Novartis Medal Lecture

Decoding the SUMO signal

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Abstract

SUMO (small ubiquitin-like modifier) emerged from the shadow of the well-established ubiquitin some 15 years ago when it was shown that a distinct conjugation pathway was responsible for SUMO modification. Since then it has been established that SUMO modifies over a thousand substrates and plays diverse roles in many important biological processes. Recognition of SUMO is mediated by short peptide sequences known as SIMs (SUMO-interaction motifs) that allow effector proteins to engage SUMO-modified substrates. Like ubiquitin, SUMO can form polymeric chains, and these chains can be recognized by proteins containing multiple SIMs. One protein that contains such a sequence of SIMs also contains a RING (really interesting new gene) domain that is the hallmark of a ubiquitin E3 ligase. This ubiquitin ligase known as RNF4 (RING finger protein 4) has the unique property that it can recognize SUMO-modified proteins and target them for ubiquitin-mediated proteolysis. Structural and biochemical analyses of RNF4 has shed light on the long sought after mechanism of ubiquitin transfer and illustrates how its RING domain primes the ubiquitin-loaded E2 for catalysis.

Key words: DNA damage, E3 ligase, really interesting new gene (RING), RING finger protein 4 (RNF4), small ubiquitin-like modifier (SUMO), ubiquitin.

Abbreviations used: APL, acute promyelocytic leukaemia; BRCA1, breast cancer early-onset 1; GFP, green fluorescent protein; HECT, homologous with E6-associated protein C-terminus; IκBα, inhibitor of nuclear factor κB α; MDC1, mediator of DNA-damage checkpoint 1; NF-κB, nuclear factor κB; PIAS, protein inhibitor of activated STAT (signal transducer and activator of transcription); PML, promyelocytic leukaemia protein; RanGAP1, Ran GTPase-activating protein 1; RARα, retinoic acid receptor α; RING, really interesting new gene; RNF, RING finger protein; SILAC, stable isotope labelling with amino acids in cell culture; siRNA, small interfering RNA; SP100, speckled protein of 100 kDa; SUMO, small ubiquitin-like modifier; SAE, SUMO-activating enzyme; SENP, SUMO-specific protease; STUbL, SUMO-targeted ubiquitin ligase; TAP, tandem affinity purification; Ubl, ubiquitin-like protein; YFP, yellow fluorescent protein.

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Introduction

Modification of proteins by covalent addition of ubiquitin is a widely utilized signal that alters the fate of the modified protein. The reversible nature of the modification provides flexibility and there are few biological processes that do not involve the conjugation or deconjugation of ubiquitin. One of the best-studied outcomes for ubiquitylated proteins is their degradation via the proteasome and drugs that inhibit this process are widely used to treat multiple myeloma.

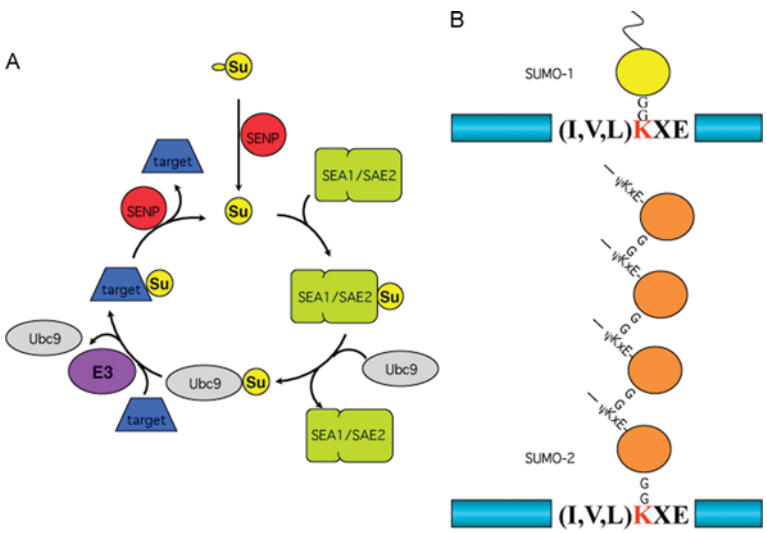
Ubiquitin conjugation is a multistep process involving three different enzymatic reactions. In the first step of the process a ubiquitin-activating enzyme (E1) utilizes ATP to adenylate the C-terminus of ubiquitin, before transferring the ubiquitin to a second active site where it forms a thioester bond between the carboxy group of ubiquitin and the thiol group of a cysteine residue in the E1 with AMP being released. The second step involves a ubiquitin-conjugating enzyme (E2) where the ubiquitin undergoes a transthiolation reaction with the carboxy group of the ubiquitin now attached to the active site cysteine residue of the E2. In humans, there are approximately 40 different E2 enzymes. The final step in which the substrate is selected is carried out by a large group of ubiquitin E3 ligases. On the basis of their mechanism of action, they fall into two different classes: RING (really interesting new gene) ligases and HECT (homologous with E6-associated protein C-terminus) ligases. HECT ligases contain an active-site cysteine residue and undergo a transthiolation reaction with the ubiquitin-loaded E2, resulting in the C-terminus of ubiquitin being linked to the active site cysteine residue of the HECT ligase. Substrate is then engaged by the E3 and nucleophilic attack of a lysine residue in the substrate on the E3 ubiquitin thioester results in the formation of an isopeptide bond between the ε-amino group of the lysine and the C-terminal carboxy group of ubiquitin. RING E3 ligases function by binding both substrate and ubiquitin-loaded E2 and facilitating ubiquitin transfer directly from the E2 on to substrate [1].

SUMO conjugation and deconjugation

By the end of 1996 and early 1997, it was becoming evident that ubiquitin was not alone and there were a number of other ubiquitin-like proteins that shared a similar core structure and could be covalently attached to other proteins.

Figure 1 | SUMO modification

(**A**) The SUMO cycle of conjugation and deconjugation. SUMO (Su, yellow) is processed by a SENP (red) before being activated and covalently linked to the SUMO E1-activating enzyme (SAE1–SAE2, green). SUMO is then transferred to the SUMO E2-conjugating enzyme (Ubc9, grey), which carries out target (blue) modification with the aid of a SUMO E3 ligase (E3, purple). Deconjugation to release free SUMO and target is mediated by a SENP. (**B**) Lysine acceptor residues subject to SUMO modification are usually found in the SUMO modification motif ΨKXE (where Ψ is isoleucine, valine or lysine). This motif is found in the N-terminal region of SUMO-2 and SUMO-3, but not in SUMO-1. Thus SUMO-2 (and SUMO-3) can form polymeric chains through conjugation at the ΨKXE motif.



The newly discovered SUMO (small ubiquitin-like modifier) had been shown to modify the nucleoporin RanGAP1 (Ran GTPase-activating protein 1) [2,3], but the pathway of modification had not been elucidated. At this time, my laboratory were investigating the signal-induced degradation of the IκBα [inhibitor of NF-κB (nuclear factor κB) α] and it had been demonstrated that this was mediated by the ubiquitin–proteasome system [4]. As the N-terminal region of IκBα had been shown to be required for signal-induced degradation, we carried out a yeast two-hybrid screen to identify those proteins that could specifically interact with this region. We were happy to find among the interactors the protein Ubc9 that had been suggested to be a ubiquitin-conjugating enzyme. Believing Ubc9 to be a ubiquitin-conjugating enzyme, we carried out a series of reactions with purified ubiquitin E1, ^{125}I-labelled ubiquitin and recombinant Ubc9, expecting to generate Ubc9 linked by a thioester bond to the labelled ubiquitin. This work was carried out by Joanna Desterro who was then a Ph.D. student, but, despite vigorous efforts, she was unable to identify the sought after Ubc9~ubiquitin conjugate. At this point, we turned to the newly discovered SUMO, but it soon became clear that SUMO could not be activated by the ubiquitin E1. We thus took HeLa cell extract and separated it into four fractions by ion-exchange chromatography. Using ^{125}I-labelled SUMO-1 and recombinant Ubc9, we tested each of the cellular fractions for the ability to catalyse the formation of a SUMO-1~Ubc9 thioester linkage. To our delight, we noted that one of the fractions could carry out this reaction. We had thus shown that Ubc9 was a SUMO-conjugating rather than a ubiquitin-conjugating enzyme and that it was likely that there was a distinct SUMO-activating enzyme present in the fraction from HeLa cells [5]. Using a genetic approach in yeast, the Blobel laboratory also noted that Smt3, the yeast homologue of SUMO, was conjugated to substrates by Ubc9 [6]. We followed this by purifying, cloning and characterizing the E1 enzyme involved in SUMO activation [7]. Whereas the ubiquitin E1-activating enzyme is encoded in a single polypeptide, the SAE (SUMO-activating enzyme) was shown to be a heterodimer composed of SAE1 and SAE2 subunits (Figure 1A). Given that we originally isolated Ubc9 as an IκBα interactor, this immediately suggested that IκBα might be a SUMO substrate. Both *in vivo* and *in vitro* studies suggested that this was indeed the case, and our studies led us to the conclusion that the acceptor lysine residue for SUMO modification was also the acceptor for ubiquitylation [8]. Whereas SUMO modification stabilized IκBα and blocked transcriptional activation by NF-κB, ubiquitylation of IκBα targeted the protein for proteasomal degradation and resulted in activation of NF-κB-dependent transcription. This paradigm of SUMO and ubiquitin modifying the same lysine residue with dramatically different consequences has been shown to be an important principle, allowing proteins to switch between different modes of activity altering biological processes ranging from DNA damage responses to disease pathology [9].

By analysing the site of modification in a number of proteins, we proposed a SUMO consensus modification site

consisting of the sequence ΨKXE, where Ψ represents a large hydrophobic amino acid and X represents any amino acid. We demonstrated further that this site constitutes a transferable signal that confers the ability to be modified with SUMO on proteins to which it is linked. The predominantly nuclear localization of both subunits of the SUMO E1, Ubc9 and SUMO suggested that SUMO modification was a nuclear process. We demonstrated that heterologous proteins carrying the SUMO consensus modification sequence are only conjugated to SUMO *in vivo* when an NLS (nuclear localization signal) was also present [10]. Using this information, we noticed that a site conforming to the ΨKXE consensus was present in the tumour suppressor p53 and we could show that p53 was SUMO-modified and that SUMO modification of p53 altered its transcriptional activity [11]. In the course of this analysis, we also identified a cluster of ubiquitylation sites required for proteasome-mediated degradation of p53 and, whereas the SUMO modification site was also a site of ubiquitylation, the redundancy in the ubiquitylation process meant that in this case SUMO modification did not interfere with ubiquitin-mediated proteolysis of p53 [12]. Work from my own and other laboratories indicted that proteins involved in transcriptional regulation were important targets for SUMO modification [13]. My collaborator Neil Perkins had demonstrated previously that the transcriptional regulators p300 and CREB (cAMP-response-element-binding protein) contained domains responsible for transcriptional repression. We demonstrated that these regions contained two copies of the SUMO consensus modification sequence. Mutations that reduced SUMO modification increased p300-mediated transcriptional activity and expression of a SUMO-specific protease or catalytically inactive Ubc9 relieved repression, demonstrating that p300 repression was mediated by SUMO conjugation. It was also demonstrated that SUMO-modified p300 recruited a histone deacetylase and that it was this histone deacetylase that was responsible for transcriptional repression [14]. It has been established in many systems that SUMO plays an important role in transcriptional repression.

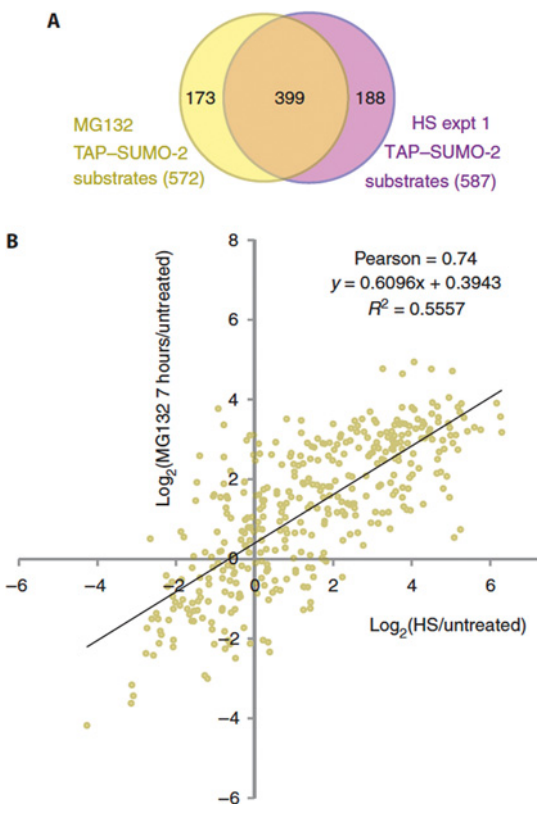
Whereas there is only a single isoform of the SUMO homologue Smt3 in lower eukaryotes such as yeast, chordates have three members of the SUMO family: SUMO-1, SUMO-2 and SUMO-3. Although SUMO-2 and SUMO-3 are 97% identical, they share only 50% sequence identity with SUMO-1 and appear to be functionally distinct. N-terminal to the ubiquitin fold domain, SUMO isoforms contain a long unstructured region. Within this region, both SUMO-2 and SUMO-3 contain a ΨKXE consensus modification site that is absent from SUMO-1. In an auto-modification reaction, SUMO-2 and -3 (Figure 1B) can use this site to form polySUMO-2 chains [15]. Although SUMO-1 cannot form SUMO polymers, it can be added to the end of a growing SUMO-2/3 chain and thus terminate chain growth [16].

Like ubiquitin and most other ubiquitin-like proteins, the primary translation product of the SUMO genes are precursor proteins that need to be proteolytically processed to expose the sequence required for conjugation. This reaction is carried out by SENPs (SUMO-specific proteases), some of which possess C-terminal hydrolase activity that can process SUMO precursors to the mature forms (Figure 1A). Precise cleavage after the second glycine residue in a Gly-Gly sequence is required to free the carboxy group that will be linked in an isopeptide bond with the ε-amino group of a lysine side chain in the target protein. After SUMO is conjugated to target proteins, it can be removed by the action of members of SENP family of proteases, that carry out both chain depolymerization and direct deconjugation of SUMO from the modified protein. This releases substrate and free SUMO that can re-enter the SUMO conjugation cycle. These proteases thus play an important role in determining the modification status of individual substrates and in the availability of free SUMO. In humans, there are six SENPs that are cysteine proteases and share a conserved catalytic core with a characteristic His-Asp-Cys catalytic triad [17]. More recently, additional proteases with specificity for SUMO have been identified that are unrelated to SENPs [18,19]. The human SENPs have been shown to be involved in diverse biological processes and fall into three distinct groups. SENP1 and SENP2 are rather similar proteases and appear to carry out SUMO processing, deconjugation of all SUMO paralogues from substrates and depolymerization of polySUMO chains. SENP3 and SENP5 are both nucleolar proteases that appear to be specific for deconjugation of SUMO-2/3, whereas the primary role of SENP6 and SENP7 appears to be in the depolymerization of polySUMO-2/3 chains [20,21].

Structural analysis of the catalytic core of the SENPs reveals that they have a common fold in which a five-stranded mixed β-sheet is apposed by two α-helices. This creates a central cleft in the protein with the active-site histidine and aspartate residues located on the β-sheet and the catalytic cysteine residue located on one of the α-helices. The dimensions of this cleft are such that it can accommodate the Gly-Gly motif of SUMO and would allow SUMO-modified substrates access to the active site as the depth of the cleft corresponds to the length of a lysine side chain. Determination of structures of covalent transition state complexes in which the C-terminal carboxy group of SUMO is linked to the thiol group of the active-site cysteine residue of the SENPs reveals how a conserved tryptophan residue of the SENP switches conformation to clamp down on the C-terminus of SUMO. Comparison of the SUMO-bound and -free forms of the SENP indicate that, whereas there are some local adjustments of structure in the active site, there are no large-scale changes in the protease as a consequence of SUMO binding. These studies indicated how the proteases recognized the SUMO component, but, as cleavage had already taken place, it was not clear how the proteases recognized intact substrates. To solve this problem, structures were determined of catalytically inactive versions of the protease domains of SENP1 and SENP2 bound to both SUMO precursors and SUMO-modified RanGAP1. In each case, the scissile peptide bond is kinked at a right angle to the C-terminal tail of SUMO and has the *cis* configuration of

Figure 2 | Changes in SUMO-2 conjugation as a consequence of proteasome inhibition correlate with those that occur after heat shock

(**A**) Protein identification overlap between this study (yellow) and a study of the change in TAP–SUMO-2 conjugation upon heat stress (HS, pink) [19]. (**B**) Scatter plot of all of the 399 proteins common to the two studies showing $\log_2$(heat-shock/untreated) (abscissa) and $\log_2$(7 h MG132-treated/untreated) (ordinate). Equations of linear regression, R^2 and Pearson correlations are shown. Note that 572 proteins were designated as 'putative substrates' for the MG132 experiment from this analysis rather than the previously reported 564 proteins (see Figure 3). This is due to the inclusion of the heat-shock data, which increased the total number of protein identifications and also increased the number of proteins from the MG132 experiment that met the 1% FDR (false discovery rate) setting during MaxQuant processing. Reproduced from Tatham, M.H., Matic, I., Mann, M. and Hay, R.T. (2011) Comparative proteomic analysis identifies a role for SUMO in protein quality control. Sci. Signaling **4**, rs4 with permission.



the amide nitrogens [22,23]. These structures were the first of any deubiquitinating enzyme or Ubl (ubiquitin-like protein) protease bound to its substrate and demonstrated how the scissile bond was manipulated into the correct orientation for the cleavage reaction.

SUMO substrates

SUMO targets were initially identified substrate-by-substrate on the basis of possessing SUMO consensus modification sequences. Initial proteomic studies indicated paralogue-specific modification, but also demonstrated that many substrates could be modified by SUMO-1 and SUMO-2/3 [24]. This is not surprising as mice deficient for SUMO-1 expression are viable, indicating that the essential functions of SUMO-1 can be carried out by SUMO-2/3 [25,26]. To obtain a more comprehensive coverage of SUMO substrates, we generated cell lines expressing close to endogenous levels of TAP (tandem affinity purification)-tagged versions of SUMO that allowed stringent purification of SUMO substrates. Coupling the use of these cell lines with SILAC (stable isotope labelling with amino acids in cell culture) allowed us to use quantitative filtering approaches as stringent criteria for the identification of SUMO substrates and to evaluate system-wide changes to SUMO modification in response to cellular stress. Gene ontology analysis of the data revealed that the almost 800 SUMO substrates are mainly nuclear and have roles relating to the packing, manipulation and expression of DNA. SUMO substrates are overrepresented among proteins involved in DNA replication, DNA repair, transcription, mRNA splicing, mRNA processing and chromatin remodelling [27]. It had been demonstrated previously that SUMO-2/3, but not SUMO-1, conjugation was increased dramatically when cells were exposed to hyperthermic stress [28]. We demonstrated that SUMO-2/3 expression was required for cells to survive a modest heat shock, and our quantitative proteomic analysis indicated that the SUMO proteome underwent a global change in response to heat stress. SUMO-2/3 was polymerized into polySUMO chains and redistributed between many of the protein groups indicated above and in addition appeared to modify proteins involved in protein folding and degradation [27]. Whereas modification by ubiquitin and SUMO-2/3 are essentially independent mechanisms, it is clear that under conditions of proteasome inhibition both modifications are increased dramatically [29]. To establish the link between SUMO and ubiquitin-mediated proteasomal degradation, we carried out a system-wide proteomic analysis of SUMO-2-modified proteins after proteasomal inhibition with MG132. SILAC-based quantitative analysis of the SUMO-2 modification status of proteins modified in response to proteasome inhibition and in response to heat stress (Figure 2) were indicative of both qualitative and quantitative similarities between the two stresses [30]. However, an important distinction between heat stress and proteasomal inhibition is that the MG132-induced increase in SUMO-2 conjugation depended strictly on protein synthesis, whereas the increase in SUMO conjugation triggered by heat stress was independent of protein synthesis. These findings indicate that the accumulation of *de novo* synthesized incorrectly folded proteins, which would otherwise be degraded by the proteasome, initiate the SUMO-conjugation response. In addition, it was apparent that proteasomal inhibition led to the accumulation of SUMO-1 and SUMO-2/3 conjugates in insoluble protein inclusions and in the accumulation of hybrid chains of Lys^{63}-linked polyubiquitin linked to SUMO-2. As Lys^{63}-linked ubiquitin chains are not thought to serve as signals for proteasome-mediated degradation,

these findings suggest novel proteasome-independent functions of SUMOs in the response of cells to the build-up of misfolded proteins [30].

SUMO recognition

Once SUMO is conjugated to targets, the SUMO signal is decoded by effector proteins that are recruited to SUMO. Recognition of the SUMO signal is accomplished by proteins that contain SIMs. SIMs are hydrophobic in nature and typically contain a core sequence of (V/I/L)X(V/I/L)(V/I/L) [31,32]. This hydrophobic cluster is accommodated in either orientation as a β-strand that inserts into a hydrophobic groove between an α-helix and a β-strand in SUMO [33]. In a number of proteins, including components of PML (promyelocytic leukaemia protein) (also known as TRIM19) nuclear bodies, the nuclear antigen SP100 (speckled protein of 100 kDa) or the E3 SUMO ligase PIAS [protein inhibitor of activated STAT (signal transducer and activator of transcription)] 1, it appears that a stretch of negatively charged amino acids flanking the hydrophobic core of the SIM enhances their affinity for SUMO [31].

Although it was initially thought that SUMO and ubiquitin modification had distinct functional consequences, it appears that the two pathways are connected by STUbLs (SUMO-targeted ubiquitin ligases) that contain both SIMs and RING domains. Whereas the SIMs allow recognition of SUMO-modified proteins, the RING domain is the hallmark of a ubiquitin E3 ligase and thus has the unique ability to catalyse ubiquitylation of SUMO-modified proteins. Originally identified in yeast (Slx5–Slx8 in *Saccharomyces cerevisiae* and Rfp1/Rfp2–Slx8 in *Schizosaccharomyces pombe*), where they function as heterodimers, the human homologue RNF (RING finger protein) 4 functions as a homodimer [34]. Whereas yeast STUbLs contain one or two SIMs, mammalian RNF4 contains four SIMs that are located in the N-terminal region. The occurrence of multiple SIMs in RNF4 indicated that RNF4 would have the capacity to engage multiple SUMO molecules. This prediction was borne out and it was shown that RNF4 bound with low affinity to monomeric and dimeric SUMO-2, but bound strongly to SUMO-2 chains. The preferential binding of RNF4 to SUMO chains is manifested in its ability to efficiently utilize SUMO chains as a substrate for RING- and SIM-dependent ubiquitylation *in vitro* [35]. The product of such a reaction is a hybrid composed of both SUMO and ubiquitin chains. Evidence for the existence of these species came from the detection by MS of SUMO peptides linked directly to ubiquitin [30,35]. Although we first reported in 2001 [15] that SUMO, like ubiquitin, could form polymeric chains, it was only with the discovery of RNF4 and its multiple SIMs that a clear function for SUMO chains emerged. Human cells, in which RNF4 expression was ablated by siRNA (small interfering RNA), accumulated high-molecular-mass forms of SUMO, suggesting that RNF4 is involved in the degradation of proteins modified with a SUMO chain *in vivo*. As high-molecular-mass SUMO conjugates also increase in abundance in the presence of 26S proteasome inhibitors [29], this suggested that SUMO modification plays a role in targeting proteins for ubiquitin-mediated proteolysis.

PML is a target for the SUMO-targeted ubiquitin ligase RNF4

PML is one of a large group of tripartite motif-containing proteins [36]. The tripartite motif is composed of a RING domain, one or two zinc-binding B-boxes and a coiled-coil domain. The original identification of PML was in APL (acute promyelocytic leukaemia), where it is fused to RARα (retinoic acid receptor α) [37,38]. Fusion of PML to RARα creates an oncoprotein that causes leukaemia as it blocks differentiation of haemopoietic progenitor cells [39]. Normally, PML is localized in a limited number of large nuclear bodies, but in cells containing PML–RARα, both PML and the oncogenic fusion protein are dispersed into many small nuclear bodies [40]. When RNF4 was depleted, the levels of PML increased and it accumulated with SUMO-1 and SUMO-2/3 in PML nuclear bodies, thus indicating that RNF4 plays a role in regulating the integrity of these nuclear structures [35,41].

APL can be treated by the administration of arsenic trioxide, which induces degradation of PML and PML–RARα [39]. Arsenic-mediated proteasomal degradation of PML and PML–RARα is preceded by the modification of PML by SUMO and the localization of SUMO-1 and SUMO-2/3 to PML nuclear bodies. In cells lacking RNF4, SUMO-1 or SUMO-2/3, arsenic administration is unable to mediate degradation of PML [35,41]. To study the arsenic-induced degradation of PML in more detail, we generated human cell lines expressing close to endogenous levels of YFP (yellow fluorescent protein)–PML [42]. This allowed real-time monitoring of PML degradation and demonstrated that, in response to arsenic, PML was rapidly recruited into PML nuclear bodies before undergoing ubiquitin-mediated degradation. In the absence of RNF4, PML responded to arsenic treatment by rapidly translocating into PML nuclear bodies, but did not undergo ubiquitin-mediated proteolysis and thus continued to accumulate in PML nuclear bodies (Figures 3A and 3B) in forms that displayed multiple modifications with SUMO, but not ubiquitin (Figure 3C). To analyse the post-translational modifications that accumulated on PML in response to arsenic, YFP–PML was isolated by immunoprecipitation with an anti-GFP (green fluorescent protein) antibody and the isolated material subjected to Western blotting with antibodies against PML, SUMO-1, SUMO-2/3 and ubiquitin. This revealed that, in response to arsenic, PML was rapidly modified with SUMO-1, SUMO-2/3 and ubiquitin (Figure 3D). PML has three sites of SUMO modification (Lys^{65}, Lys^{160} and Lys^{490}), that are required for formation of mature PML nuclear bodies and for recruitment of additional PML nuclear body components such as DAXX (death domain-associated protein) and SP100 [34]. Modification of PML by SUMO on Lys^{160} is critical

Figure 3 | Effect of RNF4 on arsenic-induced PML degradation in real time

(**A**) Time-lapse experiments were performed on HeLa YFP–PML stable cells transfected with a non-target siRNA (siNT) or an siRNA against RNF4 (siRNF4) and exposed to 1 mM arsenic for 18 h. YFP–PML was imaged in real time by fluorescence microscopy over 15 h by collecting a stack of 20 sections with the YFP channel (green) and one image with the DIC (differential interference contrast) every 15 min. The projected *z*-sections collected in the YFP channel were merged to the respective DIC image to monitor the position of PML nuclear body within the cells. Scale bar, 5 μm. (**B**) Fluorescence intensity of PML bodies was quantified by defining a region of interest containing one PML body and comparing it with a region in the nucleoplasm. Relative fluorescence intensity represents the difference of intensities between these two regions. Results are mean values from at least ten cells. (**C**) Whole-cell extracts from YFP–PML HeLa cells transfected with siRNA to RNF4 or a non-target siRNA were analysed by SDS/PAGE followed by Western blotting with a chicken anti-PML antibody to show the accumulation of PML in the absence of RNF4. Depletion of RNF4 was controlled with a rabbit anti-RNF4 antibody. (**D**) Nuclear extracts from YFP–PML HeLa cells either untreated or exposed to 1 μM arsenic for 1 h were incubated with GFP-trap beads and bound proteins were collected. Proteins were eluted from the beads and analysed by SDS/PAGE followed by Western blotting with antibodies against PML, GFP, SUMO-1, SUMO-2 and ubiquitin (Ub) to evaluate proteins bound to YFP–PML. The input represents 10% of the nuclear lysate. Reproduced from Geoffroy, M.C., Jaffray, E.G., Walker, K.J. and Hay, R.T. (2010) Arsenic-induced SUMO-dependent recruitment of RNF4 into PML nuclear bodies. Molecular Biology of the Cell **21**, 4227–4239 with permission.

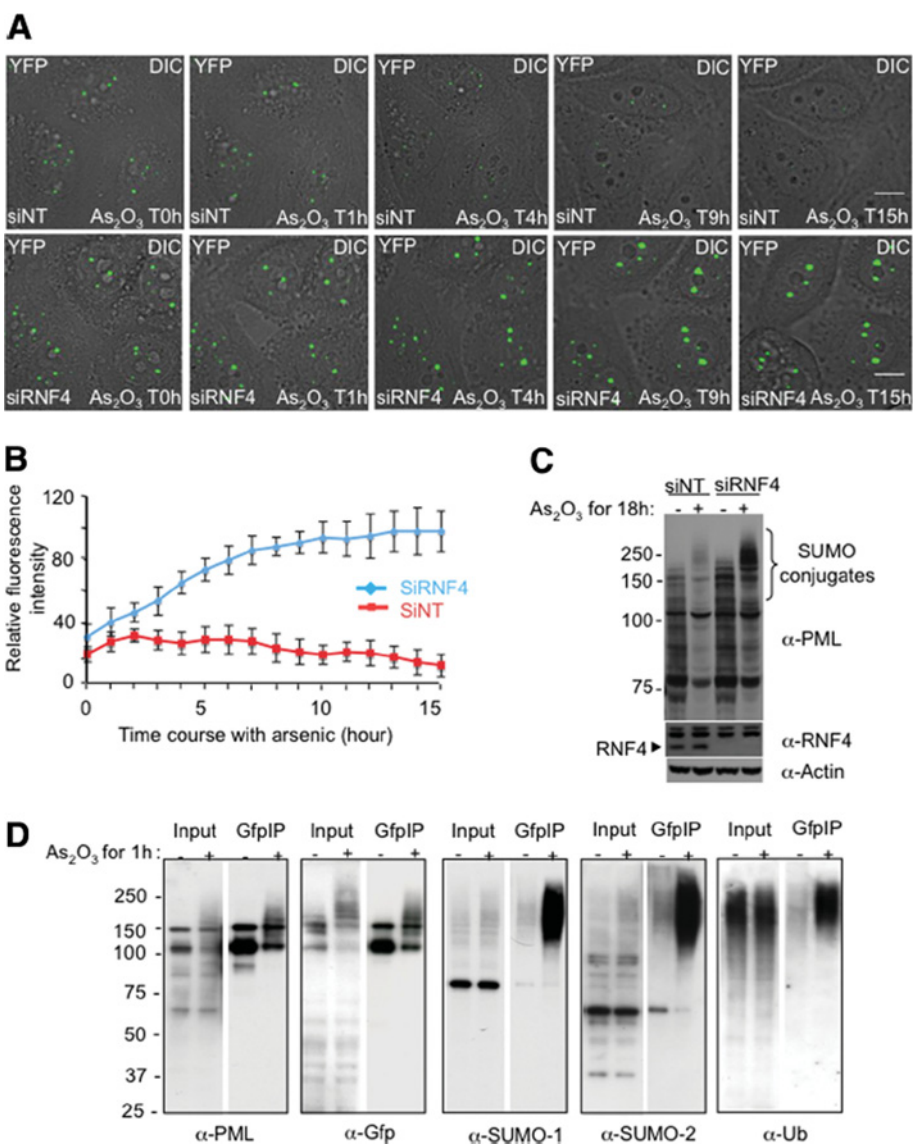
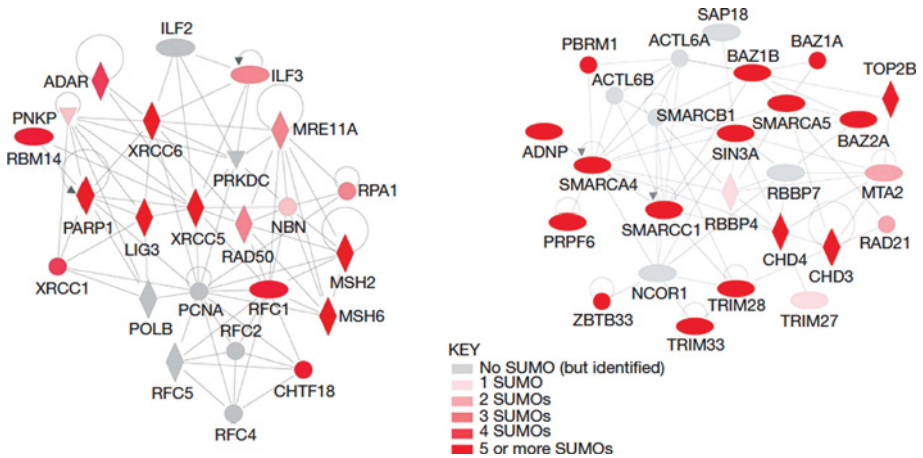


Figure 4 | Network analysis of proteins involved in DNA repair and checkpoint control

Labels are gene names (see http://www.genenames.org/ for definitions); node shapes indicate protein function: rhombus, enzyme; ellipse, transcriptional regulator; triangle, kinase; circle, other function. Lines indicate direct interactions. Nodes are coloured according to SUMOylation stoichiometry. Networks are created using 'Ingenuity pathways analysis' (http://www.ingenuity.com).



for the degradation of PML by arsenic, as mutation of this amino acid or depletion of SUMO-2/3 block arsenic-induced degradation of PML [41,43]. *In vitro*, RNF4 only catalyses ubiquitylation of PML that has undergone conjugation with SUMO-2/3 [35]. This lends support to the idea that modification with SUMO chains can trigger ubiquitylation of SUMO-modified proteins *in vivo*. Analysis of *in vitro* ubiquitylation of SUMO-modified PML by MS revealed that most of the ubiquitin was targeted to SUMO, although small amounts were found to be associated with PML. These studies identified PML as the first substrate in the pathway of SUMO-dependent ubiquitylation and proteasomal degradation (Figure 3). The identification of RNF4 as the E3 ligase responsible for the SUMO-dependent degradation of PML provides the molecular basis for the therapeutic action of a drug currently used to treat leukaemia [35].

Role of RNF4 in the DNA damage response

The DNA damage response utilizes a variety of post-translational modifications as molecular switches to co-ordinate the signalling network [44]. Although phosphorylation is clearly an important regulatory mechanism, ubiquitin and SUMO also appear to have a pervasive role in this response [27,45], although precise mechanisms of action have yet to be determined. SUMO modification of DNA damage proteins (Figure 4), driven by the PIAS1 and PIAS4 SUMO E3 ligases, is an important feature of the response to DNA damage in mammalian cells, and all three forms of SUMO (1, 2 and 3) are recruited to damaged DNA [46,47]. However, it was not clear how these signals were recognized by the effectors that bring enzymatic activities to the sites of SUMO modification. The STUbLs are highly conserved [48], and it has been shown in yeast that STUbLs have a key function in maintaining genome stability [49–52]. Recently, we, and others, have demonstrated that RNF4 plays an important role in the response of mammalian cells to DNA damage [53–56]. Cells in which RNF4 expression has been ablated are hypersensitive to DNA damage that requires homologous recombination for its repair, whereas mice deficient in RNF4 display persistent ionizing radiation-induced DNA damage. Furthermore RNF4-deficient mice show an age-dependent defect in spermatogenesis which is similar to that observed with many other mutations that affect homologous recombination [55]. RNF4 recruitment to sites of DNA damage requires its SIMs and RING domain, and proteins such as NBS1 (Nijmegen breakage syndrome 1), MDC1 (mediator of DNA-damage checkpoint 1), RNF8, 53BP1 (p53-binding protein 1) and BRCA1 (breast cancer early-onset 1) recognize the DNA damage and generate signals that in turn recruit further effectors. The recruitment of these factors to sites of DNA damage was not diminished in the absence of RNF4, but their removal from DNA-damage-induced foci was delayed. By employing proteomics to analyse SUMO substrates whose modification status changed after DNA damage, we identified MDC1 as a protein that displayed increased SUMO modification in response to ionizing radiation. It appeared that DNA-damage-induced SUMO modification of MDC1 served as a platform to load RNF4 on to sites of DNA damage where it could then ubiquitylate multiple substrates. Cells deficient in RNF4 displayed defective loading of Rad51 on to single-stranded DNA. This appeared to be a consequence of reduced recruitment of the single-strand-specific DNA-binding protein RPA (replication protein A) and the CtIP [CtBP (C-terminal-binding protein)-interacting protein] nuclease complex, with consequent inefficient

end-resection [56]. As the action of RNF4 generates a hybrid chain of SUMO and ubiquitin, this could serve as unique signal for the recruitment of DNA damage factors. Thus it was demonstrated recently that a hybrid SUMO–ubiquitin chain could preferentially bind RAP80 (receptor-associated protein 80) and thus bring BRCA1 to sites of DNA damage [57]. These studies indicate that RNF4 plays an important role in homologous recombination during the repair of damaged DNA.

Insights into the mechanism of ubiquitin transfer

RNF4 is a member of the RING domain-containing family of proteins. In human cells, there are thought to be more than 600 RING-type ubiquitin E3 ligases that influence almost all aspects of biological activity by targeting substrates for ubiquitylation. These ubiquitin ligases are modular proteins typically containing a substrate-binding domain and a RING domain that is the catalytic engine of ubiquitylation. This is exemplified by RNF4 where its N-terminal domain, containing the multiple SIMs, binds to its polySUMO substrate, whereas the RING domain is responsible for ubiquitin transfer. Although we have known that RING proteins were ubiquitin ligases since the 1990s, the mechanism by which the RING catalyses transfer of ubiquitin to substrate has remained elusive. As RNF4 is a relatively small protein (<200 amino acids) that is expressed well in bacteria, we felt that it would be an excellent model to investigate the structural aspects of RING-mediated ubiquitylation. Our initial attempts to obtain crystals of full-length RNF4 for structural analysis were unsuccessful as the N-terminal region containing the SIMs is largely unstructured in solution. Although the isolated C-terminal RING domain was inactive in substrate ubiquitylation, it was fully active in an autoubiquitylation reaction, indicating that the C-terminal RING domain alone possessed ubiquitin E3 ligase activity. Thus, as a first step in determining the mechanism of RNF4-mediated ubiquitin transfer, we determined the crystal structure of the RNF4 RING domain at 1.5 Å (1 Å = 0.1 nm) resolution. The RNF4 RING domain has the typical RING fold that has at its core two Zn^{2+} ions ligated by seven cysteine residues and one histidine residue. It was immediately obvious from the structure that the RNF4 RING domain was dimeric. The dimer interface buries 518 Å^2 of surface-accessible area of a monomer and is formed predominantly by residues from the three β-strands and the very C-terminus of RNF4. Gel-filtration analysis confirmed that both the isolated RING domain and full-length RNF4 were dimeric in solution. Mutation of residues at the dimer interface disrupted dimerization and abolished ubiquitin ligase activity. Experiments carried out with fluorescently labelled versions of RNF4 using FRET (fluorescence resonance energy transfer) revealed that individual RNF4 molecules appear to be in rapid equilibration between dimeric and monomeric states. Dimerization is required for ubiquitin E3 ligase activity of RNF4 and other dimeric RING domain E3s [58–62]. However, as monomeric RNF4 can bind both substrate and E2, this raises the question: why is dimerization essential? By mixing together inactive RNF4 dimers, one that was unable to bind substrate with one that was unable to interact with E2, it was possible to create a heterodimer with full ubiquitin ligase activity. Thus ubiquitylation could proceed *in trans* across the dimer, but it was clear that this was not an obligate requirement as ubiquitin could also be transferred to substrate from an E2 bound to the same subunit. Thus as RNF4-depenedent ubiquitylation could take place either *in cis* or *in trans*, it follows that dimerization, although necessary for ubiquitylation activity, allows flexibility in the relative orientation of substrate- and E2-binding sites. Direct interaction analysis indicated that dimeric RNF4 preferentially bound ubiquitin-loaded UbcH5a over free UbcH5a. RNF4 mutations that rendered the protein monomeric did not show this preferential binding to ubiquitin-loaded UbcH5a. In addition, incubation of RNF4 with ubiquitin-loaded UbcH5a resulted in the hydrolysis of the UbcH5a∼ubiquitin bond and likewise this activity required RNF4 dimerization. These data therefore demonstrate that RING-containing ubiquitin E3 ligases do not simply act as an inert scaffold that juxtaposes substrate and E2-loaded ubiquitin. Rather, they directly facilitate catalysis by preferentially interacting with the E2∼ubiquitin and activating the thioester bond for catalysis. Once ubiquitin has been transferred to the substrate, the weak binding of free E2 would favour its dissociation and facilitate rapid binding of ubiquitin-loaded E2, necessary for the processive synthesis of ubiquitin chains [61].

Although it was evident from these studies that the dimeric RING of RNF4 had to bind the ubiquitin-loaded E2 across the dimer, the precise mechanism by which the RING domain catalyses transfer of ubiquitin to substrate remained elusive. This was primarily because it had not been feasible to obtain a high-resolution structure of the key intermediate: the complex of E2∼ubiquitin bound to the RING. Unfortunately, structural analysis was confounded by the high reactivity of the thioester linkage in the presence of the RING domain and even the less reactive E2∼ubiquitin oxyester was rapidly hydrolysed in a RING-dependent fashion. To address this problem, Anna Plechanovova replaced the active-site cysteine residue of the E2 with lysine, such that transfer of ubiquitin from the E1 to the E2 generated an isopeptide bond between the C-terminus of ubiquitin and the side chain of the substituted lysine. This resulted in a stable intermediate suitable for structural studies that acted as an excellent mimic of the thioester-linked E2∼ubiquitin. We were thus able to determine the 2.2 Å crystal structure of the dimeric RING of RNF4 bound to ubiquitin-loaded E2 [63]. The complex contains the central RNF4 RING dimer, two UbcH5a molecules and two ubiquitin molecules related by a two-fold axis (Figure 5). Each UbcH5a molecule contacts a single RING domain and is linked by an isopeptide bond to ubiquitin located at the

Figure 5 | Structure of the RNF4 RING bound to ubiquitin-loaded UbcH5A

Surface representation of the complex. Individual RING protomers are coloured cyan and blue, UbcH5A is green, ubiquitin is orange and the isopeptide linkage between the C-terminus of ubiquitin and Lys[85] of UbcH5A is shown in yellow. Reproduced from Plechanovova, A., Jaffray, E.G., Tatham, M.H., Naismith, J.H. and Hay, R.T. (2012) Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. Nature **489**, 115–120 with permission.

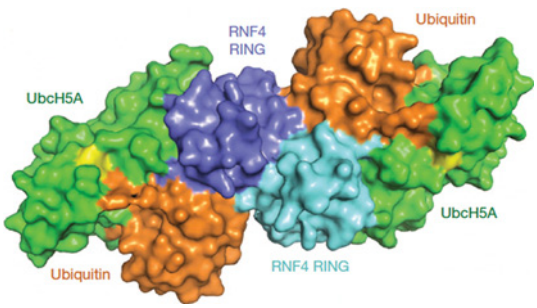
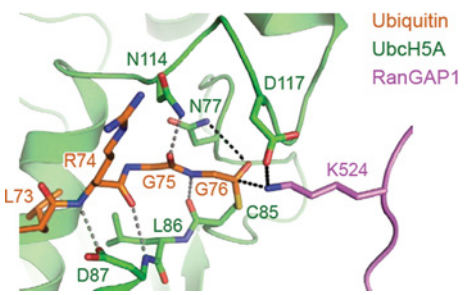


Figure 6 | Model for a RING–substrate–UbcH5A–ubiquitin thioester complex

The SUMO–RanGAP1–Ubc9–RanBP2 (Ran-binding protein 2) structure [70] provides a model for how a substrate might interact with the RING-activated UbcH5A–ubiquitin thioester. In our model of a hypothetical substrate approaching the activated thioester bond, Asp[117] in UbcH5A positions and activates the incoming substrate lysine residue for a nucleophilic attack. The resulting tetrahedral intermediate would be stabilized by a hydrogen bond with the side chain of Asn[77]. An intricate network of hydrogen-bonding interactions (grey dashes) between the C-terminal tail of ubiquitin and the active-site groove of UbcH5A lock the thioester bond in the activated configuration. UbcH5A is green, ubiquitin is orange, and substrate is violet. Reproduced from Plechanovova, A., Jaffray, E.G., Tatham, M.H., Naismith, J.H. and Hay, R.T. (2012) Structure of a RING E3 ligase and ubiquitin-loaded E2 primed for catalysis. Nature **489**, 115–120 with permission.



RING dimer interface. Notably, ubiquitin is folded back on to E2, with the Ile[44] hydrophobic patch of ubiquitin bound to the $\alpha 2$ helix of the E2. The six C-terminal residues of ubiquitin are engaged in a network of contacts with loops surrounding the active site of UbcH5a, whereas the side chain of Asn[77] in UbcH5a forms hydrogen bonds to the isopeptide carbonyl. The importance of the E2 residues surrounding the active site and lining the shallow groove that accommodates the C-terminal region of the linked ubiquitin is reflected in their high degree of conservation and by the loss of ubiquitin transfer activity when they are mutated. Each molecule of UbcH5a interacts with one protomer of the RING while both protomers of the dimeric RING are engaged by ubiquitin. In this arrangement, the ubiquitin reaches across the dimer and is locked into place by interactions both with the RING protomer to which the E2 is attached and with the opposite RING protomer. These observations explain why dimerization of the RNF4 RING is required for activity [58,61].

On the basis of the isopeptide-linked E2~ubiquitin in our structure it was possible to generate a model of the E2~ubiquitin thioester, the relevance of which is supported by mutational analysis and by the activity of the isopeptide-linked E2~ubiquitin as a competitive inhibitor of ubiquitylation. In isolation, the ubiquitin thioester-linked to the E2 can explore many different conformations and can also adopt the active 'folded-back' conformation [64–66]. Thus we envisage that E3-mediated activation would be initiated by interactions between E2 and the RING. With the E2 bound to one RING protomer, thioester-linked ubiquitin would 'fold back' on to the $\alpha 2$ helix of UbcH5a and be held in position by Tyr[193] of the other RING protomer. As a consequence, the C-terminus of ubiquitin is extended and locked into the active-site groove of the E2. This would manipulate the ubiquitin Gly[76] thioester carbonyl into an optimal arrangement for nucleophilic attack by the incoming substrate lysine residue that would form a tetrahedral intermediate on the Gly[76] carbonyl carbon. In this conformation, the transition-state oxyanion could be stabilized by a hydrogen bond to Asn[77] [63], although an alternative, but not necessarily mutually exclusive, role for Asn[77] in stabilizing the active-site loop has been proposed [67]. Asp[117] in UbcH5a is reoriented by ubiquitin binding to hold a position poised above the thioester, and mutational analysis indicates that it is not involved in RING-induced activation of the thioester bond, but plays a key role in positioning and deprotonating the incoming lysine nucleophile of the substrate (Figure 6).

Although this and other analyses [63,68,69] indicate how the structurally simple E3 ligases may function, it is reasonable to conclude that the principles of E2~ubiquitin activation revealed with RNF4 will apply to the complex multisubunit ubiquitin ligases such as the SCF (Skp/cullin/F-box) ligases and the APC/C (anaphase-promoting complex/cyclosome) that also function by RING-mediated catalysis. It is also likely that E3 ligases acting on other Ubls will employ a related catalytic strategy [70]. Thus the unifying concept is that the E3 ligase activates the E2~ubiquitin/Ubl thioester by locking the ubiquitin/Ubl in the 'folded-back' orientation and extending its C-terminal tail. The analogy is of tensioning a spring that is released by cleavage of the thioester and formation of the isopeptide bond. Although the precise molecular contacts that hold the ubiquitin/Ubl in the folded-back orientation may differ, it is the positioning of the C-terminal tail of the ubiquitin/Ubl

in the active-site groove of the E2 that is central to the mechanism. This gives a framework for understanding how E3 ligases activate the ubiquitin-loaded E2, but, as yet, we do not have a clear view of how the substrate is engaged and presented to the activated E2. Clearly, this is going to involve many further years of biochemical and structural analyses to provide a detailed understanding of how ubiquitin/Ubls are transferred to the substrate.

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