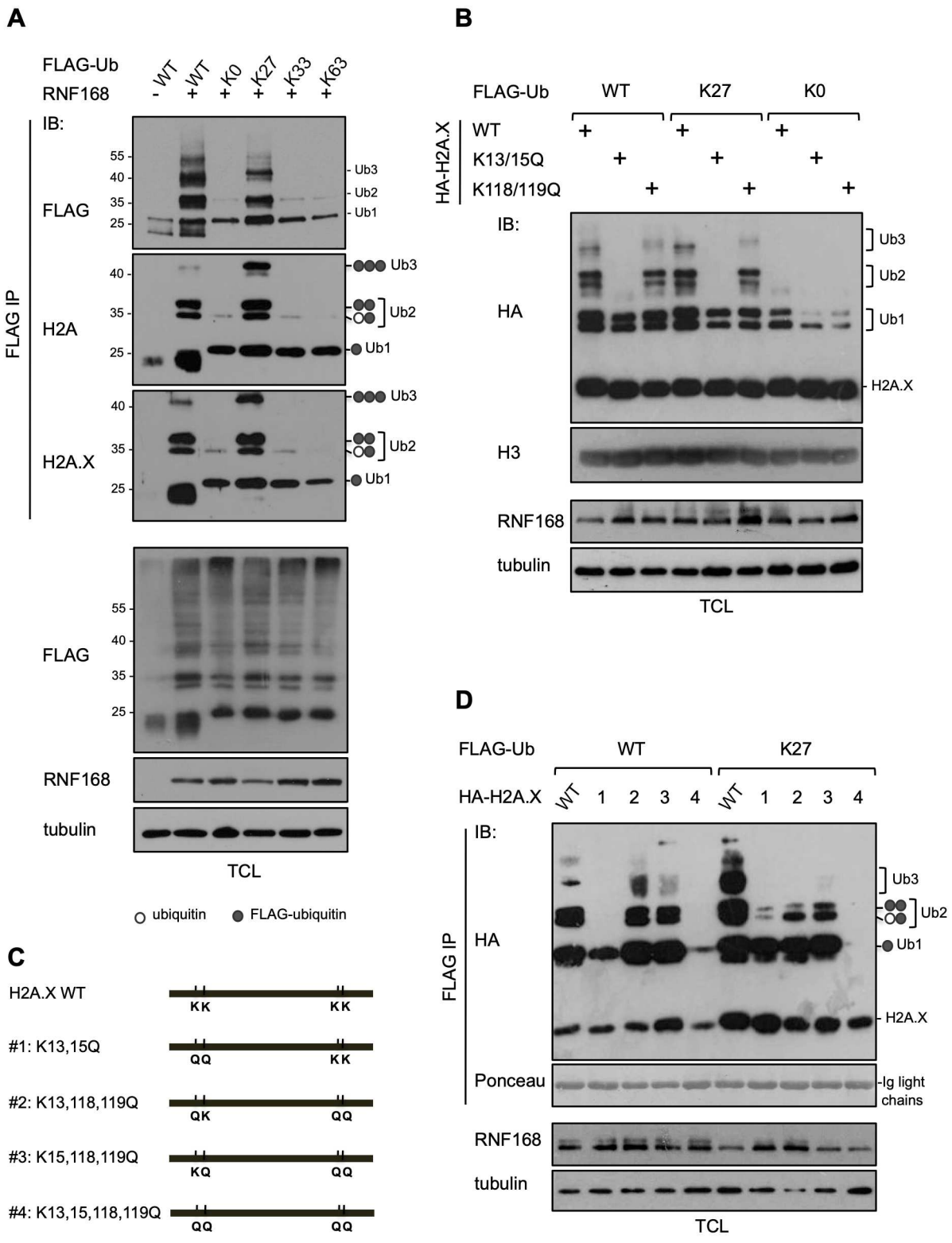

A
FLAG-Ub
RNF168
- WT
+ WT
+ K0
+ K27
+ K33
+ K63
IB:
FLAG IP
FLAG
H2A
H2A.X
55
40
35
25
Ub3
Ub2
Ub1
FLAG
RNF168
tubulin
TCL
ubiquitin
FLAG-ubiquitin
B
FLAG-Ub
WT
K27
K0
HA-H2A.X
WT
K13/15Q
K118/119Q
IB:
HA
H2A.X
H3
RNF168
tubulin
TCL
C
H2A.X WT
KK
#1: K13,15Q
QQ
#2: K13,118,119Q
QK
#3: K15,118,119Q
KQ
#4: K13,15,118,119Q
D
FLAG-Ub
WT
K27
HA-H2A.X
1
2
3
4
IB:
FLAG IP
HA
Ponceau
Ig light chains
RNF168
tubulin
TCL

Figure 4

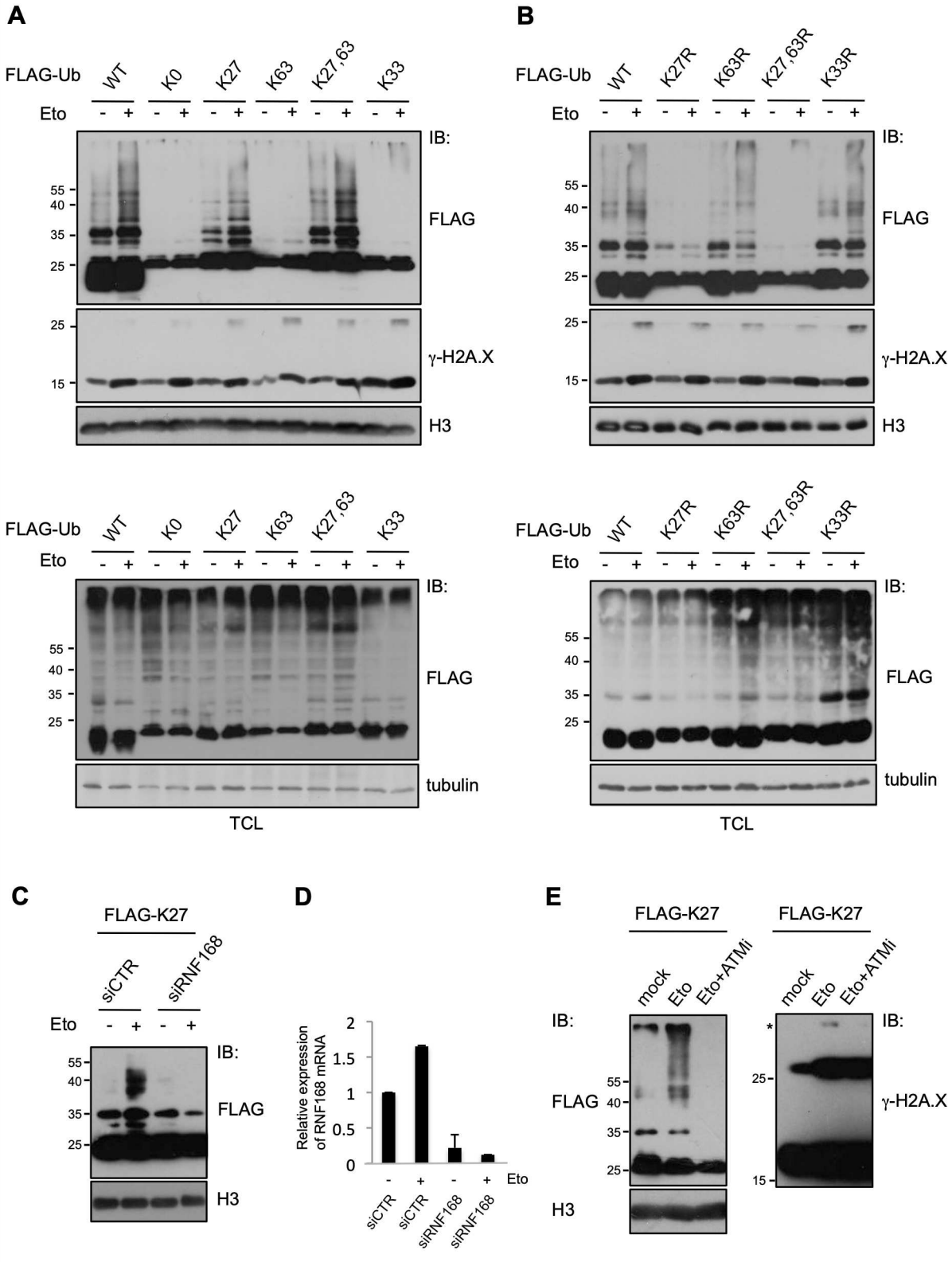

A
FLAG-Ub
WT
K0
K27
K63
K27,63
K33
Eto
IB:
FLAG
γ-H2A.X
H3
tubulin
TCL
B
FLAG-Ub
WT
K27R
K63R
K27,63R
K33R
C
FLAG-K27
siCTR
siRNF168
D
Relative expression of RNF168 mRNA
E
FLAG-K27
mock
Eto
Eto+ATMi

Figure 5

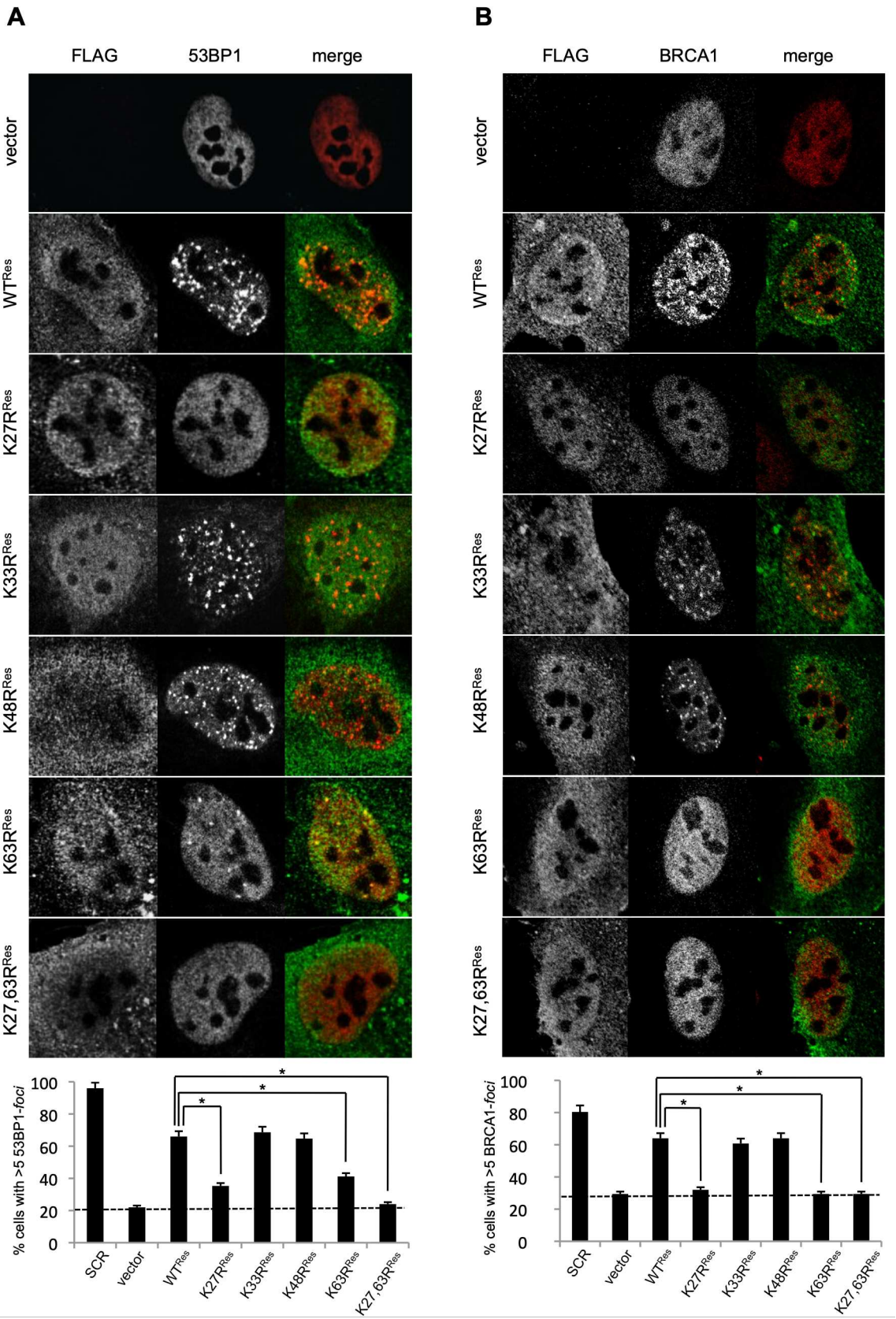

A
FLAG
53BP1
merge
vector
WTRes
K27RRes
K33RRes
K48RRes
K63RRes
K27,63RRes
% cells with >5 53BP1-foci
100
80
60
40
20
0
SCR
vector
WTRes
K27RRes
K33RRes
K48RRes
K63RRes
K27,63RRes
B
FLAG
BRCA1
merge
vector
WTRes
K27RRes
K33RRes
K48RRes
K63RRes
K27,63RRes
% cells with >5 BRCA1-foci
100
80
60
40
20
0
SCR
vector
WTRes
K27RRes
K33RRes
K48RRes
K63RRes
K27,63RRes

Figure 5

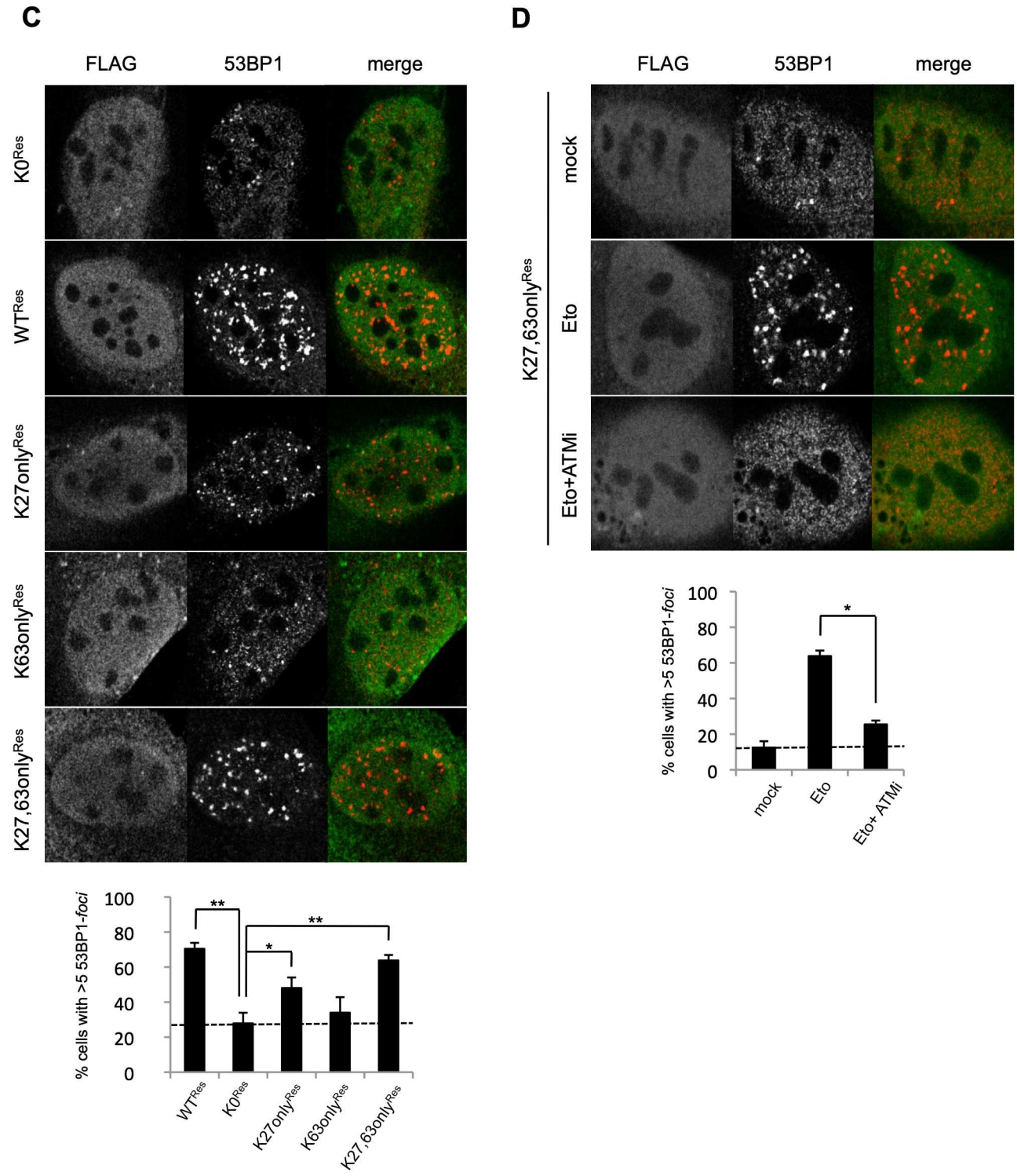
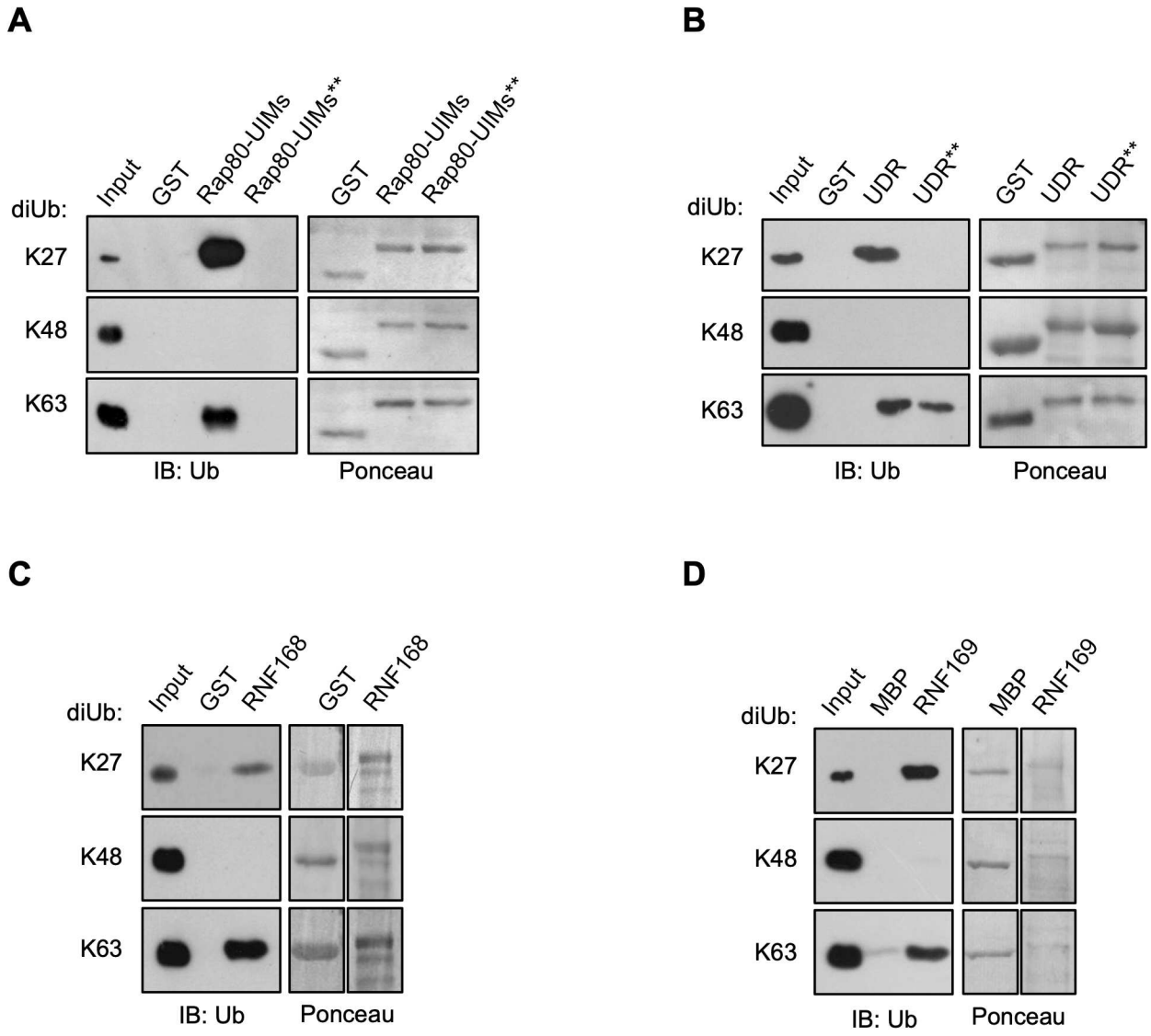

C
FLAG
53BP1
merge
K0Res
WTRes
K27onlyRes
K63onlyRes
K27,63onlyRes
% cells with >5 53BP1-foci
100
80
60
40
20
0
**
**
*
WTRes
K0Res
K27onlyRes
K63onlyRes
K27,63onlyRes
D
FLAG
53BP1
merge
K27,63onlyRes
mock
Eto
Eto+ATMi
% cells with >5 53BP1-foci
100
80
60
40
20
0
*
mock
Eto
Eto+ ATMi

Figure 6


A
Input
GST
Rap80-UIMs
Rap80-UIMs**
GST
Rap80-UIMs
Rap80-UIMs**
diUb:
K27
K48
K63
IB: Ub
Ponceau
B
Input
GST
UDR
UDR**
GST
UDR
UDR**
diUb:
K27
K48
K63
IB: Ub
Ponceau
C
Input
GST
RNF168
GST
RNF168
diUb:
K27
K48
K63
IB: Ub
Ponceau
D
Input
MBP
RNF169
MBP
RNF169
diUb:
K27
K48
K63
IB: Ub
Ponceau