Sumo paralogs: redundancy and divergencies

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1. ABSTRACT

Although sharing a common conjugation pathway, SUMO1, SUMO2/3 and SUMO4 seem to play preferential roles in the cell. Recently, many regulatory mechanisms contributing to SUMO paralogs specific modification have emerged. SUMO enzymes can discriminate between SUMO paralogs at both conjugation and deconjugation levels. Moreover, many substrates possess characteristics that promote their preference for different SUMO family members. A better knowledge of the mechanisms promoting SUMO specific modification will improve our understanding of the functions of SUMO paralogs in distinct cellular pathways.

2. INTRODUCTION

SUMO (Small Ubiquitin-related MOdifier) attachment to substrates is a reversible post-translational modification that regulates fate and function of proteins. SUMO proteins are ~10kD in size and, despite they share only around ~20% amino-acidic sequence with ubiquitin, the three-dimensional structure of the two modifiers is very similar (1-3). Covalent conjugation of SUMO proteins to their substrates requires an enzymatic cascade, analogous to the one used by ubiquitin, comprising the sequential action of three enzymes: a modifier activating enzyme (E1), one conjugating enzymes (E2s) and a member of the ligases (E3s). Many proteins modified by SUMO contain an
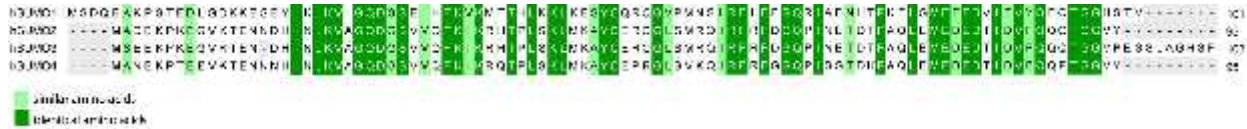
Figure 1. SUMO paralogs sequence comparison. The four human SUMO paralogs protein sequence alignment shows ~96% similarity between SUMO2 and SUMO3 sequences, whereas SUMO1 and SUMO4 proteins share ~45% and ~86% homology with SUMO2 respectively.

acceptor Lys residue within the consensus motif PsiKxE (where Psi is an aliphatic branched amino acid) (4). SUMO is expressed by all eukaryotes such as yeast, plants and animals but is absent from bacteria. Invertebrate organisms express only one SUMO, whereas plants and vertebrates encode four different SUMO proteins: SUMO1/SMT3C, SUMO2/SMT3A, SUMO3/SMT3B and SUMO4. Although in vertebrates SUMO paralogs share common conjugation properties, they also show some specificities, such as preferential substrate conjugation or subcellular distribution; nevertheless, it still remains poorly understood whether they function in unique, redundant or antagonistic ways.

Here, we discuss similarities and differences among the distinct SUMO paralogs. Starting from a general description of the SUMO paralogs, we focus on the recently discovered mechanisms that drive specificity in substrate modification and recognition. Finally, we discuss cellular functions in which SUMO1, SUMO2/3 or SUMO4 are known to play preferential roles rather than describe general functions of sumoylation.

3. SUMO PROTEINS

In humans SUMO1, SUMO2 and SUMO3 are ubiquitously expressed, whereas SUMO4 is primarily expressed in kidneys, lymph nodes and spleen (5). SUMO2 and SUMO3 are ~96% identical to each other, thus, are often referred to as SUMO2/3, whereas SUMO1 shares ~45% sequence identity with SUMO2/3 (Figure 1). The differences between SUMO1 and SUMO2/3 are mostly found in the second beta-strand and in the alpha-helix of both proteins (6). SUMO4 shares 86% homology with SUMO2 (Figure 1), but it is still unclear whether SUMO4 is conjugated to cellular proteins as the other paralogs (7).

SUMO2/3 possesses Lys residues near the amino termini used as SUMO acceptor sites, which are not present in SUMO1. Thus, the ability of SUMO2/3 to form chains efficiently, both *in vitro* and *in vivo*, through the consensus site is well established (8, 9). The role of SUMO1 was initially limited in terminating poly-SUMO2/3 conjugates, whereas only more recent studies found SUMO1 able to multimerize *in vitro* primarly via non-consensus Lys residues at the N-terminal (10). Moreover, proteomic studies have also found polySUMO1 chains through non-consensus sites *in vivo* (11, 12).

The predominant localization of SUMO2/3 is in the nucleus and PML bodies, whereas SUMO1 uniquely localizes to nucleoli, nuclear envelope and cytoplasmic foci (13-16). Furthermore, SUMO1 dynamic appears to be slower compared to SUMO2/3 and the distribution of the two paralogs changes rapidly as the cells enter G1 phase and as they enter and progress through mitosis (16).

4. CONJUGATION AND DECONJUGATION MACHINERY

For the maturation of SUMO proteins, the SUMO conjugation machinery requires the activity of a protease removing the C-terminal stretch (2-11 amino acids) after a conserved Gly-Gly motif. The mature form of SUMO is activated by adenylation at the C-terminal diglycine motif by the E1 enzyme (the heterodimer SAE1/SAE2 or AOS1-UBA2). The adenylated SUMO forms a thioester bond with a conserved Cys of the E1 enzyme and is then transferred to a Cys on the E2 conjugating enzyme (UBC9) forming and E2-SUMO thioester. Finally, UBC9 transfers SUMO to a substrate protein resulting in the formation of an isopeptide bond between the C-terminal Gly residue of SUMO and a Lys side chain of the target. Although UBC9 is able to directly interact with some SUMO substrates to conjugate SUMO to the target, E3 protein ligases often may facilitate this process catalyzing the transfer of SUMO from UBC9 to the substrate or forming a complex with SUMO-E2 and the substrate to promote specificity (Figure 2).

SUMO E3 ligases can be divided into three groups. The largest group is characterized by the presence of a SP-RING domain that resembles the RING domain in ubiquitin RING E3 ligases and includes the protein inhibitor of STAT (PIAS) family proteins in mammals, known as Siz proteins in yeast. Members of this family include Siz1, Siz2, methyl methanesulphonate-sensitivity protein 21 (MMS21) and the molecular zipper protein 3 (Zip3) in yeast and PIAS1, PIAS3, PIASxalpha, PIASxbeta and PIASy in humans. The SP-RING domain binds both the E2-SUMO thioester and the substrate bringing them into a closest position to promote SUMO transfer. Moreover, the SP-RING domain is not the only domain able to dock substrates to promote their sumoylation: indeed, also the N-terminal SAP domain of PIAS proteins has been shown to interact with LEF1 inducing its SUMO modification (17). A second type of E3 ligase is represented by the nuclear pore protein RanBP2, which binds both SUMO and UBC9 positioning the SUMO-E2 thioester bond in an optimal orientation to enhance conjugation (18, 19). Many other proteins have been identified as SUMO E3 ligases, such as the Polycomb group member PC2, the histone deacetylase HDAC4, topoisomerase I-binding RING finger protein (TOPORS), Ras homologue enriched in striatum (RHES) (20-23) and members of the diverse tripartite motif (TRIM) family (24).
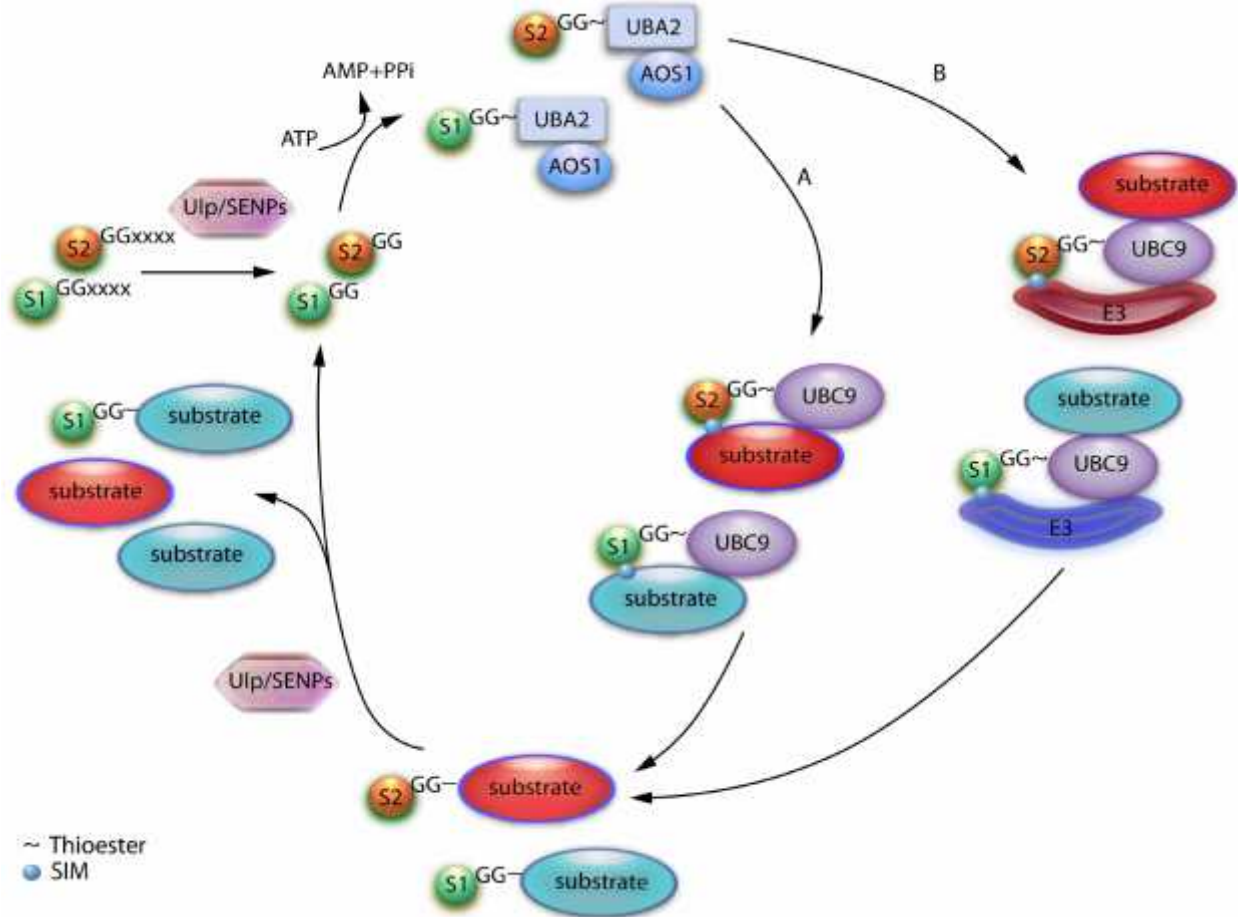
Figure 2. The SUMO conjugation machinery. SUMO (Small Ubiquitin-related MOdifier) family members SUMO1 and SUMO2/3 share the same conjugation pathway. Before their activation, they undergo processing by SUMO-specific isopeptidases (sentrin-specific proteases; SENPs) in order to expose their C-terminal Gly-Gly motif. The mature form of SUMO is activated by adenylation at the diglycine motif by the E1 enzyme (the heterodimer AOS1-UBA2), promoting a thioester bond with a conserved Cys of the E1 enzyme. SUMO is then transferred to a Cys on the E2 conjugating enzyme (UBC9) forming and E2-SUMO thioester. Some target proteins posses a SUMO interacting motif (SIM), which can facilitate their sumoylation in a paralog specific way (A). SIMs are also present on SUMO E3 ligases, which often may facilitate the transfer of SUMO from UBC9 to the substrate forming a complex with SUMO-E2 and the substrate and promoting SUMO paralog specificity (B). Finally, an isopeptide bond is formed between the diglycine motif of SUMO and a Lys residue in the substrate. Deconjugation of substrates is then performed by SENPs, which may preferentially deconjugate one paralog over the other and free SUMO may be recycled for another round of conjugation.

As mentioned, sumoylation is a reversible mechanism and the reversal of this modification is catalyzed by the action of specific proteases (Figure 2). Ulp1 and Ulp2 in yeast and six sentrin-specific proteases (SENP1-3 and SENP5-7) in humans belong to the same family of SUMO proteases. SENPs possess both isopeptidase activity and C-terminal hydrolase activity needed for the maturation of newly synthesized SUMO proteins. Only very recently, a member of a new class of desumoylases, the DeSumoylating Isopeptidase 1 (DeSI-1) has been identified. DeSI-1 seems to recognize different substrates compared to SENP (25).

It is still not completely clear whether different SUMO paralogs can be used interchangeably during conjugation to a particular substrate or whether there is substrate specificity. For some substrates paralog preference has been demonstrated; for instance the Ran GTPase-activating protein 1 (RanGAP1) is modified almost exclusively by SUMO1 *in vivo* (26), whereas topoisomerase II (27) is mainly conjugated to SUMO2/3. At the same time, other substrates, such as PML, have been reported to be conjugated to both SUMO1 (28) and SUMO2/3 (29). Considering that there is a substrate specificity, it is still not known how this specificity occurs since the same SUMO E1 and E2 enzymes are shared between all paralogs. In the ubiquitin conjugating machinery, in fact, the different E2 enzymes are responsible for both E3 ligase selection and substrate modification. In spite of the evidence that SUMO1 and

SUMO2/3 are conjugated, at least in part, to unique subset of proteins (30, 31), how different proteins are selectively modified by one paralog over the other remains unclear.

5. REGULATION OF PARALOG SPECIFICITY BY SIMs

Biochemical and structural studies have revealed that UBC9 directly recognizes the SUMO consensus site on target proteins, interacting with a pocket near the active site of the enzyme (3, 4, 32). This interaction represents a well-characterized molecular mechanism for SUMO substrate recognition. Although direct UBC9 binding cannot explain SUMO paralog selectivity, non-covalent SUMO binding may represent an alternative mechanism to confer specificity. The SUMO-interacting motif (SIM) sequences are critical to both SUMO conjugation and to SUMO-mediated effects. SIMs in receptor proteins are responsible for recognizing SUMOylated substrate (33) thereby determining intracellular trafficking, protein-protein interaction and localization of sumoylated substrates. So far, only one class of SIMs has been described (34, 35), in contrast to the many different ubiquitin-binding domains (UBDs) (36). This motif generally contains an hydrophobic core ((V/I)x(V/I)(V/I)) flanked by acidic residues (34, 37, 38) and forms a beta-strand that can bind in parallel or antiparallel orientation to an hydrophobic pocket on the SUMO surface (19, 33). It has been suggested that the negative charges flanking the core might modulate the SUMO-SIM interaction through long range electrostatic interactions with basic residues in the vicinity of the hydrophobic cleft of SUMO (37). The distribution of the basic residues surrounding the clefts differs between SUMO1 and SUMO2/3 and this may result in different electrostatic interactions between these basic residues and the acidic residues of the SIM. Phosphorylation of serine residues flanking the SIM hydrophobic core could also increase the number of negative charges (39), facilitating SUMO interaction and possibly SUMO paralog discrimination. Nevertheless, the molecular determinants of the bound orientation and paralog specificity of SIMs are still unclear. Very recently it has been suggested that antiparallel binding to the SUMO pocket tolerates more diverse sequences, whereas parallel binding prefers a more strict sequence, consisting of (I/V)DLT, that shows a preference in binding SUMO2/3 with high affinity (40).

5.1. SIMs in SUMO targets

There are many examples of target proteins whose SIMs can facilitate their SUMOylation in a paralog specific way (Figure 2). However, it is still unclear whether specific conjugation of proteins with SUMO paralogs can be regulated by cellular signaling that alter the affinity of SIMs on target proteins towards a specific SUMO paralog.

The transcriptional corepressor Daxx has been shown to possesses a SIM both important for its conjugation to SUMO and crucial for Daxx localization into PML oncogenic domains (PODs) in order to mediate transcriptional repression on many sumoylated transcription factors (41). Moreover, more recently, it has been shown that Daxx's SIM embeds into the SUMO1

groove in a parallel orientation. Furthermore, Daxx-SIM is phosphorylated on two serines by CK2. This phosphorylation markedly enhances Daxx-SIM binding affinity towards SUMO1 over SUMO2/3, increasing Daxx SUMO1 conjugation and its interaction with SUMO1-modified proteins (42).

The ubiquitin specific protease 25 (USP25) has been shown to be a preferential SUMO2/3 binding protein. Both, SUMO2/3 specific binding and conjugation depend on a SIM present on USP25. Seven amino-acid residues, including the hydrophobic core, are sufficient to confer specificity to SUMO2/3 and the acidic residues flanking the SIM enhance binding but do not alter paralog specificity (43).

The transcription repressor MBD1-containing chromatin-associated factor 1 (MCAF1) interacts with SUMO2/3 and SUMO1 and this facilitates the interaction between MBD1 and MCAF1. A SIM-containing segment from MCAF1 binds preferentially to SUMO2/3 compared to SUMO1 and this promotes the greater affinity of MCAF1 for MBD1 conjugated to SUMO2/3 than to SUMO1 (44). NMR spectroscopy and mutagenesis experiments have shown that the hydrophobic core of the SIM forms a parallel beta-sheet pairing with the beta2-strand of SUMO3, whereas its C-terminal acidic stretch seems to mediate electrostatic interactions with a surface area formed by basic residues of SUMO3 (45).

Finally, the RecQ DNA helicase mutated in Bloom syndrome (BML) is preferentially modified by SUMO2/3 both *in vitro* and *in vivo* and contains two SIMs that mediate non-covalent interaction with SUMO2/3. The non-covalent interaction between SUMO and BML is required for BML sumoylation at non-consensus sites and the preferential SUMO2/3 conjugation is determined by preferential SUMO2/3 binding (46).

5.2. SIMs in E3 ligases

Furthermore, SIMs present in E3 ligases control SUMO paralog specific modification (Figure 2). The RanBP2 nucleoporin contains an internal repeat domain (IR1-M-IR2) that catalyzes E3 ligase activity and contains two SIMs. One SIM is located in IR1 and is responsible for UBC9 binding but is not able to interact with SUMO1 or SUMO2/3, whereas the second SIM, located at the junction of the M and IR2 regions, binds to SUMO1 but not to SUMO2/3 or UBC9, indicating its specificity and high affinity for SUMO1 (47). The disruption of RanBP2 interaction with UBC9 affects only SUMO2/3 conjugation via RanBP2, indicating that an UBC9-SUMO1 thioester can be recruited to RanBP2 via specific SUMO1-SIM in the absence of strong binding with the E2 enzyme. RanBP2 peptide was shown to bind antiparallel to SUMO (19). Moreover, also the E3 ligase domain of RanB2 exhibits higher ligase activity with SUMO1 compared to SUMO2/3. Although this preference is evident, it is not known how this specificity is achieved. Thus, more recently it has been shown that both SIMs on RanBP2 show preferences for SUMO1, IR1 functions as the primary E3 ligase of RanBP2, whereas IR2 retains the ability to interact with

SUMO1 to promote SUMO1-specific E3 ligase activity (48).

The affinity and specificity of the RanBP2 SIM to SUMO1 is not dependent on post-translational modification, whereas it has been shown that the E3 ligase PIASxalpha is phosphorylated within its SIM and this modification influences its binding to SUMO1 but not to SUMO2/3. Moreover, the negative charged amino-acids surrounding the hydrophobic core seem to influence PIASxalpha binding to SUMO1 but not to SUMO2 (37). The PIASxalpha peptide was shown to bind parallel to the SUMO beta2-strand (33, 37).

Recently, the K-bZIP protein encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) has been found to be a SUMO E3 ligase. K-bZIP carries a SIM, which specifically binds to SUMO2/3 but not to SUMO1. Moreover, the ability of K-bZIP in conjugating SUMO depends on its intact SIM (49).

6. DECONJUGATION AND SELECTIVITY

Paralog specific SUMOylation is not only regulated at the level of conjugation but also at the level of protection from deconjugation (Figure 2). RanGAP1 is one of the best-studied SUMO substrates, as already mentioned, and it is preferentially modified by SUMO1 (14, 15). More recently, it has been shown that RanGAP1 modification by SUMO1 is not regulated at the level of conjugation. RanBP2, the E3 ligase most likely to affect RanGAP1 SUMOylation *in vivo* has no effect on the rate or paralog selectivity of RanGAP1 *in vitro*. This result is consistent with previous finding claiming that RanGAP1 is a unique substrate that interacts tightly with UBC9 through surfaces adjacent to the conjugation site and is thus efficiently modified in the absence of E3 ligases (3, 19). Thus, it seems that RanGAP1 is effectively modified by both SUMO1 and SUMO2/3 both *in vitro* and *in vivo* but the SUMO1 selectivity is driven by the presence of the isopeptidases SENP1 and SENP2, which specifically deconjugate SUMO2/3-modified RanGAP1 (50) (Figure 2). Moreover, RanBP2 binding to RanGAP1, through its SUMO1-specific SIM, confers additional stability and selectivity.

All six SENPs expressed in mammalian cells show paralog specificity; whereas only SENP1 has been shown to be able to react with both SUMO1 and SUMO2/3, all the other SENPs display high selectivity towards SUMO2/3. This paralog preference seems to be dependent on the variable N-terminal domain of each SENP (51). Moreover, SENP6 and SENP7 show high specificity in deconjugating SUMO2/3 chains (52).

7. CELLULAR PROCESSES AND SUMO PARALOGS

Finally, SUMOylation influences many aspects of a target protein, such as stability, localization or activity, but it is not known whether the consequences of conjugation to different paralogs are distinct or whether some redundancy exists. It has been shown that lack of SUMO1 leads to compensatory utilization of SUMO2/3, suggesting redundancy between SUMO paralogs in order to maintain sumoylation levels above critical thresholds (53, 54). Thus, it is not clear whether the conjugation of particular targets is restricted to a specific SUMO paralog or whether different paralogs can be used interchangeably. It has been demonstrated that in the presence of superphysiological concentrations of individual SUMO moieties paralog preference can be compromised (27, 55). Since in most studies protein conjugation to SUMO has been demonstrated by overexpressing one of the paralogs, paralog specificity for those substrates cannot be determined but needs to be studied under more physiological conditions. Thus, strong paralog specificity has been demonstrated under physiological conditions only for a few substrates.

7.1. SUMO1

SUMO1 was found to be important in palathogenesis, in fact disruption of the SUMO1 gene, resulting in haploinsufficiency, seems to be linked to the presence of cleft lip and palate (56). Moreover, because of its localization, both in the nucleus and in cytoplasm, SUMO1 conjugation has been proposed to regulate nucleocytoplasmatic trafficking and target protein interaction with other cellular components. Cytoplasmic modification of RanGAP1 by SUMO1 targets the GTPase to the nuclear pore complex and allows its association with RanBP2 (57, 58). In addition, the unmodified form of PML is found in the soluble nucleoplasmic fraction, whereas modification of PML promotes its compartmentalization in nuclear bodies (59). SUMO1 regulates subnuclear localization of some transcription factors, such as Sp3, heat shock trancription factor 1 (HSF1) and TEL (60-62). SUMO1 conjugation represses Sp3 activity and accumulates it in nuclear dots, while unmodified Sp3 has a more diffuse nuclear localization (60). On the other hand, for many targets transport into the nucleus seems to be a prerequisite for SUMO modification. Nuclear import was shown to be required for sumoylation of both PML and Sp100, a component of PML nuclear bodies (63, 64).

7.2. SUMO2/3

Most of the SUMO2/3 specific roles in the cell are due to its ability to form polymeric chains. Although, as previously mentioned, the formation of SUMO1 chains was also observed, it is still unclear whether they are of any biological relevance. SUMO2/3 conjugates, but not SUMO1, are known to accumulate under different stress conditions, such as heat shock, oxidative stress and ethanol addition, known factors that regulate the stress response cascade in the cell (26). Moreover, in response to heat shock SUMO2/3 polymerizes into polymeric chains and undergoes a rapid and dramatic redistribution among substrates involved in cell functions relevant for heat shock response, such as apoptosis, trafficking, folding, protein degradation, DNA replication, recombination and repair (65). Comparative analysis were conducted treating cells with the proteasome inhibitor MG132, showing a qualitative and quantitative parallelism between the responses to MG132 and to heat shock, even though

MG132 was shown to increase SUMO2/3 conjugation dependently on protein synthesis (66, 67). This implies that the accumulation of newly synthesized, misfolded proteins destined for degradation by the proteasome, triggers the SUMO conjugation response, suggesting a role for SUMO2/3 in protein quality control. Thus, SUMO2/3 conjugation might increase the ubiquitin-dependent degradation of denatured/damaged protein by the proteasome.

The link between SUMO and ubiquitin has emerged first from studies in yeast, reinforcing the importance of SIM-containing proteins in the sumoylation pathway. The RING containing E3 ubiquitin ligases Slx5 (synthetic lethal of unknown function 5) and Slx8, characterized in *S. cerevisiae*, and Slx8 and RING finger protein (Rfp) in *S. pombe* were first found to be important in the maintenance of global sumoylation in the cell (68-71). N-terminal regions of these proteins contain SIM domains that allow the Slx5-Slx8 and Rfp-Slx8 heterodimer to bind SUMO or SUMO-like domains (SLDs) then, the RING domain of Slx8 mediates ubiquitylation of the bound substrates (72). It has been recently shown that the heterodimeric RING E3 ligase complex Slx5-Slx8 is required for DNA repair at the nuclear pore complex during double-strand breaks (73). The first target identified was the DNA repair protein Rad60 in *S. pombe* (68). More recently the transcriptional regulator Mot1 has been shown to undergo Slx5-Slx8- mediated SUMO and ubiquitin-dependent proteolysis *in vivo* (74). RING finger protein 4 (RNF4) is the vertebrate homologous of Slx5, Slx8 and Rfp. Whereas the N-terminal regions of the yeast ligases contains only one or two SIMs, mammalian RNF4 contains four potentials SIMs, suggesting the possible interaction with multiple SUMO molecules. RNF4 shows strong binding preference for SUMO2 chains and efficiently ubiquitylates SUMO chains *in vitro* in a RING and SIM-dependent mechanism promoting the degradation of protein modified with SUMO chains *in vivo* (75). Thus, RNF4 is a SUMO-targeted ubiquitin ligase that has unique specificity for SUMO chains. Well-described targets of RNF4 are PARP1 (76), HIF-2alpha (77) and PML (75) but more than 300 proteins have been identified bound to RNF4 as polysumoylated in response to heat shock (78). This suggests that SUMO2/3 chains can act as a signal for the recruitment of E3 ubiquitin ligases that ubiquitylate SUMO-modified substrates targeting them for proteasomal degradation.

7.3. SUMO4

As mentioned before, SUMO4 has a unique localization in the immune system. SUMO4 gene (*SUMO4*) has been found to encode for a unique polymorphism not encoded in any of the other SUMO genes. This is a single nucleotide polymorphism (SNP) that results in an amino acid substitution of a highly conserved methionine by a valine residue (M55V). This SNP was found to be strongly associated with type 1 diabetes (T1D), an autoimmune disease resulting from pancreatic beta-cells destruction. This association has been shown in various studies among multiple Asian populations, despite controversial observation in Caucasians (79, 80). SUMO4 has been shown to conjugate to IkappaBalpha and negatively regulate NFkappaB transcriptional activity (81). The M55V substitution increases NFkappaB activity and its aberrant activation has been linked to the pathogenesis of T1D and to initiation and progression of autoimmunity.

8. CONCLUSIONS

An incomplete picture of the mechanism underlying paralog specific sumoylation still exists. UBC9 catalyses the conjugation of all SUMO family members, without discrimination. Since it recognizes the SUMO consensus site present in most of the target proteins it has a limited control over the specificity of the conjugation process, differently to E2 enzymes of the ubiquitin conjugation machinery. Thus, paralog specific conjugation has to be regulated by other factors, either at the conjugation or deconjugation levels. In the last years SUMO interacting motif, present on target proteins or on E3 ligases, have been shown to play an important role for the specific conjugation of SUMO paralogs. Although many ubiquitin binding domains have been identified for the ubiquitin pathway, so far, only one SIM has been characterized and the understanding of the role of the acidic residues flanking the SIM for the paralog specific binding still needs further investigation. Thus, the identification of novel binding motifs will help to understand their mechanism in the regulation of SUMO conjugation. Moreover, SUMO conjugation and SUMO enzyme expression and activity can be regulated by many factors, such as external stimuli (82), stress (83) and viruses (84). Thus, cellular environment and the activation of cellular signaling pathway may exert a crucial role in SUMO paralog specific conjugation. Phosphorylation, is a well established post-translational modification used in the cell to regulate signal transduction and is able to influence and control SUMO modification. Thus, considering that SUMO paralogs can play redundant and non-redundant cell functions, the knowledge of the mechanisms underlying SUMO specific conjugation should advance our understanding of its effect on cellular processes.

9. ACKNOWLEDGMENTS

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