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Crosstalk Between the SUMO and Ubiquitin Pathways

T. Hunter(✉), H. Sun

Molecular and Cell Biology Laboratory, The Salk Institute,
10010 North Torrey Pines Road, 920137-1099 La Jolla, USA
email: *hunter@salk.edu*

Abstract. Several ways in which the SUMO and ubiquitin pathways can intersect and communicate have recently been discovered. This review discusses the principles of crosstalk between SUMOylation and ubiquitination, focusing on the RNF4 family of RING finger E3 ubiquitin ligases, which specifically recognize SUMOylated proteins via their SUMO moiety for ubiquitination.

1 The SUMO Pathway

Post-translational modification adds layers of complexity to the control of protein function (Hunter 2007). Covalent modification of proteins by ubiquitin (Ub) and ubiquitin-like proteins, such as the small ubiquitin-like modifier (SUMO), is an increasingly important post-translational modification. Ub and SUMO are covalently linked through their C-terminal COOH group to an ϵ-amine group of a lysine (Lys) in the modified protein. Higher eukaryotes express four SUMO family members, SUMO1–4, encoded by different genes, with SUMO1 and SUMO2–3/4 forming two distinct groups. Newly synthesized SUMO protein is matured through proteolytic cleavage of a C-terminal peptide to expose a di-Gly motif essential for conjugation. SUMOylation occurs via a biochemical pathway analogous to ubiquitination: mature SUMO is charged via a high-energy thioester bond by the SUMO-activating enzyme (E1), a heterodimeric protein complex, and transferred to the SUMO-conjugating enzyme (E2), which catalyzes the formation of an isopeptide bond between SUMO and the target protein. SUMO ligases (E3s) provide a platform facilitating the conjugation of SUMO from E2 to the target (Gill 2004; Johnson 2004), but in contrast to the greater than 500 E3 Ub ligases in mammals fewer than ten E3 SUMO ligases are known.

In contrast to ubiquitination, where selection of the target Lys in a protein appears to be independent of primary sequence, a significant number (but not all) SUMOylation sites have the consensus sequence ΦKxE/D, where Φ is a hydrophobic residue preceding the SUMOylation acceptor Lys. This reflects a certain degree of specificity in substrate recognition by the SUMO-conjugating enzyme, Ubc9 (Bernier-Villamor et al. 2002). As with ubiquitination, both mono- and poly-SUMOylation can occur, although mono-SUMOylation is generally the rule. In addition, the branch point in poly-SUMO chains is mainly through a Lys in the N-terminal extension, in contrast to polyubiquitination, where any one of seven Lys can be used for branching and different branch points instruct different fates for the polyubiquitinated protein. While mono-SUMOylation is linked to functional modification of the target protein, the physiological significance of poly-SUMOylation is only just emerging (Hay 2005; Tatham et al. 2008). In budding yeast, SUMO chains are formed but are not essential for cell growth (Bylebyl et al. 2003); the same appears to be true in fission yeast (Prudden et al. 2007). In mammals, SUMO chains can be formed with SUMO2 and SUMO3, but not SUMO1. SUMO2 (and the closely related SUMO3 and 4) has a distinct consensus SUMOylation site ($_{10}$VKTE$_{13}$) at its N-terminus; SUMO1 lacks this motif, but in principle it may be added to a preformed SUMO2 chain (Tatham et al. 2001). SUMO itself also has the potential of being a ubiquitination target (Tatham et al. 2008); all SUMOs contain multiple Lys and may prime the formation of ubiquitin chains.

SUMO is essential for normal growth, division, and the maintenance of genome stability in eukaryotic cells. Many SUMOylated proteins are found either in the nucleus (e.g., in nuclear bodies) or at the nuclear periphery, implying an important role for SUMOylation in biological processes in the nucleus (Johnson 2004). Mutations in SUMO conjugation pathway enzymes result in sensitivity to genotoxic challenges. In fission yeast, mutations in *rad31*$^{+}$, encoding a SUMO E1 subunit, and *hus5*$^{+}$, encoding the SUMO E2 (Ubc9 ortholog), render cells sensitive to DNA damage (al-Khodairy et al. 1995; Shayeghi et al. 1997). Deletion of *pmt3*$^{+}$, the only SUMO gene in *Schizosaccharomyces pombe*, results in slow growth, sensitivity to disruption of mitosis, and increased telomere length (Tanaka et al. 1999). Pli1, the *S. pombe* ortholog of PIAS family SUMO E3s, is essential for the stability of centromeres and telomeres (Xhemalce et al. 2004). Nse2/Mms21, a component of the Smc5/6 complex, is also a functional SUMO E3 (Andrews et al. 2005; McDonald et al. 2003; Zhao and Blobel 2005).

Like many other forms of post-translational modification, such as phosphorylation, SUMOylation can tag the modified protein for novel protein–protein interactions. Therefore, SUMO-interacting domains would be expected to play a crucial role in regulating the function of SUMOylated proteins, and the identification of proteins with SUMO-interacting motifs is important for an understanding of the SUMOylation system (Hannich et al. 2005; Hecker et al. 2006). In contrast to the multiple characterized Ub-binding domains, so far only one SUMO-interacting motif (SIM, or SUMO-binding motif, SBM) is known (Minty et al. 2000; Reverter and Lima 2005; Song et al. 2004). The core of the SIM is composed of three hydrophobic aliphatic (I, L, or V) residues, arranged as V/I-V/I-X-V/I/L or V/I-X-V/I-V/I. Structural analysis shows that when a SIM and SUMO interact, these residues form a β-strand and are incorporated into a β-sheet together with SUMO's second β-strand; depending on whether the core sequence is ΦΦXΦ or ΦXΦΦ, the SIM β-strand binds to SUMO in one or the other orientation, forming either parallel or antiparallel interactions (Song et al. 2005). In this sense, the SIM motif is like SH3 domains, which can bind their peptide ligands in either orientation depending on the sequence. The SIM-binding surface on SUMO lies between its second β-strand and its α-helix. A number of hydrophobic residues in SUMO1, including F36 and V38, form a conserved hydrophobic patch to accommodate the hydrophobic side chains of the SIM (Hecker et al. 2006; Reverter and Lima 2005; Song et al. 2004, 2005). Additional residues surrounding the core SIM residues contribute to its association with SUMO, especially through electrostatic interactions involving acidic SIM residues. Residues lying outside the core may also contribute to specificity in recognizing different SUMO isoforms (Hecker et al. 2006). It seems likely that additional specificity in the interaction of SIM-containing proteins with SUMOylated targets is contributed by sequences in the target protein surrounding the SUMOylation site, and potentially interactions elsewhere with regions outside the core SIM.

2 The RING Finger Protein 4 Family: SUMO-Dependent E3 Ubiquitin Ligases

RNF4 (RING finger protein 4, also known as Snurf, small nuclear ring finger protein) was originally identified as an androgen receptor interacting protein and subsequently shown to interact with several transcription factors and regulate their activity (Kaiser et al. 2003; Lyngso et al. 2000; Moilanen et al. 1998; Wu et al. 2004). In mammals, RNF4 is expressed in proliferating tissues, such as testes and tumors, indicating a role in cell cycle and growth control regulation (Cavallo et al. 2005; Galili et al. 2000). Early studies demonstrated that RNF4 was associated with free SUMO-1 and with SUMOylated-PML protein (Hakli et al. 2005). However, the nature of this association was unknown. Recently, we and others found RNF4 to be a member of a conserved protein family with homologs in both mammals and simple eukaryotes that can bind directly to SUMO (Kosoy et al. 2007; Prudden et al. 2007; Sun et al. 2007; reviewed by Perry et al. 2008).

The RNF4 family now includes *Saccharomyces cerevisiae* Hex3 (also known as Slx5), *S. pombe* Rfp1 and Rfp2, *Dictyostelium* MIP1, *Drosophila* CG10981, and mammalian RNF4. So far no ortholog has been identified in *Caenorhabditis elegans*. All RNF4 homologs have an N-terminal SUMO-binding region and a C-terminal RING finger domain. Interestingly, the SUMO-binding region contains multiple motifs (SIMs); each resembles a canonical SUMO-interacting motif: Rfp1 and Rfp2, which are closely related, and Slx5 each has two SIMs in tandem, whereas mammalian RNF4 has four distinct SIMs. In the yeast RNF4 homologs, the two SIMs correspond to SIM2 and SIM3 in RNF4. The functional significance of the tandem SIMs in these proteins awaits fuller characterization (see below), but each of the SIMs in fission yeast Rfp1/Rfp2 can interact with SUMO independently, and the same is true for all four SIMs in RNF4 (Tatham et al. 2008). Possibly, the tandem SIMs specifically recognize poly-SUMO chains, in a manner analogous to the tandem Ub-binding motifs in Ataxin3 that specifically recognize poly-Ub. Alternatively, they could recognize two different SUMO residues in a multiply SUMOylated protein.

The RING domains in the RNF4 family proteins can be classified into two groups. Like many RING fingers, the RNF4 RING and MIP1 RING are themselves active Ub E3 ligases. In contrast, the RING domains of the yeast RNF4 homologs – Rfp1, Rfp2, and Slx5 – all lack E3 activity, and instead recruit Slx8, an active RING finger Ub ligase, through a heterodimeric RING–RING interaction, to form a functional E3 complex. As a result, all members of the RNF4 family can act as Ub ligases with the potential to specifically target SUMO-conjugated proteins. Several studies have recently shown that RNF4 family proteins can ubiquitinate target proteins in a SUMO- and SIM-domain-dependent manner in vitro, including GST-SUMO fusion proteins, Rad60, Rad52 and PML (Ii et al. 2007a,b; Prudden et al. 2007; Sun et al. 2007; Tatham et al. 2008; Uzunova et al. 2007; Xie et al. 2007). Another characteristic of RNF4 family proteins is a C-terminal motif consisting of three hydrophobic amino acids (L/I-Y/F-L/I/V/Y/F—the central Y/F seems to be critical), located at the end of the RING domain. This motif is present in Rfp1 and Rfp2, Slx5, MIP1, and RNF4. Deletion of these three amino acids abolishes activity in vivo (Sun et al. 2007), suggesting that it serves a critical function. One possibility is that this tail is critical for proper RING–RING interactions, as is the case for Mdm2 and MdmX, another heterodimeric RING finger E3 ligase (Poyurovsky et al. 2007; Uldrijan et al. 2007), where the hydrophobic tails of both Mdm2 and MdmX form an essential part of the RING–RING dimer interface (Linke et al. 2008).

In lower eukaryotes, RNF4 family proteins are involved in transcriptional regulation and gene silencing (Darst et al. 2007; Wang et al. 2006) and are crucial for maintaining eukaryotic genome integrity and surviving genotoxic stress, suggesting they act in DNA damage repair pathways (Burgess et al. 2007; Kosoy et al. 2007; Mullen et al. 2001; Prudden et al. 2007; Sun et al. 2007; Torres-Rosell et al. 2007; Xie et al. 2007; Zhang et al. 2006). *S. pombe* Rfp1 and Rfp2 are essential for cell proliferation, and their mutation results in a plethora of phenotypes due to loss of genome integrity (Prudden et al. 2007; Sun et al. 2007). In *S. cerevisiae*, Slx5 and Slx8 are essential for DNA damage repair during cell cycle progression; they interact genetically with Sgs1, a RecQ family DNA helicase (Mullen et al. 2001; Zhang et al. 2006). *slx5*Δ and *slx8*Δ mutations are also both synthetically lethal with *sgs1*Δ (Mullen et al. 2001; Zhang et al. 2006) and interact genetically with the telomerase gene *(tlc1)*, such that *slx5*Δ and *slx8*Δ enhance the senescence phenotypes of the *tlc1* mutant (Azam et al. 2006). Interestingly, Slx5 associates with components of the Smc5/Smc6 complex in both *S. cerevisiae* and *S. pombe* (Hazbun et al. 2003; Prudden et al. 2007). In addition, Rad60, which associates with Smc5/Smc6 and has two functional C-terminal SUMO-like domains (Boddy et al. 2003; Raffa et al. 2006), was identified in yeast two-hybrid screens for Rfp1/Rfp2 interacting proteins (Sun et al. 2007). Rad60 can be ubiquitinated by Rfp/Slx8 complexes in vitro, suggesting that it may be a physiological target (Prudden et al. 2007). Another DNA repair protein, Rad52, can also be ubiquitinated in vitro by Slx5/Slx8 complexes (Xie et al. 2007). In summary, in lower eukaryotes, RNF4 family proteins are needed for transcriptional regulation and gene silencing (Darst et al. 2007; Wang et al. 2006) and for genotoxic stress survival, suggesting they have a prominent role in DNA damage repair pathways (Kosoy et al. 2007; Mullen et al. 2001; Prudden et al. 2007; Sun et al. 2007; Torres-Rosell et al. 2007; Xie et al. 2007; Zhang et al. 2006). Therefore, SUMOylated subpopulations of DNA repair proteins are likely targets for RNF4/Rfp/Slx5-mediated ubiquitination in vivo. Degradation of SUMOylated forms of these proteins may be necessary to balance their activity and to prevent excessive processing of certain DNA structures generated by the DNA repair machinery, and possibly for restart of replication once repair has been completed.

RNF4 family proteins are linked to the SUMOylation pathway both biochemically and genetically (Burgess et al. 2007; Hannich et al. 2005; Hazbun et al. 2003; Prudden et al. 2007; Sun et al. 2007; Uzunova et al. 2007; Wang et al. 2006). Slx5 interacts with SUMO in yeast two-hybrid screens (Hannich et al. 2005; Uetz et al. 2000). Loss of function mutations in *slx5* and *slx8* suppress a temperature-sensitive *mot1* mutant *(mot1-301)*, which encodes an inhibitor of TBP in *S. cerevisiae* (Wang et al. 2006). Significantly, this unbiased genetic approach identified almost exclusively components of the SUMOylation pathway, including both subunits of E1, E2, and two SUMO proteases, together with Slx5 and Slx8. In *Dictyostelium*, DdMIP1, the RNF4 homolog, was found to interact with MEK1 through its SIM in yeast two-hybrid assays. Interestingly, DdMIP1 can promote the ubiquitination of activated MEK1 kinase in a RING finger-dependent manner, and the loss of DdMIP1 results in accumulation of SUMOylated MEK1 during cAMP-induced *Dictyostelium* chemotaxis (Sobko et al. 2002). Therefore, a conserved function of the RNF4 family proteins could be to destabilize SUMOylated transcription factors and activated protein kinases via RING finger-mediated ubiquitination.

In mammalian systems, RNF4 was originally identified through its ability to interact with the androgen receptor and enhance the transcriptional activation by steroid hormone receptors (Moilanen et al. 1998). However, early work demonstrated that mammalian RNF4 was associated with SUMO and SUMOylated proteins, including PML, the major scaffold of nuclear structures known as nuclear bodies. Two recent studies have identified SUMOylated PML as a target for RNF4 (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008). siRNA-mediated depletion of RNF4 in HeLa cells results in accumulation of poly-SUMOylated proteins, containing both SUMO1 and SUMO2. Overexpression of RNF4 results in increased SUMOylation of PML and degradation. Since RNF4 ubiquitinates poly-SUMO1 or poly-SUMO2 chains in vitro more efficiently than mono-SUMO, it has been proposed that RNF4 polyubiquitinates PML molecules containing branched SUMO chains. RNF4 stimulates the formation of Ub adducts to multiple Lys in SUMO both in vitro and in vivo, and catalyzes formation of Ub chains branched at several Lys, including K11, K48, and K63; of these, only K48-branched chains are known to be recognized by the proteasome to mediate degradation. Arsenic trioxide is used therapeutically for the treatment of acute promyelocytic leukemia (APL). One form of APL is driven by a chimera between PML and RARα resulting from a t15;17 chromosome fusion, and arsenic trioxide treatment causes rapid degradation of PML-RARα in APL cells, as well as PML in normal cells. Recent results show that this depends on SUMOylation of K160 in PML, with a preference for SUMO2 (Lallemand-Breitenbach et al. 2008; Tatham et al. 2008). Degradation of PML in presence of arsenic trioxide requires RNF4, based on the diminished effect of arsenic trioxide in cells depleted for RNF4 or expressing dominant-negative ligase-deficient or SIM-deficient RNF4 mutants. It is currently unclear, however, exactly how arsenic trioxide treatment triggers RNF4-mediated degradation of SUMOylated PML. Overexpressed RNF4 localizes to nuclear bodies, which contain several other SUMOylated proteins, including Daxx, and it is possible that RNF4 also mediates ubiquitination of these proteins. In summary, SUMOylated PML is the first bona fide in vivo target for RNF4, which is ubiquitinated and degraded in a SUMOylation-dependent fashion. Whether other RNF4 targets require polySUMOylation is unclear, and it remains possible that monoSUMOylated proteins can be targeted, perhaps if they contain multiple SUMO residues. Certainly, in fission yeast no phenotype is observed in cells in which SUMO cannot form branches (Prudden et al. 2007), and, in contrast to cells lacking RNF4, SUMOylated proteins do not accumulate, which implies that polySUMOylation is not essential for RNF4 function in mediating metabolism of SUMOylated proteins, at least in this organism.

Consistent with Rfp/Slx8 being responsible for degradation of SUMOylated proteins, genetic depletion of Rfp1/Rfp2 or Slx8 in fission yeast causes accumulation of SUMOylated proteins, that are lost when the Pli1 SUMO E3 ligase is mutated (Kosoy et al. 2007; Prudden et al. 2007; Sun et al. 2007). Nevertheless, there are other possible explanations; for instance, Rfp/Slx8 might regulate activity of the SUMO-specific proteases Ulp1 and Ulp2. Clearly what is needed is the identification of the SUMOylated proteins to which Rfp/Slx8 binds in vivo and a demonstration that the level of one or more of these proteins is elevated in cells lacking Rfp/Slx8 function (e.g., *rfp1*Δ*rfp2*Δ cells). This demonstration may be difficult, because it is likely that only a very small fraction of the population of any target protein is SUMOylated, meaning that only a slight change in protein level may be observed. Our original identification of Rfp1 (Sun et al. 2007) came through a yeast two-hybrid interaction with Ark1, the fission yeast ortholog of the Aurora mitotic kinases, and we have preliminary evidence that the level of Ark1 is modestly elevated in *rfp1*Δ*rfp2*Δ fission yeast, suggesting that a population of Ark1 may be a Rfp/Slx8 target. Nevertheless, since monoubiquitination and polyubiquitin chains branched at K63 dictate other fates for the modified proteins, one should not rule out the possibility that RNF4-mediated ubiquitination might have consequences for its target proteins other than proteasomal degradation.

In summary, the RNF4 family shows a remarkably high degree of functional conservation, with human RNF4, being able to complement the combined loss of Rfp1, Rfp2, and Slx8 in fission yeast, in a manner that requires both the SIM region and the RING finger. This implies that SUMOylated proteins are the critical targets, but one should leave open the possibility that RNF4 may also have non-SUMOylated targets for ubiquitination. Finally, in addition to RNF4, it has recently been reported that SUMOylation can also target the HIF-1α transcription factor for polyubiquitination and degradation by the VHL-containing cullin-Rbx1 E3 ligase (Cheng et al. 2007). The SUMOylated HIF-1α is directly recognized by the VHL substrate specificity subunit, but whether the SUMO moiety is itself bound by VHL is not known.

3 Principles of Crosstalk Between SUMOylation and Ubiquitination

There are several general principles through which the SUMO and Ub pathways can intersect and communicate.

1. Ub and SUMO E3 ligases can modify the same target Lys in a substrate protein, such that one modification will preclude the other and vice versa. In this manner, SUMOylation might prevent ubiquitination-dependent degradation of a protein or binding by a Ub-binding domain (UBD) protein. This type of Lys target competition appears to be the case for PCNA, IκBα, NEMO, ERα, and p53. In the case of PCNA K164 can either be monoubiquitinated or SUMOylated, and this regulates the type of DNA repair occur-

ring at a lesion (Andersen et al. 2008). Monoubiquitinated PCNA is recognized through UBZ/UBM domain-mediated binding of translesion synthetases, which are needed for DNA replication across residues with large adducts; SUMOylated PCNA is recognized by the Srs2 helicase, whose binding displaces Rad51 and prevents inappropriate homologous recombination. Monoubiquitinated PCNA can also be polyubiquitinated to form K63-branched chains. Whether the stoichiometry of SUMOylation and ubiquitination at a single Lys is ever high enough for competition to be a physiological regulatory mechanism is unclear, but, as is found with PCNA, ubiquitination and SUMOylation of the same Lys can have different consequences, and in this sense they are competitive.

2. (Poly)SUMOylated proteins can be targeted for ubiquitination by RNF4 family E3 ligases through direct recognition of the SUMO moiety (Tatham et al. 2008). This can result in proteasomal degradation of SUMO, or possibly other fates.
3. SUMOylation may also target a protein for polyubiquitination through another type of E3 ubiquitin ligase, as has been shown for HIF-1α, which is polyubiquitinated by the VHL-CRL cullin-RING E3 ligase when it is SUMOylated, through a direct interaction between SUMO-HIF-1α and the VHL protein (Cheng et al. 2007).
4. SUMO itself can be polyubiquitinated, with the attached Ub chains being branched through K11, K48, and K63 (Tatham et al. 2008). This may be a mechanism for degrading SUMO attached to SUMOylated protein chains or may serve some other purpose.
5. Conversely, enzymes in the ubiquitination pathway can be regulated by SUMOylation. For instance, the ubiquitin-conjugating enzyme E2-25K is SUMOylated at K14, and this interferes with interaction of the upstream E1, and charging of E2-25K with Ub (Pichler et al. 2005). In addition, the USP25 Ub-specific protease contains a SIM, and the binding of SUMO-Ubc9 to the SIM results in SUMOylation close to its two Ub-interaction motifs (UIMs), which are required for efficient hydrolysis of ubiquitin chains, and this SUMO residue thereby impairs binding to and hydrolysis of Ub chains (Meulmeester et al. 2008).

4 Conclusions and Challenges

Given that there are more than ten other ubiquitin-like proteins (UBLs) in addition to SUMO, it is entirely possible that analogous crosstalk exists between the Ub pathway and the other UBLs. A major challenge remains the identification of the physiological targets for the highly conserved RNF4 family of proteins, which apparently play roles in many fundamental cellular processes. In addition, since RNF4-ligated Ub chains on SUMO itself and on target SUMOylated proteins are potentially branched at K11, K48, and K63, this raises issues as to which of the RNF4 targets are proteasomally degraded via K48 chains, and which have other fates through K11 and K63 chains. Another unanswered question is how RNF4 recognizes its SUMOylated substrates. Does this require polySUMOylation, and perhaps simultaneous binding of multiple SUMO residues in the chain by the RNF4 multi-SIM, or rather are there monoSUMOylated protein targets, which might be recognized through additional contacts between the substrate and the RNF4 protein. Crosstalk between the ubiquitin and SUMO pathways has emerged only in the past few years, and additional connections between these two pathways are certain to be revealed over the next few years.

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