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Review

SUMOylation and cell signalling

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SUMOylation is a highly transient post-translational protein modification. Attachment of SUMO to target proteins occurs via a number of specific activating and ligating enzymes that form the SUMO-substrate complex, and other SUMO-specific proteases that cleave the covalent bond, thus leaving both SUMO and target protein free for the next round of modification. SUMO modification has major effects on numerous aspects of substrate function, including subcellular localisation, regulation of their target genes, and interactions with other molecules. The modified SUMO-protein complex is a very transient state, and it thus facilitates rapid response and actions by the cell, when needed. Like phosphorylation, acetylation and ubiquitination, SUMOylation has been associated with a number of cellular processes. In addition to its nuclear role, important sides of mitochondrial activity, stress response signalling and the decision of cells to undergo senescence or apoptosis, have now been shown to involve the SUMO pathway. With ever increasing numbers of reports linking SUMO to human disease, like neurodegeneration and cancer metastasis, it is highly likely that novel and equally important functions of components of the SUMOylation process in cell signalling pathways will be elucidated in the near future.

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1 Introduction

Transient post-translational modifications like acetylation, phosphorylation and ubiquitination are a fast and very efficient way for the cell to respond to intra- and extracellular stimuli, and are thus favoured in cell signalling cascades. A more recently identified transient protein modification is the attachment of a SUMO peptide, the process of which is often referred to as SUMOylation. This has also been shown to occur in signalling pathways, initially within the nucleus and, more recently, also in other parts of the cell. SUMO attachment has been implicated in a number of cell processes, such as transcription, nuclear transport, DNA repair, mitochondrial activity, plasma membrane ion channels, cell cycle and chromatin structure. Although its function is as diverse as its substrates, one generalisation could be that modification of a protein substrate by SUMO alters its interactions with other protein and DNA molecules. At any given time, only a very small amount of substrate is modified, usually around 1%. This may be one reason why SUMO and the modified forms of substrate proteins were only discovered recently. Since its identification, however, a great many proteins that are modified by SUMO species and an equally great number of pathways in which SUMO partakes are now being revealed, and the various effects of the modification are beginning to be elucidated.

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Abbreviations: **DRP-1**, dynamin-related protein 1; **HSF1**, heat shock factor 1; **MAPL**, mitochondrial-anchored protein ligase; **PIAS**, protein inhibitors of activated STAT; **PML**, promyelocytic leukaemia protein; **pRB**, retinoblastoma protein; **SENP**, sentrin-specific protease; **shRNA**, short hairpin RNA; **SUMO**, small ubiquitin-related modifier; **Ubc9**, ubiquitin-like protein SUMO-1-conjugating enzyme 9

2 The SUMO modification pathway

Originally reported just over a decade ago as a protein related to Ubiquitin, the small ubiquitin-related modifier (SUMO) has been since shown to be present in all metazoans and take part in a number of diverse cellular functions, the number of which is still extending [1]. Although SUMO only shares 18% of overall sequence identity with ubiquitin, the general structure fold is conserved, where the C terminus of SUMO is almost super-imposable on the equivalent region of ubiquitin [2, 3]. SUMO proteins have a distinct overall surface charge distribution compared to ubiquitin, and the function of SUMO modification is completely different, and sometimes counteractive to the ubiquitin pathway. SUMO can occupy available lysine residues and inhibit ubiquitin attachment, thus protecting proteins from breakdown, or it can promote proteasome-mediated degradation, possibly by recruiting and/or regulating enzymes that control degradation.

The SUMO protein sequence is around 100 amino acids, with a molecular mass of ~11 kDa; it is highly conserved in all eukaryotic cells, and is present in all tissues and developmental stages of higher organisms. It has been shown to be essential for cell viability both at the organism level and in cells in culture [4]. Different organisms contain different numbers of SUMO species, with a single SUMO present in yeast (Smt3p) [5], *Drosophila* (SMT3) [6], and *Caenorhabditis elegans* (smo-1) [7], and four SUMO peptides in mammals, termed SUMO-1, SUMO-2, SUMO-3 and SUMO-4 [8–11]. SUMO-4 is the most recently identified gene and has an 86% similarity to SUMO-2. Its mRNA transcripts are mainly present in kidney, lymph system and spleen, but show limited expression compared to the other SUMO species [8]. Since no native SUMO-4 protein has yet been detected in any tissue, it has been suggested that SUMO-4 might be an expressed pseudogene [12]. In addition, a proline in a position at its C terminus instead of a glutamine, results in resistance of SUMO-4 to hydrolysis by SUMO-specific proteases and it can thus not be processed to a mature form capable for conjugation [13]. SUMO-1 shares 50% sequence identity with SUMO-2 and -3, while SUMO-2/3 share a 95% identity. Also, SUMO-2/3 possess consensus SUMOylation sites at their N-terminal tails that allow formation of poly-SUMO chains, in contrast to SUMO-1, where these sites are absent, along with the capacity for chain formation [14]. The differences between the isoforms also translate to functional activity, as the function of SUMO-2/3 is almost indistinct, whereas SUMO-1 has a dissimilar function. In the case of intrinsic transcriptional repression ability, for example, SUMO-2 and -3 show stronger inhibitory activity compared to SUMO-1, the activity of which appears weaker [15, 16]. Furthermore, while there are pools of free SUMO-2/3 available within cells, SUMO-1 is rarely found unattached [17]. Certain proteins show a preference in conjugation to either SUMO-1 or SUMO-2/3, but others can be modified equally well by both SUMO species.

Attachment of SUMO to target proteins takes place using a similar mechanism to ubiquitin attachment, where the enzymes that catalyse SUMO complex formation are analogous to the ubiquitin pathway. SUMO modification enzymes are specific for this peptide and not interchangeable with the equivalent for ubiquitination. SUMOylation occurs via the formation of an isopeptide bond between the glycine residue at the C-terminal end of SUMO to the e-amino group of an internal lysine residue within the substrate [18]. The SUMO peptide is initially translated as a precursor, with a short sequence extending past a conserved GG motif that defines the carboxyl end of the mature protein. Proteolytic cleavage of this sequence converts SUMO to its mature form. SUMO-specific peptidases, for example members of the sentrin-specific protease (SENP) family, catalyse this step. SUMO-activating enzymes, also called E1, activate the mature SUMO in an ATP-dependent reaction. Active SUMO is then transferred onto the E2 SUMO-conjugating enzyme Ubc9 [19–21]. Conjugation of SUMO to target proteins occurs through Ubc9 with the aid of an E3 ligase [22, 23] (Fig. 1). Substrate specificity for SUMO is conferred by both Ubc9 and various E3 ligases, the former by forming the covalent attachment of SUMO to its targets, while the latter probably interacts with other areas of the substrate and provides more specificity [24].

SUMO attachment is a reversible and highly transient modification. The same enzymes that facilitate the initial maturation of SUMO molecules also catalyse the cleavage from their substrates. Six human SENP family proteins, SENPs 1–3 and 5–7 have been shown to be SUMO-specific proteases [25]. Unlike the enzymes catalysing SUMO attachment, SUMO proteases show little similarity to the equivalent enzymes in the ubiquitin pathway, but appear closely related to viral proteases [26]. The differential subcellular localisation of the SENP proteins, most likely dictated by non-conserved N-terminal sequences, is thought to provide the specificity for the SUMO-substrate complexes they regulate [27, 28]. SENP1 is localised mainly in the nucleus with little, albeit persistent, cytoplasmic presence [29, 30]; SENP2 is associated with the nu-

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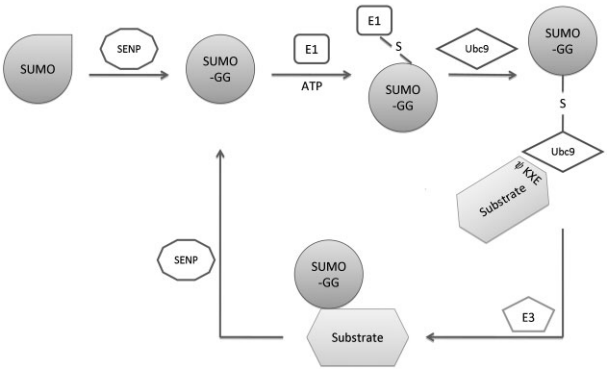

Figure 1. Brief schematic representation of the SUMO pathway. SUMO is conjugated to and de-conjugated from target proteins in a series of steps that involve enzymes corresponding to those of the ubiquitin pathway. Mature SUMO is produced from the precursor protein, it is then activated by E1 enzyme, and passed on, through subsequent thioester bonds, from the E2-conjugating enzyme Ubc9 onto the substrate protein. Release of SUMO from the complex is catalysed by a SUMO hydrolase of the SENP family.

clear pore [31]; SENP3 and SENP5 are nucleolar [32], while separate reports place SENP6 in both the nucleoplasm and the cytosol [33, 34]. Although the SUMO isoforms and the SUMOylation target proteins described so far are predominantly nuclear, increasing numbers of enzymes of the SUMO conjugation pathway as well as SUMO-target complexes are being identified in other parts of the cell, like the cytosol, the plasma membrane and the mitochondria [35–37]. Specificity in SUMO complex targeting may be achieved through the subcellular location of each protease [38].

A short amino acid sequence motif within the target protein frequently serves as a recognition site for SUMO attachment. This recognition site is conserved and presents the consensus sequence ψKXE/D [39, 40], where ψ is a large hydrophobic amino acid, like leucine, isoleucine or valine; K is the lysine residue at which the attachment takes place, and X is any amino acid followed by a glutamic (E) or aspartic (D) acid. At the same time this sequence was identified as the SUMOylation consensus, independent studies pinpointed it as a SUMOylation-dependent transcriptional inhibitory motif in transcription factors [41, 42]. A number of alternative SUMO modification sequences have also been described, in which for example the amino acid before the lysine can be a different one from the three consensus residues. Furthermore, SUMOylated proteins have been described that either do not contain the consensus site, use a different motif for SUMO conjugation [16], or maintain SUMOylation after the recognition site has been mutated. More extensive SUMO conjugation motifs have also been described; it has been suggested that acidic residues downstream of the core SUMOylation motif have a role in enhancing specificity for substrates [43]. SUMO attachment sites are often found in the vicinity of activation or repression domains of target proteins, as for example in Msx1 [44].

SUMOylation alters the relations of the modified target with other macromolecules, through providing novel interaction surfaces. Thus, besides the sites for SUMO conjugation, other sequences have been identified that mediate interactions between SUMOylated complexes and their protein partners. A conserved SUMO-interacting motif (SIM) has been found on proteins that associate with SUMO-conjugated moieties, including the promyelocytic leukaemia protein (PML) and members of the protein inhibitors of activated STAT (PIAS) family [16, 45]. Mutation of these sites abolishes their interaction with the SUMO-target complex. In addition, the surface of SUMO that the SIM of interacting proteins binds is an essential region required for transcriptional repression when SUMO is recruited to promoter regions [15, 46].

3 Links with phosphorylation/acetylation/ubiquitination

Other post-translational protein modifications have been associated with the SUMO pathway. The availability of certain proteins for SUMOylation has been shown to be dependent on their phosphorylation status, like for example GATA-1 (GATA-binding protein 1, globin transcription factor 1), myocyte-specific enhancer factor 2A (MEF2A), Smad nuclear interacting protein 1 (SNIP-1), and heat shock factors HSF1 and HSF4b. The HSF1 factor, which is phosphorylation-deficient, cannot become modified by SUMO, as this protein needs to undergo a conformational change, mediated by phosphorylation of a serine residue, to allow SUMO attachment [47]. Moreover, the phosphorylation of these heat shock factors is heat inducible and may be directly associated with the inducible SUMOylation of HSF1 [48]. A phosphorylation-dependent SUMOylation motif (PDSM), which greatly resembles an extended consensus SUMO attachment site, ψKxExxSP, has been described for such proteins [49]. Furthermore, histone deacetylases, including HDAC2 and HDAC6, have been shown to preferentially interact with SUMO-modified substrates [50, 51]. It has been suggested that deacetylation of histones by HDAC enzymes may make more lysine residues available for SUMOylation [52]. A SUMOylation switch based

on acetylation/deacetylation has been described in the p53-HIC1-SIRT1 regulatory loop [53]. Hypermethylated in cancer 1 (HIC1), a protein involved in the p53 tumour suppression pathway, has been shown to be both SUMOylated and acetylated, possibly on the same lysine residue, as lysine mutants that are SUMOylation deficient show much lower levels of acetylation compared to the wild-type protein. Deacetylases with roles in the p53 pathway, sirtuin 1 (SIRT1), a NAD-dependent deacetylase, and HDAC4, appear to promote deacetylation and SUMOylation, respectively [53]. Increased SUMOylation of H4 correlates with decreased acetylation of the gene when Ubc9 is targeted to the promoter region [54]. An association between SUMOylation and the ubiquitin pathway is perhaps not surprising due to the close relation between the two proteins. SUMO-1 overexpression results in reduced levels of p63 [55]. Since mutating the SUMO acceptor sites of p63 appears not to have any effect on protein levels, this down-regulation of the protein is not dependent on its own SUMOylation state and is more likely to result from changes in the SUMOylation of other regulatory proteins [56]. In the case of Smad4, when its SUMO attachment sites are mutated, the lysine mutant displays increased stability compared to wild-type protein due to reduced poly-ubiquitination [57]. Because SUMO peptides can use the same lysine residues as ubiquitin, SUMOylation of a substrate protein may lead to protection from degradation through the ubiquitin pathway. However, at the same time, if these sites are disrupted, ubiquitin attachment may also be hindered, and thus lead to increased stability. This effect is different to impaired SUMOylation due to increased activity of SENPs, where the lysine residues of the SUMO sites are still intact and available for interaction with ubiquitin. One of the rare cases where SUMOylation promotes protein catabolism involves the promyelocytic leukaemia gene product (PML) that concentrates in nuclear bodies, and the product of its fusion to the retinoic acid receptor a (PML-RARa) that occurs after a chromosomal translocation in patients with acute promyelocytic leukaemia [58–61]. Induction of SUMO modification of PML and PML-RARa upon arsenic treatment recruits a ubiquitin E3 ligase (ring finger protein 4, RNF4) onto the nuclear bodies, where these proteins reside. RNF4, a small nuclear protein, contains a number of SUMO-interacting motifs in its sequence and preferentially interacts with PML and PML-RARa proteins modified by poly-SUMO-2 chains. Along with RNF4, SUMO isoforms, ubiquitin, the 20S proteasome and specific transcription factors are also recruited to PML nuclear bodies, and PML and PML-RARa are degraded through SUMOylation-dependent ubiquitination [62, 63].

4 Involvement of SUMO in different cellular pathways

SUMO modification has been frequently associated with transcriptional regulation, mainly through promoting transcriptional repression, but also in some cases aiding activation, depending on the specific substrate and biochemical pathway [64]. In fact, the majority of SUMOylation targets reported to date have been sequence-specific transcription factors and the essential role of the SUMO pathway has been conclusively supported in all aspects of transcription regulation, from promoter binding to recruitment of co-factors [18, 52, 65]. Nevertheless, it would be fair to say that the function of SUMOylation extends a lot further than transcription control and covers a great variety of cellular processes, including genome integrity, DNA repair, protein trafficking, activity of voltage-gated ion channels and protein stability [4, 37]. In addition, since this review appears in this special issue of the Biotechnology Journal, it seems important to mention the applications that SUMO has had in the field of Biotechnology. N-terminal fusion of proteins, even ones that are difficult to express, to native and truncated forms of SUMO results in greatly increased expression levels and improved solubility, both in bacterial [66–69] and eukaryotic expression systems [70–72]. The SUMO part of the fusion protein can be retained and removed at will, through the use of appropriate SUMO proteases, thus making it possible to closely regulate the activity of enzymes or toxic proteins.

Most recently, important roles for the SUMO pathway have been elucidated in areas that may provide stronger links between SUMOylation and the process of aging, including the activity of the mitochondria, the major tumour suppressor pathways and signalling pathways leading to senescence. Further support to the significance of this modification for maintaining normal cell and tissue homeostasis is provided by the fact that defects in components of the SUMOylation pathway, either individually or synergistically, have been linked to an increasing number of disease phenotypes in humans [73–79]. Below, we present these areas in more detail.

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5 SUMO and mitochondrial activity

Mitochondria have been traditionally called the powerhouses of the cell as they provide most of the energy (in the form of ATP) needed for cell metabolism. The morphology of the mitochondria is crucial to their normal function and mutations that affect the shape of these organelles have been linked to problematic cell homeostasis and neurodegenerative conditions [80]. Normal mitochondrial morphology is maintained through a balance between the processes of fusion and fission, and these processes are essential for the maintenance of healthy cell metabolism and homeostasis. Mitochondrial fusion forms a network of interconnected organelles that maintains mitochondrial DNA and facilitates transfer of electron potential between different areas of the cell [81]. Mitochondrial fission ensures equal division of material between daughter cells after mitosis and provides the cell with extra energy under specific circumstances, as for example during spermatogenesis in *Drosophila* [82] and during the early stages of apoptosis [83]. The idea that mitochondrial activity and the regulation of the equilibrium between fission and fusion in response to signalling cascades is maintained by dynamic post-translational modifications has been positively regarded for a while, as transient protein modifications present perhaps the easiest way to control systems that go through rapidly changing states.

The first line of evidence that SUMOylation is involved in mitochondrial function came 5 years ago, when mitochondrial dynamin-related protein 1 (DRP-1) was reported to interact in a yeast two-hybrid screen with Ubc9 and SUMO-1 [84]. DRP-1 is a large dynamin-like GTPase that is transported from the cytosol to the mitochondrial membrane during the process of mitochondrial fission and functions to facilitate the fragmentation of the mitochondria. Fluorescence immunostaining experiments showed SUMO-1 signals to co-localise with DRP-1 at the site of mitochondrial fission, while overexpression of SUMO-1 protects DRP-1 from degradation (as does the presence of *N*-ethylmaleimide, a SUMO-1 hydrolase inhibitor) and induces fragmentation of mitochondria, as shown by the specific organelle morphology [84]. More recent studies have conclusively demonstrated the modification of DRP-1 by SUMO-1 and identified the site of interaction at specific residues [85]. Furthermore, high numbers of SUMO-1-conjugated proteins were identified in the mitochondrial part after cell fractionation, thereby suggesting that SUMOylation is important in various aspects of mitochondrial activity.

In more recent years, the specific enzymes that catalyse the SUMO conjugation/ de-conjugation cycle of DRP-1 have been identified. SENP5 has been shown to be able to hydrolyse the SUMO-1/DRP-1 conjugate [86], while the mitochondrial-anchored protein ligase (MAPL) has been identified as the complex-specific E3 ligase [35]. SENP5 has been shown to be essential for normal mitochondrial morphology and function, as both overexpression and silencing of this protease have profound mitochondrial phenotypes. Overexpression of SENP5 rescues the mitochondrial fragmentation caused by SUMO-1 up-regulation, while blocking of SENP5 by short hairpin RNA (shRNA) results in extensive mitochondrial fragmentation and much lower rates of mitochondrial fusion compared to control cells. In addition to problematic mitochondrial morphology, shRNA-SENP5 cells show a significant rise in the production of reactive oxygen species (ROS), and it has been suggested that this high concentration of free radicals may actually be the cause of the decrease of mitochondrial fusion in these cells [86]. When DRP-1 is down-regulated in these cells, ROS production returns to normal levels, fragmentation is hindered and mitochondria appear fused into a network. Besides DRP-1 regulation, SENP5 showed a broader specificity for a number of mitochondrial SUMO-conjugated proteins, further supporting its importance in mitochondrial function. The protective role of SENP5 in mitochondrial morphology and metabolic status is specific, as another member of the SUMO protease family, SENP2, has no effect on mitochondria morphology or substrates [86].

MAPL has been identified as a protein ligase that contains a RING domain, is anchored to the mitochondrial outer membrane and has a role in mitochondrial fragmentation [87]. Recently, it has been shown that MAPL is a SUMO-specific E3 ligase and the first mitochondrial one [35]. Experiments with synthetic peptides as well as native mitochondrial SUMO targets support this role. Down-regulation of MAPL via shRNA leads to a significant decrease in SUMOylation, while there is almost no effect on the ubiquitination state of the cells, unless enzyme concentrations reached very high levels that are believed not to be biologically relevant. Therefore, MAPL specifically and preferentially catalyses SUMO conjugation and is not a ubiquitin ligase [35]. Furthermore, shRNA against MAPL hinders global cell SUMOylation, but the mitochondrial fraction of the cells is the mostly affected compared to the nuclear and cytosolic parts, suggesting that many of the SUMO target proteins that MAPL specifically modifies may be present in mitochondria. More data on the specific SUMO-

ylated proteins that are affected by such global down-regulation of the SUMO pathway through MAPL are needed to further elucidate the function of SUMOylation in the mitochondria.

6 SUMO and environmental stress

SUMO modification has been shown to be susceptible to environmental effects, especially different types of cellular stress [88]. Such factors include oxidative stress, osmotic stress, heat shock, exposure to ethanol and viral infection [17, 89–92].

Initially SUMO-2 and -3 were mainly associated with cellular responses to stress, with reports that showed altered substrate interactions after stress signals [93, 94] and very recent experiments showing that they are essential for cell survival after heat shock [95]. SUMO-1 has now also been demonstrated to play a role in stress response as its expression increases during hypoxia [96]. Exposure of cells to oxidative stress led to modification of the targeting patterns of the SUMO peptides, which were de-conjugated from original complexes and re-distributed to new substrates, including anti-oxidant proteins and proteins involved in signalling during DNA damage. Reports on the response of the SUMO pathway to oxidative stress have been contradictory, with some suggesting stress results in an increase in SUMO modification of target proteins, while others demonstrate a disappearance of SUMO-conjugated species under similar conditions [94, 97]. Experimental design may play a role in these results, but since SUMOylation has been associated with a variety of diverse cellular functions, it may also be that different mechanisms of conjugation and de-conjugation are in place for different signalling and transcription regulation pathways. For example, inducing oxidative stress by treating cells with hydrogen peroxide resulted in increased levels of SUMO-2/3-modified p53, while levels of p53/SUMO-1 conjugates appeared unaffected [98]. The observation that substrate proteins are not all affected at the same time or with the same speed may support this theory [88]. Mimicking oxidative stress conditions in cell-based assays with low, and thus perhaps more physiologically relevant, concentrations of reactive oxidative species inhibits the activity of SUMO-conjugating enzymes. This leads to a general decrease in SUMOylation of substrate proteins, including essential transcription factors [88]. This effect was shown to be specific to the SUMO modification process, since the ubiquitin pathway was not affected. Such stress can occur *in vivo* after exposure to UV, ionising radiation, chemotherapeutic agents and hyperthermia [99]. Cellular responses to elevated temperature have also been linked to SUMOylation through the modification by SUMO of HSF1, as mentioned before [47].

Another likely influence on the SUMO process is viral infection [100, 101]. Gam-1, a viral protein from an avian adenovirus, can target E1 and E2 SUMO ligases for degradation and thus inhibits cellular levels of SUMO modification [100]. Down-regulation and loss of SUMOylated substrates leads to an up-regulation of cellular transcription and is thought to enhance viral replication. This association is made perhaps more interesting by the fact that, as mentioned earlier, the SUMO-specific hydrolases are much more closely related to viral proteases than to the corresponding enzymes in the ubiquitin pathway [102].

7 SUMO in cellular senescence and ageing

A number of senescence-associated proteins have been identified as targets for SUMO attachment and, more recently, SUMO peptides and enzymes that participate in the SUMO pathway have been linked with the process of senescence. The SUMO isoforms, a SUMO-specific E3 ligase and a SUMO hydrolase have been shown to have either positive or negative effects on the induction of cellular senescence (Fig. 2). In addition, elevated numbers of SUMOylated proteins have been shown to accumulate in senescent cells compared to normal replicating cells [103], while levels of SUMO isoforms and associated enzymes appear to decrease with age in a different tissue type [104].

The different substrate specificity frequently observed for SUMO-1 and SUMO-2/3 species means they can take part in different cascades within the cell, even within the same greater pathway. Overexpression of SUMO-2/3 in cultured cells has been shown to induce premature senescence, observed by slow cell growth and early growth arrest of these cells [98], while SUMO-1 up-regulation does not appear to affect the process of cellular senescence [105]. This is perhaps not surprising, considering that SUMO-2/3 is thought to be the isoform(s) mainly and most frequently associated with the response to stress. However, it is interesting that under normal conditions SUMO-1 appears mainly conjugated to target proteins within the cells, while unconjugated pools of SUMO-2/3 species are abundant and available to be used as required.

When cells experience some form of stress, the p21/p53 and p16/retinoblastoma protein (pRB) response pathways are usually activated [106, 107].

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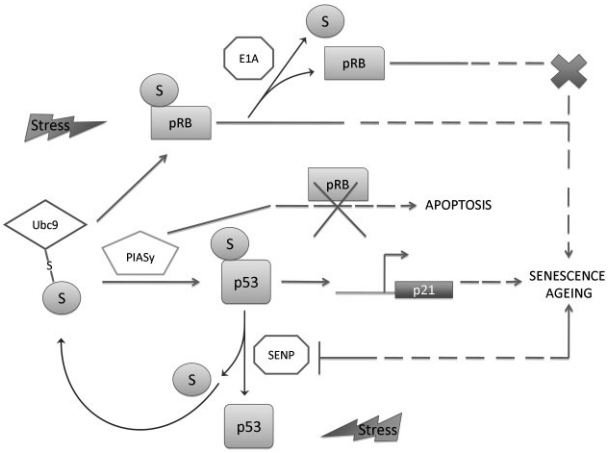

Figure 2. Components of SUMOylation in stress-response pathways leading to cellular senescence and ageing. Upon intra- or extracellular stress, the SUMOylation cycle of p53 is crucial to downstream signalling cascades that lead to senescence and ageing. Disturbances at both the SUMO conjugation and de-conjugation steps can impede the normal function of these pathways and block the onset of senescence. SUMOylation of pRB can influence the decision of the cell to undergo senescence or apoptosis. The SUMO E3 ligase PIASy and SUMO proteases of the SENP family also appear to have important roles in these pathways.

Various genes that have been associated with the induction of senescence have been shown to be part of either the p53 or pRB pathways [108]. The major proteins in these signalling cascades, p53 and pRB, greatly influence the cell's response to the stress factor(s), *i.e.* if a cell will enter senescence or undergo apoptosis, as both mechanisms are used to manage unsolicited cell growth [109]. p53 and pRB can inhibit unsolicited cell growth through pathways regulated by post-translational modifications, including phosphorylation, acetylation, ubiquitination and SUMOylation, as both proteins are substrates for SUMO-1 and SUMO-2/3 modification [110–113]. SUMO-1 attachment promotes gene transactivation by p53, as shown by up-regulation of p21 expression, a p53 target gene [98]. Moreover, components of the SUMO pathway control pRB repression of E2F-regulated target genes [114]. pRB is normally de-SUMOylated by E1A, a viral oncoprotein [111]; it has been suggested that this may be a strategy used by viruses to suppress cell senescence. Overexpression of SUMO-2/3 surpasses this de-SUMOylation, leaving pRB-SUMO-2/3 conjugates free to stimulate senescence [98].

RNA interference on p53 and pRB cancels the senescence phenotype of SUMO-2/3-overexpressing cells, thus supporting the idea that the effect of SUMO-2/3 modification on senescence occurs via p53- and pRB-mediated pathways [98]. p53-induced up-regulation of p21 is a known pathway for activation of senescence [115]. During SUMO-2/3 overexpression, the transcriptional activity of p53 is significantly enhanced and p21 is clearly up-regulated; however, the levels of p53 protein remain unchanged. Since p21 is under the transcriptional control of p53, this increase in p21 protein levels may be due to the altered SUMOylation state of p53, modified by SUMO-2/3 [98].

During replicative senescence the levels of endogenous PIASy, a member of the PIAS protein family of SUMO E3 ligases [116], are significantly increased compared to presenescent cells, as are levels of hyper-sumoylated proteins [114]. No other member of the PIAS protein family appears to have similar activity. High levels of PIASy can result in either cellular senescence or apoptosis, depending on the state of p53 and pRB within the cell. The E3 ligase activity of PIASy is essential for senescence stimulation and mutation of the ligase active site on the protein abolishes the effect. The E6 oncoprotein inhibits the SUMO ligase activity of PIASy and blocks induction of senescence [114]. Interestingly, an extended lifespan is observed in cells that overexpress mutant forms of PIASy with an inactive E3 ligase site. Mouse embryo fibroblasts that are deficient in PIASy show a significant delay in the onset of senescence after appropriate signalling. Even after induction of p53 expression by a pro-senescence signal, for example through oncogenic RAS, p53-target genes p21 and murine double minute 2 (MDM2) are not up-regulated in the absence of PIASy. In parallel, PIASy can induce p53-dependent apoptosis during pRB deficiency; inactivation of pRB by hyper-phosphorylation is not enough to give similar results and the effect of PIASy is not there when pRB is present, even in an (as far as we know) inactive state [114]. The different cellular responses to PIASy, depending on the pRB status, suggest a possible role for PIASy as one of the factors influencing the cell's decision to undergo senescence or apoptosis. It might be that PIASy is involved in altering the binding affinity between pRB and its co-factors, through pRB SUMOylation. It has been suggested that hypophosphorylated pRB enlists its co-repressors together with components of the SUMO pathway to the site of genes promoting proliferation. A number of these co-factors could be themselves targets of SUMOylation and these interactions could stabilise the pRB repressor complex. Also, as it has been shown that SUMO attachment increases affinity between protein partners [117], the presence of SUMO-modified pRB repressor group on DNA could provide a high affinity site for the recruitment of proteins involved in chromatin remodelling and reorganisation [103]. This process could

ultimately lead to the silencing of genes involved, also facilitated by the activity of histones, which are substrates for SUMOylation and have major roles in transcriptional repression in their modified state [54, 118].

Senescence may also be induced by down-regulation of a number of SUMO proteases of the SENP family. SUMO proteases may thus be required for the proliferation of normal human cells and have important roles in age-related phenotypes. After many cell passages, when human fibroblasts undergo replicative senescence, SUMO-containing PML bodies accumulate in the nucleus; this effect is also seen when SUMO proteases Senp1, Senp2 and Senp7 are repressed. Particularly Senp1 deficiency triggers premature senescence through the p53 signalling pathway [119], as the p53 pathway needs to be intact for the full result of Senp down-regulation to be exerted. It is thus very likely that Senp1 has a role in maintaining the balance in the cell after exposure to stress and perhaps preventing premature senescence.

8 SUMO in human disease

Disturbance of the SUMO modification pathway has recently been associated with a number of disease phenotypes in humans. Induction of SUMOylation has been shown for cerebral ischaemia and it has been suggested that this process may have a role in defining the outcome of neurons exposed to such conditions [120]. Other neurodegenerative diseases have been implicated in the SUMO pathway, including multiple system atrophy and Huntington's disease (HD), where SUMO peptides have been shown to localise within the protein aggregates characteristic for these conditions. In HD, SUMO modification of mutant huntingtin, which contains polyglutamine repeats, significantly inhibits its aggregation and promotes cell death [121]. This process is facilitated by Rhes (Ras homologue enriched in striatum), a small guanine-binding protein that shows E3 ligase activity and greatly enhances SUMOylation of mutant huntingtin and other proteins [121, 122]. Kennedy disease, or spinal and bulbar muscular atrophy, is also characterised by a polyglutamine expansion in the N terminus of the androgen receptor. Recent experiments have shown that SUMOylation hinders aggregation of these mutated protein forms, in a mechanism that is independent on androgen receptor transcription regulation [123]. The Parkin protein also appears to have a role in regulating the turnover of an E3 SUMO ligase [124]. These findings strongly suggest that the SUMO pathway may have a protective role against neurodegeneration. Furthermore, compromised SUMOylation has been linked to some cancers, through regulation by SUMO of nuclear receptor-mediated gene expression, for example the androgen and estrogen receptors with roles in prostate and breast cancer, respectively [42, 125]. The regulation of a number of other genes with roles in cancer development has been linked to SUMOylation [78], and a role for SUMO modification in cancer metastasis has been suggested through association of the SUMO pathway with the regulation of transcription of *KAI1*, a metastasis suppressor gene [126, 127].

Naturally occurring mutations in a number of genes have been associated with defects in SUMOylation of the mutant proteins, like for example missense mutations in the T-box transcription factor TBX22, present in patients with X-linked cleft palate (CPX) [128], and missense and nonsense mutations in p63 that disrupt the SUMOylation site(s) [55]. In the cases where mutation positions tested were distant from the SUMO attachment site(s), it may be that conformational changes caused by these single substitutions disrupt the availability of the SUMO attachment site or cause mechanical problems that disturb the interactions with the SUMO peptide and/or the SUMO-conjugating enzymes. Furthermore, other proteins with a direct association to craniofacial defects have been shown to be SUMOylated, such as MSX1 and special AT-rich sequence binding protein 2 (SATB2) [44, 55, 56, 129], and SUMO modification has been shown to be necessary for their normal function. In the case of SATB2, SUMO-1 attachment has been shown to directly regulate its activation potential and its localisation into the nucleus [129], while mutations in the SUMO acceptor sites of p63 result in a significant increase of the transactivation potential of the protein and affect the transcription regulation of its target genes, although they do not appear to disturb its nuclear localisation [56]. Mutation of SUMO attachment sites for both TBX22 and SATB2 compromise their function, while several other SUMOylated proteins have been shown to retain their transcriptional activity when their SUMOylation sites are disrupted, for example LEF1, p53, Msx1 and STAT1 [113, 130–132]. In the case of Msx1, interaction of the protein with PIAS1, an enzyme that acts as an E3 ligase in the SUMO modification pathway, appears to modulate the function of Msx1 as a transcriptional repressor, its ability to bind DNA elements in a target promoter sequence and inhibit terminal myocyte differentiation, while no significant functional defects are observed with mutations in SUMO attachment sites [133]. This supports the

theory that SUMO modification can affect target protein function through different mechanisms, and hints to the fact that certain proteins that are involved in one signalling cascade may share the same SUMO regulatory mechanism.

9 Conclusions

For most of the SUMO target proteins described so far only a small fraction of the total substrate protein present in cells appears to be SUMOylated, and very few examples of substrates appear to be modified at a greater percentage; for example 50% of Ran GTPase-activating protein 1 (RanGAP1) is modified within the cell at any given time [134]. The modified substrate may represent the functionally active form, at least for a certain part of a biochemical pathway. It has been suggested that SUMO modification is a very transient state, where target proteins get modified and un-modified perhaps as part of a loop, rather than specific protein molecules staying stably modified within a cell. Attachment of SUMO peptides to substrates may facilitate a specific function (for example interaction with DNA, other proteins and co-factors), and specific peptidases de-conjugate the SUMO molecules from substrates when this function is complete, effectively freeing them up inside the cell for the next round of modification [135]. In addition, it may also be possible that the unmodified proteins have a different role within the cells, besides the one conferred on them upon SUMOylation. It is becoming increasingly clear that the nature of such protein modifications is essential for cells to maintain the balance between various activities that need be completed, and be able to respond to internal and external stimuli in rapidly and efficiently, when time is limited and of great importance. One can also imagine that it must be much faster and more energy-efficient for the cell when the same protein can fulfil different roles within one or more signalling pathways, be ready for alternate protein-protein interactions and translocate to various cell parts, only by going through rounds of such transient "on-off" modification.

Elucidating the relationship between environmental factors, the SUMO pathway and the networks of proteins that are influenced by this post-translational modification may be crucial for our further understanding of the signalling cascades that underlie complex cell and tissue states, like metabolism, cell death and ultimately, human disease.

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